FOOD FORENSICS AND TOXICOLOGY

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Food Forensics and Toxicology

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Preface

Deliberate tampering of foods (food adulteration, changes to originality and composition, etc.) for whatever purpose, be it for ensuring unfair business advantages in winning markets for economic gain, or unlawful tendencies that result in trespassing on legal binding standards, and crimes harming other human beings, are on the increase. The tendencies to either add or subtract food components, replace food ingredients with inferior ones, or label inferior food products with those of higher grade components, have become a serious problem and are affecting people in various ways almost everywhere.

In some cases, food adulteration has involved the deliberate introduction of harmful substances into foods (chemical and biological poisonous agents), where foodstuffs such as fruits, seafood, milk, dairy products, and water have been poisoned and therefore have directly affected the health of consumers either individually or as a large part of the community, causing disease and illness, abnormalities such as neurological impairment, allergies, and even death. There is a heavy penalty to be paid by criminals who cause food poisoning by food adulterations, as there are now laws that govern the quality of foods, together with standards, guidelines, and regulations that have to be adhered to by food producers, food processing industries, vendors, and food distributors.

This book covers different types of cases that encompass food forensics and food toxicology. The book also surveys different methods and techniques that are useful in providing the evidence required to be presented in food forensics cases. The book will be of relevance to colleges and universities where forensics science modules are being taught, or to academics who are involved in research activities in food forensics or food toxicology. The book may also be useful in food forensics laboratories, to researchers in food forensics, and government departments that deal with health and the general public at large.

List of Acronyms and Abbreviations

2,4-D
: 4-Dichlorophenoxyacetic acid

2,4,5-T
: 2,4,5-Trichlorophenoxyacetic acid

3PBA
: 3-Phenoxybenzoic acid

4F3PBA
: 4-Fluoro-3-phenoxybenzoic acid

AAS
: Atomic Absorption Spectroscopy

AC
: Affinity Chromatography

AChE
: Acetylcholinesterase

ACP
: Ascorbyl-palmitate

ADA
: Aliphatic dicarboxylic acid

ADI
: Acceptable Daily Intake

AdSV
: Adsorptive stripping voltammetry

AFM
: Atomic Force Microscopy

ALT
: Alanine aminotransferase

AM
: Atrazine mercapturate

AMP_PCR
: Anchored microsatellite primed-PCR

ANN

Artificial Neural Networks

ANOVA
Analysis of Variance

APCI
Atmospheric Pressure Chemical Ionization

API
Atmospheric Pressure Ionization

AST
Aspartate aminotransferase

ATR
Attenuated Total Reflection

BChE
Butyrylcholinesterase

BDDE
Boron-doped diamond electrode

BFE
Bismuth film electrodes

BHA
Butylated hydroxyanisole

BHT
Butylated hydroxytoluene

BOTX
Botulinum toxin

BSDA
Backward Stepwise Discriminant Analysis

BSE
Bovine Spongiform Encephalopathy

BSEs
Backscattered electrons

CA
Chromosomal aberrations

CART
Classification and Regression Trees

CCD
Charged Coupled Detector

Charged Coupled Detector

CD
Circular Dichroism

CDT
Cytolethal Distending Toxin

CE
Capillary Electrophoresis

CEC
Capillary Electrochromatography

CGE
Capillary Gel Electrophoresis

CIEF
Capillary Isoelectric Focusing

CITP
Capillary Isotachophoresis

CK
Creatine kinase

CM
Carboxylmethyl

CMC
Carboxylmethyl cellulose

CMP
Caseinomacropeptide

CNE
Carbon nanotube electrodes

CNS
Coagulase-Negative Staphylococci

CPB
Clostridium perfringens Beta toxins

CPE
Carbon paste electrodes

CPE
Clostridium perfringens Enterotoxins

CPLA
Polylactide aliphatic copolymer

CP-MAS
Cross Polarization Magic Angle Spinning

CPS
Coagulase-Positive Staphylococci

CSIA
Compound-specific isotope analysis

CSV
Cathodic stripping voltammetric

CTAB
Cetyltrimethyl Ammonium Bromide

CYP
Cytochrome P

CZE
Capillary Zone Electrophoresis

DA
Discriminant Analysis

DAD
Diode Array Detector

DAP
Dialkyl phosphates

DBCA
3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid

DCCA
3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid

DDD
Dichlorodiphenyl dichloroethane

DDE
Dichlorodiphenyl ethane

DDT
Dichlorodiphenyl tetrachloroethane

DEAE
Diethyl aminoethyl cellulose

DEET
N,*N*-diethyl-m-toluamide

DEP
Diethyl phthalate

DEPT
Distortionless enhancement by polarization transfer

DG
Dodecyl gallate

DG
1,2-diacylglycerol

DLS
Dynamic Light Scattering

DMSO
Dimethylsulfoxide

DNA
Deoxyribonucleic acid

DRIFTS
Diffuse Reflectance Infrared Fourier Transform Spectroscopy

DSBs
Double Strand Breaks

DSC
Differential Scanning Calorimetry

ds-cDNA
Double Strand Complementary DNA

DSC
Differential Scanning Calorimetry

DTA
Differential Thermal Analysis

DTG
Differential Thermogravimetry

ECD
Electron Capture Detector

EELS
Electron Energy Loss Spectroscopy

EIA
Enzyme Immunoassay

EI-MS
Electron Ionization Mass Spectrometry

ELISA
Enzyme Linked Immunosorbent Assay

ELSD
Evaporative Light Scattering detector

EMR
Electromagnetic Radiation

ENPs
Engineered nano particles

EOF
Electroosmotic Flow

EPN
Ethyl Paraoxon

EPR
Electron Paramagnetic Resonance

ESCA
Electron Spectroscopy for Chemical Analysis

ESEM
Environmental Scanning Electron Microscopy

ESI–MS
Electrospray Mass Spectrometry

ETX
Epsilon toxin

F4
Flow Field-Flow Fractionation

FAAS
Flame-AAS

FCMs
Food contact materials

FFF
Field-Flow Fractionation

FINS
Forensically informative nucleotide sequencing

FIR
: Far-Infrared

FITC
: Fluorescein isothiocyanate

FMOC
: 9-Fluorenyhlmethyl chloroformate

FP
: Fluorescence Polarization

FSCE
: Free Solution Capillary Electrophoresis

FSDA
: Forward Stepwise Discriminant Analysis

FTIR
: Fourier Transform Infrared Spectroscopy

GBS
: Guillain-Barré syndrome

GC
: Gas Chromatography

GC FIS/NPD/FPD
: Gas Chromatography Flame Ionization Detector/Nitrogen Phosphorus Detector/Fluorine Phosphorus Detector

GFAAS
: Graphite Furnace-AAS

GGT
: Gamma glutamyl transferase

GM
: Genetically Modified Foods/Organisms

GPC
: Gel Permeation Chromatography

GSTP1
: Glutathione S-Transferase Polymorphisms 1

HA
: Hemagglutinin

HCA
: Hierarchical Cluster Analvsis

HCB
Hexachlorobenzene

HCH
Hexachlorocyclohexane

HDC
Hydrodynamic chromatography

HFB
Heptafluorobutylated

HFBA
Heptafluorobutyric acid

HFBI
Heptafluorobutyrylimidazole

HMDE
Hanging mercury drop electrode

HMF
Hydroxymethylfurfural

HPAECPAD
High Performance Anionic Exchange Chromatography – Pulsed Amperometric Detector

HPAI
Highly Pathogenic Avian Influenza Virus

HPLC
High Performance Liquid Chromatography

HPMC
Hydroxypropylmethylcellulose

HPTLC
High Performance Thin Layer Chromatography

HUVECS
Human umbilical vein endothelial cells

IACE
Immunoaffinity capillary electrophoresis

IC
Ion Chromatography

ICP

Inductively Coupled Plasma

ICP-OES
Inductively Coupled Plasma Optical Emission Spectroscopy

IDT
Initial decomposition temperature

IEC
Ion Exchange Chromatography

IMPY
2-Isopropyl-4-methyl-6-hydroxypyrimidine

IRMS
Stable Isotope Ratio Mass Spectrometry

ISFET
Ion-selective field effect transistors

ISSR
Inter-sequence simple repeat

ITX
Iota toxin

kNN
k-Nearest Neighbours

LC-Q-TOF-MS
Liquid Chromatography-Quadruple-Time of Flight-Mass Spectrometry

LDA
Linear Discriminant Analysis

LDH
Lactate dehydrogenase

LDPE
Low-density polyethylene film

LIF
Laser-induced Fluorescence (detector)

LOS
Lipo-oligosaccharide

LPAI
Low Pathogenic Avian Influenza Virus

LPS
Lipopolysaccharide

Lipopolysaccharide

MALDI-TOF-MS
Matrix-assisted Laser Desorption Time of Flight Mass Spectrometry

MALS
Multi Angle Light Scattering

MANOVA
Multivariate Analysis of Variance

MBTFA
N-methyl-bis(trifluoroacetamide)

MCF
Menthylchloroformate

MCHO
Mean Corpuscular Hemoglobin Concentration

MDA
Malathion dicarboxylic acid

MDM
Mechanically deboned meat

MEKC
Micellar Electrokinetic Chromatography

MES
2-(*N*-morpholino)ethanesulfonic acid

MFE
Mercury film electrode

MIR
Multiple Internal Reflection Spectroscopy

MLR
Multiple Linear Regression

MMA
Malathion monocarboxylic acid

MN
Micronuclei

MPK
Mitogen-activated Protein Kinase

MP-PCR
Microsatellite primed-PCR

MPV
Mean Platelet Volume
MRDT
Maximum rate of decomposition
MRI
Magnetic Resonance Imaging Microscopy
MRM
Mechanically recovered/reclaimed meat
MS
Mass Spectrometry
MSM
Mechanically separated meat
NA
Neuraminidase
NAD
Nicotinamide Adenine Dinucleotide
NADPH
Nicotinamide Adenine Dinucleotide Phosphate
NDGA
Nordihydroguaiaretic acid
NIR
Near Infrared
NMR
Nuclear Magnetic Resonance
NPs
Nano particles
NTE
Neuropathy Target Esterases
OG
Octyl gallate
OIDN
Organophosphate-Induced Delayed Neuropathy
OPA
o-phthalic anhydride

OPIDP
Organophosphate-Induced Delayed-Polyneuropathy

OPS
Oriented polystyrene

PAGE
Polyacrylamide Gel Electrophoresis

PARAFAC
Parallel Factor Analysis

PB
Basic Polymerase

PBDE
Polybrominated diphenyl ethers

PCA
Principal Component Analysis

PCB
Polynuclear Chlorinated Biphenyls

PCR
Polymerase Chain Reaction

PCL
Polycaprolactone

PCR
Partial Components Regression

PDO
Protected Designation of Origin

PET
Polyethylene terephthalate

PFE
Polymer film electrode

PFGE
Pulsed-Field Gel Electrophoresis

PFO
Perfringolysin O

PFPA
Pentafluoropropionic anhydride

PG
Propyl gallate

PGI
Protected Geographical Indication

PHA
Polyhydroxy-alkanoates

PHB
p-hydroxy benzoic acid

PHB
Polyhydroxybutyrate

PHBV
Copolymer polyhydroxybutyrate valerate

PHMG
Polyhexamethylene guanidine

PLA
Poly(lactic acid)

PLC
Phospholipase C

PLS
Partial Least Square

PLSR
Partial Least-Squares Regression

PNP
Para-nitrophenol

PON
Paraoxonase

POPs
Persistent Organic Pollutants

PP
Polypropylene

PS
Polyester

RAIRS
Reflection-Absorption InfraRed Spectroscopy

RAMs
Randomly amplified microsatellites

RAPD
Random amplified polymorphic DNA

RBC
Red blood cells

RFLP
Restriction fragment length polymorphism

RI
Refractive Index

RIA
Radio Immunoassay

RNA
Ribose Nucleic Acid

RNPs
Ribonucleoproteins

RP-HPLC
Reversed-Phase High Performance Liquid Chromatography

RRS
Roundup Ready Soy

RT-PCR
Real Time Chain Polymerase Reaction

RT-PCR
Reverse Transcriptase Chain Polymerase Reaction

SANS
Small-Angle Neutron Scattering

SaPIs
S. aureus Pathogenicity Islands

SARS
Severe Acute Respiratory Syndrome

SAXS
Small-Angle X-ray Scattering

SBCEE
Surface-bound crown ethers electrodes

SCC
Staphylococcal Cassette Chromosome

SCE
Sister chromatid exchange

SCGE
Single Cell Gel Electrophoresis

SDA
Stepwise Discriminant Analysis

SDS-PAGE
Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SEC
Size Exclusion Chromatography

SEM
Scanning Electron Microscopy

SEs
Staphylococcal enterotoxins

SEs
Secondary electrons

SESANS
Spin Echo Small-Angle Neutron Scattering

SFE
Supercritical Fluid Extraction

SFG
Sum Frequency Generation Spectroscopy

SFP
Staphylococcal food poisons

SIMCA
Soft Independent Modelling of Class Analogies

SLN
Solid lipid nanoparticles

SNP
Single Nucleotide Polymorphism

SPAR
Single primer amplification reaction

SPE
Screen printed electrodes

SPE
Solid Phase Extraction

SPM
Scanning Probe Microscopy

SPT
Skin Prick Test

ss-DNA
Single Strand DNA

SSR
Simple sequence repeats

STM
Scanning Tunneling Microscopy

STRs
Short tandem repeats

TAPS
N-Tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid)

t-BDMS
ter-butyldimethylsilyl

TBHQ
Tert-butylhydroquinone

TCBS
Thiosulphate Citrate Bile Salt Sucrose

TCPY
3,5,6-Trichloro-2-pyridinol

TDFs
Transcript-derived fragments

TDH
Thermostable Direct Hemolysin

TDH-TRH
Thermostable Direct Hemolysin-related hemolysin

TDPA
3,3'-thiodipropionic acid

TDS
Time Domain Spectroscopy

TEM
Transmission Electron Microscopy

TFA
Trifluoroacetates

TFAA
Trifluoroacetic anhydride

TGA
Thermal Gravimetry Analysis

TGA
Thermogravimetric

THBP
2,4,5-trihydroxybutyrophenone

THz
Tetrahertz

TIS
Transmission Infrared Spectroscopy

TLC
Thin Layer Chromatography

TMA
Thermomechanical Analysis

TMS
Trimethyl silyl

TNF
Tumour Necrosis Factor

TPC
N-trifluoroacetyl-L-prolyl chloride

TSG
Traditional Speciality Guaranteed

TXRF
Total X-ray Reflection Fluorescence

UVVis-
Ultraviolet-Visible (UV-Vis)

VAMP
Vesicle associated membrane protein

VNTRs
Variable number of tandem repeats

XMT
X-ray Microtomography

XPS
X-ray Photoelectron Spectroscopy

1
Food Forensics: Introduction

“Forensic” is derived from the Latin, with its meaning referring to law. Hence the definition of forensic science hinges on this meaning as a branch of science that applies scientific principles and methods to public domain cases related to criminology and civil law. It deals with the whole process of gathering and examining information that can be presented as evidence in courts of law, to enable the execution of law enforcement in relation to criminal or civil laws.

The application of science and scientific principles to point beyond reasonable doubt to criminals and crimes has been in existence for centuries. In the past, the strategy to catch and deal with criminals had many limitations, mainly due to the lack of standardized methods, which led to numerous flaws that allowed criminals to evade punishment due to lack of sufficient evidence. In those ancient times, criminal acts were investigated based on the testimonies of witnesses and from personal confessions. There was no application of any of the scientific methods and techniques which make use of the scientific principles and concepts that are available to us today. In these modern times, forensic science, as it is applied in a diverse number of fields, employs highly sensitive and selective methods and techniques in analyzing evidence obtained at a crime scene, even where the specimen presented as evidence is at the trace level. Among the areas of disciplines which make use of scientific principles to investigate foul play are in foods and foodstuffs.

Given its importance to life, food will remain the most fundamental of our needs, because it is the source of all the energy needed to carry out life’s activities, including those which define the very characteristics of life. Food is essential to our physical well-being, and it is the main item that all the cares of the day are invested towards. Food is the source of energy needed for tissue repair, muscle movement and also plays an important role in the whole process of growth. One may dare to say that without food there is no life! On the other side of the same coin, food that is unfit for human consumption may be used to target and terminate life. For this reason, food is one of the items prone to be used or abused to threaten life.

There are many ways and instances where food composition has been compromised or tampered with, bringing negative effects to consumers.

Normally the introduction/incorporation of either microbial (bacterial, fungal or viral) metabolites or chemical agents, compromises the safety of food that is meant for consumers and this constitutes a food fraud case. There are two main types of fraudulences as far as food is concerned. One such example involves a complete replacement of the entire authentic food product with a substitute product. This kind of fraud is termed as “crude fraud” and in many cases it involves expensive, highly moveable items such as alcoholic beverages. Another type of fraud that is practiced within the food business/industry is known as “sophisticated fraud”, in which some food components are manipulated by either substituting quality ingredients with inferior ones or the entire food product is subjected to dilution (e.g. addition of water to milk, brine to frozen meat, glycerol to wine, etc.).

Again, of late, there have been many food scares, scandals and fraud cases reported widely that not only pose a large risk but have also caused loss of life globally. For example, the advent of genetic engineering technology in food as well as in food industries has introduced genetically modified foods/organisms (GMO), which could pose a potential risk to human existence, though not yet proved scientifically. Apart from the GMO issue, there have been plenty of other incidences, such as the outbreak of bird/avian and swine flu (H1N1, H5N1, H7N3, H7N7, H7N9, H9N2, etc.), and the outbreak of foot-and-mouth disease and bovine spongiform encephalopathy (BSE), also known as Mad Cow Disease. Other microbes, such as the strain of *Escherichia coli* (*E. coli* O157), are harmless resident flora in the gut of cattle but can cause disease in humans through the consumption of meat and meat products containing this bacteria strain. *Clostridium botulinum*, a spore-forming bacterium, produces botulism toxins that are fatal to consumers of food items containing this toxin.

There have also been numerous reports linking the incorporation of illicit food additives (which in some cases are not listed on the labels properly) in food products, and other malpractices by food producers and food industries. These reports have alerted the public and raised the awareness of the composition of food products, thus the high demand for tighter scrutiny, guidelines and regulations on additives in foods.

Incidences related to foul play in foods in terms of processing, labeling, distribution, food poisoning and intoxication, etc., have signaled law enforcers, nutritionists and health bodies to introduce more laws, set regulations and draw up guidelines to safeguard the health and well-being of consumers. Due to this, foodstuffs and food products are now analyzed and investigated to see if they comply with the requirements set for quality, nutritive contents, adulteration,

compliance against legally set guidelines and regulations, and also whether the labeling requirements are accurate. These considerations are also meant for research and development purposes to improve the quality of food products.

All governments, as well as national and international agencies, have set regulations, guidelines and recommendations in place to ensure the quality and safety of foods and food products. Such regulations, guidelines and recommendations have to be observed and adhered to by all suppliers and food industries responsible for providing consumers with foods. The enforcement of rules and regulations ensure the wholesomeness and safety of foodstuffs, and satisfy the public need which insists that food suppliers and food industries inform consumers about the state and nutritional composition of their food products, to enable individuals make their choices with regard to their preferences or to provide an environment with fairness, in cases where there is more than one competing company or entity, to avoid any possibility of economic fraud.

Food regulatory bodies, and national and international agencies, have specified some standards to be observed by food suppliers and food industries with regard to quality and compositions of food products. Some of these standards are legally extremely strict and it is mandatory that they are adhered to by all responsible parties, while some others tend to be more flexible.

The strict standards which are mandatory include the quality standards in which quality specifications regarding stability, types and classes of color, mass/or volumes, tenderness, etc are being considered. Standards of identity are also mandatory by regulations and specifications regarding type, composition, ratio and amounts of various ingredients required for a specified food product, which must be made known so that where a threshold limit is set, the guidelines should be clear that it has not been violated. For example, in some foodstuffs, levels of fats are controlled by law and should not exceed certain specified levels. The standards of fill-of-containers are also mandatory, in which case a measure of fill/mass has to be known to avoid fraud or treachery of any sort. In this category of standards, the means or how to ascertain the fill/mass has to be made known as well. Flexible standards may as well include grading of food products.

Authorities responsible for food regulations also enforce a requirement that the origin and authenticity of food products be correctly included in the labeling, together with the labeling specifying food ingredients and their composition. This information is vital for the prevention of economic fraud and for ensuring that the correct type of food is supplied to the target people. For example, people

of a particular religious group, or those who need food with certain ingredients, for instance, fat, sugar, salts, etc., at certain amounts. For this reason, food and food products need to be analyzed and ascertained for their safety and quality.

In some cases, microbial attack on food not properly stored leads to the presence of toxic metabolites. Therefore, storage conditions and techniques are some of the issues that are scrutinized, especially for the types of foods and food products that need to be transported to distant places or those which are not to be consumed immediately.

The presence of undesirable chemical and biological molecules prompts the enforcement of monitoring schemes to ensure safety and quality of foods and food products. The monitoring scheme has to have scientific methods and techniques in place that are sensitive and selective enough to exclude all other undesirable or non-targeted matrix molecules as well as to enrich the analytes of interest. The enrichment step is necessary because. in many instances, the target analytes are in trace amounts or dissolved/incorporated into a larger mass/volume of food products which causes the analyte to be highly diluted.

Normally, the analytical strategy involves the development of highly selective and sensitive methods that are capable of thorough clean-up and pre-concentrate the analytes before their introduction into the analytical system, such as HPLC, GC, AAS, ICP, *etc.* The data obtained from these analyses are meant to provide information regarding the safety of the foodstuffs or if a particular food item contains harmful microbes such as Salmonella or their metabolic by-products, or the presence of chemical molecules such as pesticide residues, or even the presence of some other foreign contaminants. Foodstuffs for human consumption imply that they are free from all such things, and food manufacturers as well as suppliers have a duty to ensure food safety by carrying out routine analysis of food products. Moreover, food industries are at all times faced with stiff competition from each other and are thus obliged to perform these routine checks to remain competitive and win more markets.

Generally, strict regulations and guidelines necessitate food industries to use methods and techniques that are reliable and which are capable of providing low detection and quantification limits to be able to comply with guidelines and regulations. Testing and analysis should normally be done strategically, before, during and after the manufacturing and in some cases, long after the manufacturing to ensure stability under different sets of storage conditions, such that the final food product possesses the same properties and qualities including shelf life, appearance (color), flavor and texture in addition to chemical, physical

and biological properties.

However, naturally foodstuffs do undergo deterioration and spoilage over time and under some conditions where the chemistry of raw material ingredients unpredictably changes, thus affecting the whole food product. Under such circumstances, what is required is to understand the chemistry of the ingredients and the mechanism by which each plays its role in relation to the final food product. This knowledge helps to predict the behavior of various ingredients under different sets of conditions and thus control the production processes accordingly, in order to avoid unnecessary food deterioration. For example, food color of some products, such as chips made from potatoes, is highly dependent on the concentrations of reducing sugars present in the potatoes during the manufacturing processes. If the reducing sugars are present at high concentrations in the potatoes, it will cause more browning of the potatoes as time goes on (Msagati, 2012). This implies that those responsible in the preparation have to ensure that the potatoes used have the required concentration of reducing sugars to avoid color changes with time or during the frying process.

Currently, the global trend with respect to attitudes towards foods has been highly shaped by the opinion of consumers, due to changes in consumer preferences towards the types of foods that are seemingly healthier. In some cases the origin of food, cost or those seen as exotic, have been an important factor for consumer preference. This trend has shaped the whole food industry, which has to conduct research and development for either the improvement of the existing products or formulate new food products that satisfy the changing demand of consumers. In this scenario, food industries engage in research activities that investigate the mechanisms of how various food ingredients and components work and also the roles they play individually or when introduced to others. The investigation process also encompasses the whole industrial processes that take place or occur during food manufacturing and how they affect the quality and composition of food, even after a long period of time when subjected to various physical/environmental conditions such as temperature, humidity, storage, *etc*. In other words, food development of new products always requires the knowledge of the food ingredients as well as the processing operations and how they interact to give food the desired quality.

On the other hand, the analytical procedures required by food analysts need to establish factors that will reveal the information about composition, physico-chemical properties of food, *etc*. This is due to the fact that food components (composition) tell how safe or nutritious the food is. The composition of many

foods is complex, being made up of many different ingredients (vitamins, proteins, carbohydrates, fats, fibers, minerals, etc.) and in different ratios, both in terms of the types of atoms (e.g. C, H, N, O, S, Na, Se, etc.) and molecules (e.g. H_2O, sugars, essential fatty acids such as omega-3, essential amino acids, etc.). For food manufacturers and distributors to comply with regulations, they are thus obliged to declare compositions, ratios, levels and types of all the ingredients in their products.

Similarly, the attributes of foods in terms of their physical characteristics as measured by their rheological properties (stability, taste, color/optical, flavor, etc.), are very important to consumers, as they affect their appeal and choices. This shapes the food industry since it requires that foods and food products be designed to contain the qualities that will appeal to consumers and at the same time comply with regulations and guidelines. The design and operating conditions must also ensure food stability under all conditions without affecting attributes such as color, smell/odor, feel or texture. Moreover, the design and operation processes during food manufacturing have to be such that they maintain the structure of the food and food products.

Food analysis will therefore require different types of analysis, methods and techniques, due to a variety of information and measurements that may be needed to ascertain the quality and safety of a particular food product. There are many sources where the information for the appropriate technique for measuring or ascertaining a particular food property can be obtained. For instance, there are specific books, journal publications and suppliers of instruments, reagents, chemicals and materials used in the analysis of foods. There are also official methods and techniques published by authorized bodies that deal with food analysis and quality assurance, as well as public domains such as the World Wide Web (internet).

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2
Food Provenance and Food Fingerprinting: Authentication and Traceability of Foods and Food Products

Is it important to know the origin of the food we eat? This may be a question to which some insist on concrete answers, though for some it may not matter whether the food they eat comes from west, east, north, or south. Generally speaking, there has been an interest in where food comes from, and how it reaches the consumers (knowledge of mechanisms and routes of distribution and supply chains). Some food products are in demand throughout the year, thus necessitating a well-known supply chain and this requires the knowledge of the origin of such food items be provided. Moreover, with concerns arising from environmental pollution, which directly negatively affects the whole of food safety, the food preference for many consumers is tilting towards organic foods (those that are grown with no application of long-lasting agrochemicals and fertilizers). All these factors have prompted authorities to introduce rules and regulations to govern the traceability mechanisms of food products, which will give confidence to consumers about the authenticity, quality, integrity, safety, and provenance of the food products of interest. At this juncture, there is a need to define the term "origin" as far as food provenance is concerned. The origin of the food's location refers to the geographical location where the food in question is either produced/obtained/found. In cases where the same food product is found in more than one geographical location, then the location where the last substantial transformation is recorded will be considered to be its geographical location of origin for that particular food product.

Introduction

In the modern day, consumers of various foodstuffs and food products demand to be provided with clear, accurate, precise, and succinct information and data about the origin and specification of the food products they consume. Authorities have put in force strict regulations that such information be outlined in the labels and labeling, which should adhere to the rules and regulations set by describing properly the geographical origin and authenticity of all that is claimed in terms

properly the geographical origin and authenticity of all that is claimed in terms of quality and composition on the packaging labels. The rules, guidelines, and regulations that have been set speak volumes about the legality of the whole issue about food provenance and fingerprinting, with laws being enacted to prosecute the culprits, offenders, and transgressors.

Food Fraudulence and Adulteration

Food fraudulence refers to deliberate and intentional actions and tendencies to illegally tamper by substituting, adding, or mislabeling of foodstuffs, food ingredients, or even food packaging for whatever gain (Elliot, 2013). Food fraudulence involves all practices that lead to an intentional or pre-meditated addition or substitution of an ingredient in order to either raise its value or lower production/processing costs (Shears, 2010 ; Spink, 2013). Practices such as dilution of food products to levels that reduce their nutritive value or to an extent that risks the health of consumers and/or masking the effects of dilution by adding other inferior substances or replacing quality ingredients with either weaker/inert ones, false branding/misbranding or mislabeling, etc., are all part of food fraudulence (Ellefson *et al*., 2013 ; Elliot, 2013). This term "food fraudulence" also encompasses other tendencies, such as giving false, inaccurate, or misleading information or statements regarding a particular food product (Ellefson *et al*., 2013). In the context of this topic, food fraudulence will not deal with other forms of food fraudulency tendencies such as those that deal with economically motivated kinds of fraudulency, misbranding, smuggling, tax evasion, and food counterfeiting practices. This topic will only deal with intentional food fraudulency tendencies with motivations that affect food quality and food safety, which endangers the health of consumers.

For any food product to be considered fraudulent or adulterated it must contain the following:

- any substance or ingredient, or an added ingredient, that may cause a health hazard to consumers;
- an ingredient or component (added externally or internally) that is regarded as spoiled/decomposed, regardless of whether this ingredient was formed during processing, handling, or storage, or it came from a disease that has affected the plant or animal from which the food originated, from the container/packaging, or stores where the foodstuff was kept/stored;
- does not contain any valuable component whether omitted, damaged,

concealed, abstracted, or present in an inferior form;

- harmful ingredients such as additives (coloring agents, flavor enhancers, etc.);
- wrong measure of fill (volume, mass/weight, bulkiness) or strength (e.g. color intensity, flavor sharpness, etc.);
- does not contain proper information, such as food items sold under a different identity (origin, type of food, etc.) and foods imitating others or foods with wrong definitions.

However, caution should always be taken when defining the cause of the incidences in food fraudulence cases. In the scenario where it is proved beyond reasonable doubt that there has been an unintentional and deliberate action of food contamination or adulteration through either improper food handling, or during processing, or spoilage by microorganisms or addition of ingredients that will poison the food products, all these will constitute what is termed as issues related to food safety. The situation will only be regarded as food fraudulence when it has been proven that there has been an intentional cause for the presence of adulteration in the food for whatever reason, especially the economic gain.

The common aspect between the two terms is that both food fraudulence and food safety result in health risks to consumers. When there has been an incidence of either food fraudulence or food safety endangering consumers' health, efforts are normally invested to address (prevent or reverse) the effects of intentional or deliberate adulteration that was caused for whatever motive, and this is defined as food defense. On the same note, cases that involve unintentional food adulterations (food spoilage or deterioration), which result in economic losses for the individuals or industry, constitute what can be termed as food quality issues. Food quality can be caused by various physical and chemical properties of the food. Now the efforts to prevent or measures put forward as intervention or responses to address food quality phenomena are known as food protection.

Bearing in mind the consequences of all these phenomena, authorities normally devise strategies and plans to prevent food adulteration incidences well before they take place. Among the strategies that are put in place are the enforcement of effective programs of risk-based intervention strategies well in advance and also the institution of rapid reaction measures to address effects that can be caused by food fraudulence once it is discovered. For convenience, food fraudulence can generally be categorized into a number of groupings as follows:

- counterfeiting: in which case the perpetrators infringe the legal owners'

Intellectual Property, and produce a similar food product and package using similar packaging and without adhering to the standards;

- simulation: is another food fraudulence category that is similar to counterfeiting, where the design and formulation of simulated food products gives them a similar look of the real food product, but in reality it is similar and may have slightly different formulas or ratios of ingredients, and in most cases the simulated food products fail the compliance test in terms of quality assurance;
- adulteration: also a food fraudulence category that involves the addition of a foreign ingredient to a finished food product, for example adding brine to frozen meat and products, or adding melamine in milk;
- tampering: another category of food fraudulence that involves misusing either legitimate food products or food packaging, *e.g.* by changing the information, such as expiry period.

Currently, there is an ongoing fight against food fraudulence that is ever growing and expanding, covering almost all parts of the world. There is a widespread tendency of adulterations where foods, foodstuffs, food additives, and food supplements have been found to be contaminated or without the expected quality or labeling. The results of food adulteration cost the food industry greatly and affect consumers both health-wise and economically. The sources of adulteration may be traced from the geographical identity of the place of origin of that particular food product, the brand name and method of food processing, or even the time it has been stored. Food fraudulence is currently attracting the interest of many stakeholders, both in private as well as public sectors, as an emerging risk that is threatening society. This is partly due to the complex nature, trends, and principles that govern distribution and global food supply chains. Unscrupulous food manufacturers and distributors entertain the practice of tampering with the ingredients of foodstuffs by cutting down levels or replacing altogether some of the ingredients in order to maximize profit (Elliot, 2013). The availability of numerous reports and publications suggests that there are major and serious food adulteration and contamination practices occurring, frequently and regularly (Ellefson *et al*., 2013).

For example, according to an article that appeared in the *Washington Post* on Tuesday, 30 March 2010; A01, which was entitled “FDA pressured to combat rising ‘food fraud’” by Lyndsey Layton, *Washington Post* Staff Writer, reports on fraudulence involving sheep’s milk cheese that was later discovered to be

cow's milk; another case was sturgeon caviar that was later proved to be Mississippi paddlefish (source: http://phe.rockefeller.edu/news/wp-content/uploads/2010/04/FDA-pressured-to-combat-rising-food-fraud.pdf; accessed 11 September 2014). This article also pointed to instances where some honey-makers tended to dilute honey products with either sugar beet or corn syrup. Other instances involved the sale of frozen catfish fillets claiming to be other brands such as red snapper, *etc*. but they were actually from Vietnam (source: http://phe.rockefeller.edu/news/wp-content/uploads/2010/04/FDA-pressured-to-combat-rising-food-fraud.pdf; accessed 11 September 2014).

In general, deception in the food industry has been around for a long time, although the extent of its impact on consumers' health or the economic sector is difficult to ascertain. There are contributing factors such as the current trend toward urban migration, as well as globalization that play a role in its widespread increase, both in terms of magnitude and extent (Ellefson *et al*., 2013). These factors (urbanization and globalization) encourage a special trend of food supply chain to sustain the huge population concentrated in urban centres. To control such a complex food supply chain in the current global economy with limited resources, creates room for criminals to practice unscrupulous business deals with food products. As time goes by, they come up with new ways of practicing fraudulence, which creates more challenges to deal with the problem.

Scientific Methods and Strategies for Verification of Food Fraudulence Cases

There are a variety of profiling methods that generate useful data (those chemical signals obtained from analytical instruments that can be de-convoluted to give the composition of food), as well as many other chemical and biochemical techniques that are available to food forensic scientists, to ascertain any of the already mentioned food fraudulence cases. The advantage of profiling methods is that they can be applicable to complex samples such as foods and are also capable of performing a wide range of scientific measurements simultaneously. Among the chemical and biochemical techniques that are used to verify food adulterations or fraudulence are chromatography (e.g. liquid chromatography (LC), gas chromatography (GC), etc.); mass spectrometry (MS); nuclear magnetic resonance (NMR); infrared spectroscopy (IR); enzyme-linked immunosorbent assays (ELISA); and polymerase chain reaction (PCR), etc.) (Pomeranz and Meloan, 1994). Data from these techniques can further be

subjected to statistical analyses, using multivariate techniques such as partial least square (PLS) or principal component analysis (PCA), to provide more in-depth information and variability of parameters that can reveal abnormal trends of behavior or lack of consistency.

Scientific Verification of Geographical Indications and Designation of Origin of Foods

There are three main types of geographical description or schemes for foods that play a significant role to protect the identity and names of foods, as well as other agricultural products. There is a scheme known as “Protected Designation of Origin” (PDO), which applies to those foodstuffs and food products originating/produced and/or prepared/processed in a particular geographical location using acceptable/known and standardized methods and techniques (Gonzalez *et al*., 2009).

The second type of scheme or geographical description of foods is known as a “Protected Geographical Indication” (PGI), which signifies that there is a connection with the geographical location or area in at least one step of either production, preparation, or processing of a particular foodstuff (Gonzalez *et al*., 2009). This second description of the geographical description (PGI) seems to be of more importance than PDO, due to the fact that it associates the food with a particular geographical location.

The third scheme is known as “Traditional Speciality Guaranteed” (TSG), which reveals the traditional nature or character in terms of either ingredients (composition) or the procedures followed during production.

Processes, Steps and Procedures to Distinguish the Geographical Origin of Foods

The identification of the origin of foods or the effects of different food production processes is not straightforward. The process must involve the identification of specific markers that distinguish food products, in order to ensure that there is no possibility of having either a false negative or a false positive. There must also be in-depth scientific knowledge of the chemical composition of foods, effects, or contribution of environmental factors (biological and physico-chemical factors), and other important differences between the target food items of interest to other similar food products found in

other geographical locations. This calls upon the presence of analytical chemists who will establish procedures of correct identification of the exact discrepancy that defines the fraudulence in question (fraudulent food product and the type of fraudulence).

Analytical chemists will need to play an important role in developing appropriate methods that will reveal the exact chemical composition, as well as isotopic abundances of the elemental composition of that particular food product or other specific chemicals/molecules, spices, or flavors that may form part of the composition of the food products in question. Analytical chemists will also need to develop methods and strategies to identify products that may be formed during food processing or production, thus fingerprinting products by using appropriate analytical and bio-analytical techniques, including chromatography, spectroscopy, or mass spectrometry. Measurements of this kind of work require large sample sizes, as well as the application of statistics for validation, otherwise the results may not be considered authentic enough.

Geographical Identification, Quality Verification and Authentication of the Origin for Fruit Nectars, Fruit Juices, Vegetable Juices, and Non-alcoholic Beverages

The term “fruit juice” refers to a liquid derived from a fruit, while “fruit nectar” refers to an unfermented food product obtained by adding water to a fermentable food product. Generally, fruit nectars may contain additives such as sweeteners or honey. On the other hand, the general term “non-alcoholic beverages” includes products such as soft drinks, energy drinks, bottled water, carbonated drinks, and sports and isotonic drinks. Fruit juices, vegetable juices, fruit nectars, and other non-alcoholic beverages are normally verified on the basis of the composition of the additives incorporated for the purposes of adding or enhancing color and those that are added as flavoring or flavor enhancers. Also, these food products are verified on the basis of water content that forms part of the naturally derived fruit juice. Generally, for nectars and juices prepared from fruit, there are regulatory standard requirements that are supposed to be adhered to before the certification of the product for commercialization (sale). These include the requirement that they should possess and retain the same characteristic qualities of aroma (flavor and flavor sharpness), as well as the appearance (color and color intensity) to that of the fruit from which the product

(fruit or nectar) was processed.

Another decisive factor that is crucial in the authentication or identification of the origin of fruit juices, vegetable juices, and nectars, is the measure of the main quality parameters that are normally required to be displayed on the label (showing composition). Verification of the measure of various ingredients in the fruits juices, vegetable juices, and nectar as claimed in the labeling, is always mandatory in order to prove compliance with the regulations. For instance, with some adulterations involving fruit juice blending, where a high-quality juice can be blended with an inferior one (adulteration for economic gain), the information as well as the measure and quality of ingredients as it may appear on the labels may be different from the actual chemistry of the juice.

The problem of food adulteration related to juices and nectars has prompted the need for the development of analytical methods, techniques, and regimes to analyze and verify the identity and authenticity of fruit juices and fruit nectars.

The development of analytical procedures for this purpose has been faced with challenges due to the complex nature of these food products, as well as the variability of fruit juices and nectars in terms of their composition. Another challenging consideration comes from the fact that the analysis requires skilled personnel as well as advanced analytical instruments. For fruit/vegetable juices and fruit nectar, the detection of identity, adulteration, and authenticity involve the detection of mainly the types and ratios of organic acids, amino acids, sugars, or other sweeteners, antioxidants such as polyphenols and flavonoids, as well as inorganic elements, and these compounds can thus be used as indicators of adulteration.

For example, one of the nutritive compositions in fruit is sugars, which is one of the components that actually impart to the fruit and juices the characteristic taste, aroma, and flavor. Sugars also play other roles, including the role of an indicator of the conditions for storage. The abundances and ratios of these sugars is characteristic to fruits, such that if there is any significant difference, then the situation will represent a possible scenario of adulteration. This is because any attempt to add either a sweetener, syrup, sugar, water, or an inferior type of juice blend, will alter the ratios of various sugars and their abundances, thus confirming a fraud scenario. Sucrose (glucose and fructose), as well as sorbitol (a sugar alcohol), are the main types of sugars that are normally analyzed to confirm a fraud situation for fruit juices, vegetable juices, and nectars. The abundance and ratios of these sugars are qualitatively and quantitatively different for different types of fruit or vegetable juices and are dependent on a number of

factors such as species, variety, geographical location where the fruits are grown (environment), and the maturation level of the fruit at the time of harvest. For example, pineapples, grapes, oranges, and apples from the same geographical location and maturation will have different profiles and ratios of sucrose, glucose, and fructose. Normally, glucose and fructose are found in higher levels in grapes and at the same time, sucrose in grapes is generally lower. The situation is the opposite in the case of apples, pineapples, and oranges. Sorbitol is generally lower in many fruits and fruit juices and where needed can only be added, in which case it has to be within the regulations and guidelines or else this will constitute an act of adulteration.

Sugars are the building molecules of carbohydrates and thus when analyzing sugars, methods used for carbohydrate analyses are normally applicable for sugars as well. The most common method involves the use of a high performance anion exchange chromatographic system equipped with a pulsed amperometric detector (HPAEC-PAD). Where necessary, this can further be confirmed by the use of stable isotope methods to ascertain the results for authenticity and identification of adulteration. This type of chromatography is normally preferred for the separation of anionic species, which can either be anions in their natural form or those that can be ionized at high pH values, for example sugars and carbohydrate. Where species that ionize at high pH are analyzed using HPAEC, the mobile phase for such kinds of species will constitute mainly hydroxide-based eluent solutions, because they are capable of producing anions from these analytes (e.g. sugars), which can form charges in neutral pH environments. The stationary phases (column packing materials) for HPAEC are normally prepared from non-porous resins, in which microbeads with anion-exchange moieties are attached to cation exchange resin particles to impart stability at all pH values. Moreover, the preference of non-porous polymeric resins as stationary phases for HPAEC comes from the fact that they minimize band broadening in chromatograms, thus highly improving the separation of analytes.

After separation, they can be detected without the need for derivatization using a pulsed amperometric detector (PAD) in which various (specific) electrochemical potentials are applied to a working electrode for a period of time, causing oxidation-reduction processes of the sugar's hydroxyl groups at the surface of the working electrode. The redox processes at the electrode surface will cause losses in protons, which will result in currents being generated and the resulting current will be proportional to the magnitude of the hydroxyl groups of the sugar samples.

Stable Isotope Ratio Analysis (SIRA) of Sugars in Fruit Juices and Fruit Nectars: Isotope Signature

By definition, stable isotopes are the non-radioactive atoms, with nuclei having the same number of protons but a different number of neutrons. Chemically, isotopes behave like other major elements such as carbon, hydrogen, oxygen, nitrogen, and sulfur, although there are some differences in terms of certain physico-chemical properties that can be explained from the measure or magnitude of their mass differences. It is these differences that are exploited to enable the separation of isotopes from the major elements during many physical processes or during the course of a variety of chemical reactions (Cui *et al*., 2011 ; Lin and Ke, 1995). In the process of separating isotopes from other major elements, there are two main isotopic effects that are generally encountered, which include:

- the equilibrium fractionation that takes place during the isotopic exchange reactions, which triggers the conversion of a liquid phase into the gaseous phase;
- the second isotopic effect is termed the kinetic fractionation and involves unidirectional reactions characterized by reaction rates that are sensitive to certain positions of the masses of atoms of one of the reactants. Isotopic effects can be quantified (have got magnitudes) as well as the standard unit known as delta (δ), which can be expressed in terms of discrimination magnitude values (defining the deviation of a fractional factor, Δ, from unity) expressed as capital delta (Δ), defined by Equation 2.1:

$$\Delta = a - 1. \quad (2.1)$$

The mathematical equation that expresses the isotopic composition (δ), which is defined as the isotope ratio measured against a certified standard, is given by Equation 2.2:

$$\delta X(‰) = \left(Rsam / Rstd - 1 \right). \quad (2.2)$$

where δX = isotope ratio expressed in delta units as measured against a certified standard; *Rsam* = absolute isotope ratios of the sample being analyzed; and *Rstd* = absolute isotope ratios of the certified standard.

As defined previously, a stable isotope means that the isotope is non-radioactive

As defined previously, a stable isotope means that the isotope is non-radioactive. Examples of the stable isotopes include those of carbon, hydrogen, nitrogen, sulfur, and oxygen. The use of isotopic analysis to ascertain the authenticity, identification of geographical origin, or the detection of adulterations is attractive, because it provides a marker isotopic identity that reveals the profile and distribution of certain stable isotopes and chemical elements in the given food product sample (e.g. sugars, juices, etc.).

The justification for the use of isotopes in foods of both plant and animal origin comes from the fact that there is a strong correlation between the isotopes present/found in plants as reflected in their various sources (air: CO_2, NO_2, NH_4, SO_2, etc.), soils, and water (H_2O) to the processes that control the plants' assimilation of the same elements as well as to the environment where the plants are growing. Considering plants as examples, the fraction patterns of carbon dioxide diffusion (CO_2) is what actually governs the content and fraction of the carbon-13 ratio absorption in the plants in that particular geographical location. The geochemistry of groundwater at specific geographical locations plays an important role in dictating the composition, abundance, and ratio of oxygen isotopes. How rich the soil is, in terms of nutrients (N and P), may determine and govern the availability and fraction ratio of nitrogen or phosphorus isotopes. The occurrence of natural disasters and phenomena such as volcanic eruptions, or anthropogenic activities such as the use of fertilizers in agriculture, contribute to the profiles of sulfur isotopes. Also, the age of the underlying geological structure and rocks of a particular location have an influence on the ratio of strontium-87 and strontium-86 isotopes. For example, if the ratio of strontium-87 to strontium-86 is less than 0.706, then the geology of that location is characterized by young basalt rocks and the plants growing in this particular location will have the same ratio of strontium isotopes. The geographical location characterized by old crystalline rocks will have the isotopic ratio of strontium-87 to strontium-86 greater than 0.701 (Brereton, 2013).

Generally, the isotopic trends in various geographical locations will have an influence on the vegetation and thus be reflected in the chemical composition of the flora and fauna of that particular location. This will therefore enable the fingerprinting and geographical identification of the food product's origin.

On the other hand, the trend and profile of trace element composition from a particular geographical location is normally characteristic and specific to that area and therefore can also be used for fingerprinting and geographical identification of origin for various species. To sum up, we can say that a combination of the profiles for element composition and multi-element stable

isotope analysis may offer a stronger and more reliable approach for the determination of the geographical origin of food products.

There are a number of analytical instruments that are normally used for the measurement of stable isotopes, and these include the isotope ratio mass spectrometry (IRMS), which gives a measure of variations in isotopic ratios after isotopic fractionation, as well as Fourier transform infrared spectrometry (FTIR), which is mainly used for the measurement of deuterium. The IRMS uses differences in terms of the charge-to-mass ratio to separate the various isotopes of elements that have been fractionated. The isotope ratio of the same elements differ from place to place, because the ratios of isotopes are affected by various factors such as weather patterns, humidity, altitude, latitude, vegetation, topography, *etc*. For example, in geographical locations that experience low levels of humidity, the $_{18}O$ isotope is also low as most of it is lost through precipitation phenomena and this affects the water composition fraction that gets into the plants and animals in the same magnitude, while $_{16}O$ evaporates and adds to the atmospheric oxygen. The variability of isotopic ratios will also be reflected in the plants through to the fruits and the juice or nectar that is made from them. The analysis of stable isotopes will therefore verify the geographical origin from where the food product (fruits or vegetables) used to make juice came from. It will also reveal whether there is an unusual isotopic ratio, which can be an indication of adulteration.

Stable Isotope Ratio Analysis (SIRA) and Multi-element Stable Isotope Analysis Techniques

As already pointed out, stable isotopes have become attractive in the process of identifying the origin of food products. The reasons behind this approach come from the fact that there is a systematic variation in terms of the distribution of stable isotopes globally. There is a relationship between the variation of stable carbon and hydrogen isotopes as observed with the photosynthetic mechanisms in plants, as well as the pattern and trend of the application of agrochemicals (particularly chemical fertilizers). These factors play a crucial role in shaping the uniqueness in terms of the distribution pattern and profile of stable isotopes in various geographical locations. In addition to this, climatic and environmental factors add to the uniqueness of the profile and pattern distribution of stable isotopes, making them unique for a particular region/location, thus making it possible to create isotopic landscapes known as isoscapes.

For this reason, stable isotopes can be used reliably to provide accurate information about the geographical origin of food products, detection of adulteration of food products, and identification of the difference between organic and conventional farming approaches (Kelly, 2003 ; Kelly and Bateman, 2009 ; Maggi *et al*., 2011). The isotopes that are widely or commonly used include carbon-13 (^{13}C), nitrogen-15 (^{15}N), sulfur-34 (^{34}S), deuterium (^{2}H), and oxygen-18 (^{18}O).

For both plant-based and animal-derived food products, stable isotope ratios and their abundances has been a useful tool in quality assurance, as well as verification of geographical origin of food products. The water chemistry of a particular geographical location is known to be characteristic to that specific area and tends to influence the ratio of oxygen-16 to oxygen-18 isotopes ($^{16}O/^{18}O$), as well as of that of hydrogen to deuterium ($^{1}H/^{2}H$ or H/D) (Maggi *et al*, 2011). On the other hand, the geology, soil chemistry, and the feed are also known to characterize the pattern of isotope ratios of a particular geographical location of carbon-13 to that of carbon-12 ($^{12}C/^{13}C$), strontium-86 to that of strontium-87 ($^{86}Sr/^{87}Sr$), sulfur-32 to that of sulfur-34 ($^{32}S/^{34}S$), and nitrogen-14 to that of nitrogen-15 ($^{14}N/^{15}N$) (Kelly and Bateman, 2009).

The sample preparation for the analyses, which involves ^{13}C and ^{15}N isotopes, normally requires the samples (usually placed in clean tin sample holders) to be converted to high purity CO_2 gas and N_2 gas respectively before their introduction to the gas chromatograph (GC) coupled to isotope ratio mass spectrometer (GC-IRMS) (Kelly, 2003). The high purity gas products are then treated at high temperatures in an environment rich in oxygen (excess O_2), and then an inert gas (helium preferably) is used to purge the combusted gas into combustion catalysts (normally oxides of chromium and copper wire) to affect the oxidation of other unwanted combustion by-products such as sulfur and halogen derivatives. The combustion process results in a number of gases such as carbon dioxide, oxides of nitrogen, and water vapour, and these are catalytically reduced at a high temperature in a process that eliminates oxygen and also reduces oxides of nitrogen to nitrogen gas. Water and carbon dioxide are normally removed from the reaction system using magnesium perchlorate and other selective carbon adsorbents. In order to quantify and qualify the gases of interest, nitrogen and carbon dioxide, separation and detection using a gas chromatograph coupled to IRMS is normally employed. The qualifier ions for carbon dioxide are $m/z = 44$, 45, and 46, while those for nitrogen are $m/z = 28$,

29, and 30 (Maggi *et al*., 2011).

The analyses that involve ^{34}S isotopes, like those for ^{13}C and ^{15}N, require a sample preparation step that produces a high-purity sulfur dioxide (SO_2) before introducing the sample to GC-IRMS. In this analysis, the sample of interest is also placed into clean crucibles/sample holders made of tin and then subjected to high temperatures in a furnace, and the combustion is allowed to proceed in an environment rich in oxygen. The combustion gaseous products are then passed (using an inert helium gas) to the oxides of tungsten and zirconium oxide combustion catalysts and then they are subjected to the reduction process using copper wires. Water in the reaction mixture can be filtered through a water selective Nafion membrane, which allows only water to pass through and the SO_2 is separated and quantified using a gas chromatograph coupled to IRMS (GC-IRMS), where the qualifier ions monitored are m/z = 64, 65, and 66 (Kelly and Bateman, 2009).

For the deuterium (^2H) and ^{18}O isotope analyses, samples of interest have to be converted to high-purity hydrogen (H_2) and carbon monoxide (CO) gases respectively, before analysis using GC-IRMS. The samples are normally placed into clean sample holder/crucibles/capsules made of silver and then subjected to high temperatures in a furnace in which glassy carbon is also introduced in order to convert them into H_2 and CO gases. The resultant water product is removed using magnesium perchlorate, while CO_2 is removed using CO_2 selective adsorbents. The pure H_2 and CO_2 are then analyzed using GC-IRMS. The qualifier masses for H_2 are m/z = 2 and 3, while those of CO_2 are m/z = 28, 29, and 30 (Kelly and Bateman, 2009).

For geographical identification (GI), the mean ratios values, such as Δ^2H‰, $\Delta^{18}O$‰, *etc.* are correlated to the values of the same isotopes found at the latitude of production regions, as well as the parallel relationship to the Meteoric Water Line.

Multi-element Pattern Analysis: Targeted Analysis Methods for Food Provenance: Examples for Honey, Fish, and Meat Products

Where the analyte to be analyzed is predefined and known prior to the analytical work, the method is termed as targeted analysis. There are a number of

analytical instrumentations that may be involved in targeted analysis, such as hyphenated techniques involving chromatography and mass spectrometry (e.g. GC-MS, GC-MS/MS; LC-MS, LC-MS/MS, etc.) and also others such as nuclear magnetic resonance (NMR). Both the test and certified authentic/genuine samples are analyzed and the results compared and where there is any suspected case for non-authenticity or adulteration, it will be revealed by the data from comparison of the two samples (test sample vs. certified sample). For example, in meat industries, there are several ways of binding off-cuts and minced meat, as well as trimmings into meat products with features like that of steak meat. In this process, binders (glues) may be obtained from either blood or other neutral binding agents that have been proved not to impart any foreign color or odor characteristics to the processed meat product. In the case where blood is used as a binding agent, the blood preferred is normally bovine or porcine, such that the fibrin protein (from blood plasma) together with thrombin (enzyme responsible for blood clotting) are mixed with the meat for binding. However, the process is sometimes adulterated by blending undeclared products from different animals in the manufacture of meat products. A similar scenario also occurs in the processing of fish products. This type of fraud may in a large part be intended for economic gain as the binders are normally permitted by law as part of the food additives.

A tandem LC-MS/MS is a tool that may be used to detect such kinds of fraudulence cases. In the analyses, the target samples will be the plasma samples from bovine as well as porcine (Grundy *et al.*, 2013). The analysis is based on the fact that fibrin proteins from different animal species differ in terms of the mass spectral characteristics and this will be evident from the fragmentation pattern obtained using LC-MS/MS for different species. However, proteomics-based LC-MS/MS methods, though useful in the identification of unique proteins preset in food products, may not be suitable as methods for the authenticity test, but are more suitable for highly processed meat where DNA fragments have already been denatured by the process. These methods are thus suitable for the forensic identification of both undeclared and declared components incorporated into food products.

Another sensitive and reliable method for the detection of meat adulteration is the real-time chain polymerase reaction (RT-PCR). This is a DNA-based biochemical technique that analyses DNA fragments extracted from meat samples such as trimmings, cut-offs, or lasagne (Chisholm *et al.*, 2005). This technique can tell with certainty whether there is contamination of meat (e.g. beef) with horse meat, pork, *etc.* Alternatively, this test is also used to detect

adulteration from the incorporation of banned substances in meat, such as hormonal substances or anti-inflammatory agents such as phenylbutazone. RT-PCR techniques can be useful in detecting possible undeclared genetically modified foods or other undeclared mixed food products such as mixed rice (e.g. high-quality rice mixed with low-quality rice) (Fumiere *et al*., 2013), mixed milk (buffalo milk mixed with cow's milk) (López-Calleja *et al*., 2004), and meat that came from some specific breeds (Conyers *et al*., 2012).

In PCR-based methods, a specific sequence of the DNA fragment of interest is amplified and then copied for analysis using a variety of methods such as those based on electrophoretic principles. Species specific based PCR methods are useful in the verification of food products, due to the fact that the specific DNA sequences/fragments that are targeted are specific to that particular species and can easily be detected and identified, even where the food product is contained in a mixed heterogeneous pool of other DNA fragments from various other species. Moreover, PCR methods may also be useful in providing DNA signatures that can be identified in the DNA profile of different plant species where animals graze off the forage fed to other animals or of microbial populations colonizing a particular location (environment). The microbial population in milk for example can be fingerprinted using PCR-based methods.

However, PCR-based methods sometimes suffer from the effects of PCR inhibitors that may be present/added into food products, because the PCR inhibitors tend to suppress the amplification of the target DNA. In some cases, the food processing protocols may result in highly degraded DNA material, making it difficult to amplify and analyze. In these cases, extensive dilution may be desirable in order to minimize or eliminate the effects of PCR inhibitors. The dilution may be applied in cases where there is a large DNA sample size or otherwise very sensitive detection methods have to be employed.

Other methods with potential application in the determination of animal proteins for the purpose of geographical identification of the origin of the species include immunological-based techniques such as the enzyme linked immunosorbent assay (ELISA) (Reaney and Bremer, 2013); classical microscopy based methods (van Raamsdonk *et al*., 2013); as well as near infrared microscopy (NIR) (Fernandez *et al*., 2013). The ELISA is based on the specificity of the antibody antigen reactions. The preference will be to use a combination of all these methods and techniques in order to obtain in-depth information about the sample and more convincing evidence (Bremer *et al*., 2013). Otherwise, another approach, the gelatine method has been considered as confirmatory for

establishing provenance of meat (especially skin and bone parts of the animal) from the feeds consumed by the animals.

Non-targeted Metabolomic Methods for the Determination of Maturation, Expiry, and Shelf Life of Meat Products

In the meat industry, the quality of meat can be enhanced by allowing the meat and meat products to mature, a process that may imply maintaining the ageing process of meat at a particular set of temperatures, for a longer period of time. This creates room for deception, where labeling may fraudulently and intentionally indicate false claims about the ageing or expiry date of that meat product. Therefore, metabolomics methods have been developed and optimized to test and verify the information given in the labeling regarding the ageing of meat and the conditions at which the aging process was carried out. To do this, a non-targeted analysis of the test samples is normally conducted using techniques such as ^{1}H-NMR to establish a profile of metabolites such as amino acids (leucine, phenylalanine, alanine), as well as others such as nicotinamide or nicotinic acid, which normally vary depending on the meat maturity and the physical conditions (e.g. temperature) to which the meat was subjected. The same approach can be suitable for the verification of the expiry date of meat products.

Apart from meat and fish, different types of honey are the other food product that can easily be adulterated. For example, the composition of chestnut honey contains kynurenic acid as a unique and characteristic compound of this product (Donarski *et al.*, 2010). There are unique and characteristic compounds in the composition of honey, which include dihydroxyacetone and methylglyoxal. Different types of honey products are known to have antimicrobial as well as biocidal activity. Fraudulence in the honey industry may be caused by some unscrupulous tendencies to enhance the levels of beneficial components such as methylglyoxal by various means including heating. However, heating also generates other compounds such as hydroxymethylfurfural (HMF), which are unwanted, and the authorities have set threshold values of 40 mg/kg for HMF in unheated honey. Monitoring of compliance to the standards and authenticity can be performed using a number of techniques, including nuclear magnetic resonance (NMR) and high performance liquid chromatography coupled to infrared spectroscopy (HPLC-IR).

Methods for Dairy Products Authentication

Physico-chemical Property Measurements and Chemometrics

There are a number of authenticity tests that may be carried out for dairy-related products such as milk and cheese. Some of these are simple and straightforward and can be done essentially in any laboratory. Physico-chemical methods in the detection of dairy products such as cheese, milk, *etc.* involve the measurements of parameters such as total and soluble nitrogen, pH, moisture, salt content, *etc.* The limitations of this approach can be described by the fact that the analysis involves the measurements of general parameters that result in broad, bulky, and general observations, making it difficult if not impossible for fingerprinting. However, when this analytical approach is done in conjunction with chemometric multivariate statistical techniques, it becomes possible to classify or discriminate the authenticity of dairy products.

Chromatography, Mass Spectrometry and Chemometrics

Chromatographic methods, such as high performance liquid chromatography (HPLC) and gas chromatography (GC), utilizing their traditional detectors such as fluorescence, diode array (for HPLC), and flame ionization detector (GC), have been useful in the determination of dairy products authentication. Both HPLC and GC can also be coupled to a mass spectrometer to strengthen the quality of results obtained, because the use of mass spectrometry can result in unique and characteristic fragmentation patterns for the fingerprinting of the specific components in the dairy products, thus confirming the authenticity. In addition, chemometric tools such as principal component analysis (PCA) and hierarchical cluster analysis (HCA), strengthen further the validation of the discrimination of samples.

The HPLC analysis of dairy products (e.g. cheese, raw milk, and pasteurized milk) normally involves the process of proteolysis in order to break down the proteins into simpler components and amino acids. This process is then followed by the ethanolic extraction of the proteolysis components before the separation and the detection of both the ethanol-soluble and ethanol-insoluble extracts using HPLC coupled to an appropriate detector, including the mass spectrometer. HPLC analysis also involves the detection of amino acids to evaluate the proteolysis process and also because the measure of amino acids determines the ageing of the dairy products. The HPLC method together with chemometric

tools offers the possibility to discriminate dairy products based on ripening stages and how the dairy products have been treated.

The shortcomings of the HPLC method for complex samples such as those of dairy products come from the fact that it certainly requires stringent and extensive sample preparation steps to get rid of hydrophobic undesirable components of the matrix, making the procedure laborious and time-consuming. Moreover, the interpretation of the amino acid profiles from the results obtained from the HPLC analysis is complex and requires a competent chromatographer to understand them. Therefore, incorporation of statistical multivariate tools to discriminate the observations is always crucial.

Another chromatographic method that can be useful for the authentication of dairy products is head space gas chromatography that can also be coupled to a mass spectrometer. This technique is mostly applicable to the volatile fraction of the samples, and as far as dairy products are concerned, terpenes are the components of interest, which are volatile enough to be suitable for GC analysis. The profiles of terpenes can differentiate between the grazing and non-grazing feeding system of the animals from which the milk was obtained. This is because the composition of plants used for feeding the animals (cows) has a significant influence on the profiles and composition of terpenes, which will then be reflected in the milk and other milk-derived dairy products such as cheese. Thus, the profiles of terpenes can be a useful tool for the fingerprinting of dairy products.

Advanced Spectroscopic Methods: Vibrational Spectroscopy

Many food products contain components that can interact with the electromagnetic radiation (EMR) and display unique and characteristic spectral data to reveal important information about the functional groups and molecular bonds present in the food products. Therefore, the use of spectroscopic techniques for fingerprinting of food products has become attractive, particularly because these methods do not need stringent sample preparation steps. Spectroscopic methods are also known to be non-destructive and capable of recording data from even small/tiny particles (nano-gram to pico-gram ranges). These attributes are essential in the food forensic analyses for authenticity and verification purposes.

Among the spectroscopic techniques that are generally used in the analysis of

food products are ultra violet-visible (UV-Vis) spectroscopy, fluorescence spectroscopy, tetrahertz spectroscopy (THz), infrared (IR) spectroscopy, Raman spectroscopy, Mössbauer spectroscopy, and nuclear magnetic resonance (NMR). Spectroscopic methods are based on interactions between electromagnetic radiation (EMR) and sample to generate scientific information (data) about the components of a particular food product. The information or data obtained from spectroscopic studies are mainly about the functional groups, molecular composition, the types of chemical bonds present, structure, *etc.*

There are 17 regions/bands in the EMR spectrum where matter can interact with light to give a display of different types of spectral patterns and behavior (Belton, 1997). Some of the EMR regions, for instance the very high frequency band of gamma rays and hard X-rays that are capable of exciting the nuclear transitions, may not be of importance in food analysis, as the transitions result in very little information about the chemical environments of the sample. However, these bands can be used to reveal information regarding nuclear states of the atoms in the food sample and can thus be suitable for elemental analysis. The lower frequency soft X-rays and high frequency ultraviolet radiation can be used to excite transitions of the cores of the electrons when they are directed toward the sample (food product). However, these bands are also insensitive to the chemical environment of the food product (sample), resulting in information mainly about elemental composition and thus not of much help in verification or authentication of food products. On the other hand, the lower energy ultraviolet radiation is known to be sensitive to the chemical environment and may thus be of use in the analysis of food products (Belton, 1997). The sensitivity of lower UV radiation to the chemical environment of the samples may be attributed to the fact that the energy of the band able to excite transitions of the valence electrons normally takes part in chemical bonding. The problems associated with this band (lower UV) comes from the fact that the transitions are normally broad, thus affecting negatively the magnitude of chemical sensitivity.

Among the most important EMR regions, as far as food forensic analysis is concerned, is the near infrared (NIR), because in this region the molecular transitions take place through the overtones and combinations of chemical bond vibrations and not electronic transitions that are excited (Belton, 1997). This gives a reason for it to be used in food analyses and forensic investigations of food products. Another useful region of EMR for food analyses is the mid-infrared (MIR), which like NIR experiences good sensitivity to chemical environments due to molecular vibrations occurring when the radiation is directed to the food samples (Belton, 1997). The spectral data obtained from this

MIR-EMR region provide valuable information regarding the molecular composition of the food product, as well as the physical properties of the absorbing species present in that food product (Belton, 1997). Just as in other infrared methods, mid-infrared spectroscopy is to the transition of the vibrations of molecular chemical bonds. As described above, some molecules, especially those that are symmetrical like O=O, C=C, or N=N, are not IR active due to the relative high photon energy environments in IR instruments. This makes it necessary to use Fourier transform techniques as well as efficient sample preparation methods. This approach is useful in the analysis of food samples, especially those that are opaque and also those that would otherwise require stringent sample preparation methods (Belton, 1997).

Vibrational spectroscopy can be useful in the analyses of food components as an analytical technique, especially when it is in combination with chemometric multivariate tools (e.g. principal component analysis, etc.), in which case the method (vibrational spectroscopy) can offer unparalled results. These methods (vibrational spectroscopic methods) can also be suitable for conformational studies to discriminate food products on the basis of protein structure differences or polysaccharide patterns.

Other bands, such as the far-infrared (FIR) and microwave, are normally useful in providing information on low frequency vibrations and the rotational motion of samples that are subjected to EMR magnitudes falling in these regions. In addition to this, microwave is the region that is associated with electron paramagnetic resonance (EPR), where transitions related to the magnetic states of electrons take place. These regions may not be particularly useful in food forensics, but may be of use in analyses involving transition metals and also synthetic and free radicals.

Generally, infrared spectroscopy and Raman spectroscopy have many features in common, such as microspectroscopic capabilities (they all can analyze samples that are in the nano-or pico-gram range), and they are all non-destructive, *etc.* Despite this similarity, IR and Raman have limitations as well as attractive features that make an analyst prefer one over the other, depending on the application. For instance, when the sample to be analyzed is hydrated or contains moisture (water), water is always a nuisance in IR measurements taken in the mid-infrared regions, then Raman will be preferred for the analysis of this sample rather than IR. Again, Raman spectroscopy is based on the molecular polarizability of the molecule, while IR is based on the dipole moment of the molecules, in which case only molecules with dipole moments such as C=O,

O─H, *etc.* will be IR active and not the homopolar molecules such as O═O, C ═C, N═N, etc., which are very active on Raman.

The radio frequency region of the EMR is another useful band for food forensic scientists, as it displays good chemical and physical sensitivity to the samples of food products. This region is the one where experiments on the nuclear magnetic resonance (NMR) are carried out. The usefulness of this region (radio frequency) to food analysis is due to the fact that the band width of the nuclear transitions obtained are not broad but narrower, if compared to the wavelength used in the excitation process.

Another vibrational spectroscopic technique that may have applications in food forensics is Mössbauer spectroscopy which, like NMR spectroscopy, is associated with good sensitivity to the chemical and physical environment (food samples), because the change in frequency is normally very small when compared to the frequency originating from the exciting radiation.

From the facts narrated above, it is clear that only a few regions of EMR will be useful to the food forensic scientist or those regions that may actually be considered as tools that can be instrumental in the identification, verification, authentication, interrogation, or detection of the materials presented as evidence in food forensic related cases. Actually, the spectroscopic techniques that have been widely reported include infrared (NIR and MIR), Raman spectroscopy, NMR, and tetrahertz (THz) spectroscopy.

However, the main challenge when using vibrational spectroscopic methods in food forensic analyses is the complexity of food items originating from various sources (either plant or animal origins). This will result in complex spectra that will challenge the analyst to assign the functional groups correctly. However, when the analysis is involving the use of NMR, the challenge may be eliminated because NMR is capable of filtering the data/information such that signals for individual nuclei are recorded at the same time (Belton, 1997). Moreover, if other nuclei (apart from carbon-13 or protons) are chosen, the observed spectra generated from the NMR are always simple, making it easier for interpretation. For example, if a phosphorus-31 nucleus is chosen for NMR analysis of food samples, then the NMR will only select signals generated by phosphorus-containing species, making it possible to identify inorganic phosphorus species, the organic phosphorus (in forms of esters, phosphorus bound to serine, etc.) due to the simplicity of the spectrum.

Choosing the appropriate nucleus in NMR analysis is possible because NMR

spectroscopy is not elemental but rather an isotopic-based technique, therefore the method can be more effective by performing either isotope substitution in the case for those low natural abundant isotopes or replacing a resonant nucleus with a non-resonant nucleus for the purpose of enhancing the signal (Belton, 1997). For example, if the food sample contains water that seems to mask the signal, water ($^{1}H_2O$) can be replaced with heavy water ($^{2}H_2O$) and because deuterium (^{2}H) displays less intense resonance, the resonance due to nuclei in food samples will be clearly observed. Alternatively, in food forensic analysis, it may be advantageous to incorporate isotopic nuclei that are rare in the system to be analyzed, as this can ensure isotopic selectivity in the NMR signal selection process (Belton, 1997).

In all these instances, the sample state that is analyzed by NMR is assumed to be in solutions and that isotopic nuclei are considered for selection. However, the application of NMR is not just limited to samples in solutions where only highly mobile nuclei molecular species will generate signals in a limited range of motional states or to only provide information for isotopic signals in a sample. NMR also provides information for samples that exist in solid states (not in solutions). However, solid samples have wider line-widths, such that it is impossible to visualize the signals using ordinary or even high resolution NMR machines. The analysis of a solid sample is normally carried out using a special NMR technique known as cross-polarization magic angle spinning (CP-MAS). In CP-MAS experiments, only those nuclei that have long motional correlation times are used to provide the signal for the sample. This technique, as the name suggests, involves the transfer of polarization from nuclei with high ratios of magnetic dipole moment (magnetogyric nuclei) to ones with low magnetogyric ratios. An example of cross-polarization can be a transfer from a proton to carbon nuclei. This technique (CP-MAS) can therefore be applied to food samples such as flours (wheat, corn), *etc.* to discriminate between proteins and lipids.

Sampling Approaches for Vibrational Spectroscopy Techniques

From the analytical chemistry point of view, sampling and sample preparation is the most important step in the analytical procedure and normally is the step that determines parameters such as detection limit, precision, accuracy, *etc.* Sample preparation steps are meant to exclude interfering molecules and other non-target

species, thus leaving the analyte of interest in its pure form and also pre-concentrate or convert the analyte of interest into the form that is compatible with the requirements of the analytical instrument.

In food forensic related analyses, where samples are presented to provide evidence in a court of law for a fraudulence case, it is imperative that appropriate, robust, selective, and sensitive sampling and sample preparation techniques be employed. These sampling and sample preparation methods need to be capable of on-site use, must be versatile and applicable to a wide range of analyte forms, and in different geographical locations. Some sample preparation protocols are applicable to bulky sample size, others macro-and micro-sample size. Some of the vibrational spectroscopic techniques such as Raman spectroscopy (an emission/scattering process) are not complicated in the sense that they require minimal sample preparation when compared to infrared, which has extensive sample preparation procedures.

Sampling methods for Raman measurements may be grouped into three main categories, which include remote sampling, also known as a non-invasive method, which is done through a fibre-optic probe. Another involves the integrated Raman and optical spectroscopy, while the final sampling method is known as back-scattering, based on using either 80-or 90-degree sampling geometry.

Phenols, Polyphenols and Phenolic Flavonoids

Fruits and vegetables are known to be rich in phenols and flavonoids, which play an important role as antioxidants and radical scavengers. In the plant kingdom, there are numerous classes of phenols and flavonoids, with many containing one or more aromatic systems having hydroxyl functionalities attached to their rings and side chains. Among the most abundant phenolic antioxidants, which also give a color characteristic to some plants and fruits, are the anthocyanins. These anthocyanins are also regarded as a measure of quality of juices obtained from fruits or vegetables. There are several anthocyanins that are used to ascertain the geographical origin or authenticity of fruit juices and fruit nectars. Among these are the aglycone derivatives of anthocyanins known as anthocyanidins. Other anthocyanins that are normally used for this purpose include the hydroxycinnamic acids, ferulic acids, sinapic acids, and p-coumaric acids. Other antioxidants that are also analyzed for geographical identification and authenticity include vitamins (A, C, E, and beta-carotene), as well as selenium (an inorganic element) and caffeine.

Nearly all these components of the phenolic antioxidants and vitamins can be analyzed using high performance liquid chromatography with a UV-Vis-DAD detector. This is because the compounds contain aromatic systems with some being conjugated, thus containing chromophores giving them the ability to absorb UV-Vis radiation.

Organic Acids in Fruit Juice and Fruit Nectar as Indicators for Geographical Origin

Organic acids such as quinic acid, fumaric acid, tartatic acid, malic acid, *etc.* form part of the natural composition of fruits. The abundances and ratios of these organic acids are normally different for each fruit and very characteristic to different fruit species and the juices processed from them. The relative concentration of these naturally occurring acids in fruits is highly dependent on the species or variety, degree of ripeness, and season. For example, tartatic acid is found in abundance in grapes, thus characteristic to grape juice and therefore regarded as an indicator and a test confirmation for grape juice authenticity. Similarly, in cases where adulteration involving dilution or spiking of a higher-quality and expensive juice using grape juice is suspected, the authenticity testing will target the detection of tartaric acid.

Malic and quinic acids are the other organic acids found naturally in fruits, which are representative and characteristic to apples and apple juice. In cases of adulterations involving apple juice, measurements and detection of these two organic acids is always performed, as their concentrations will be different from the normal or natural levels.

The abundances and ratios of quinic/citric acids, quinic/malic acids, as well as citric/malic acids is characteristic to cranberry and the juice processed from this fruit. Where authenticity is questioned, the measurements are performed for these acids.

In the case of orange juice, the representative characteristic organic acids are citric and isocitric acids. The abundance and ratio of citric/isocitric is normally used to test for authenticity and the measure gives an indication of adulteration. The concentration of isocitric acid in oranges is normally very low but constant. The ratio of citric/isocitric acid greater than 130 is normally taken as an indicator of adulteration resulting from dilution involving citric acid. In other words, isocitric is the actual indicator for adulteration.

Adulteration of pomegranate juice can be indicated by the test involving

measurements of quinic and tartaric acids.

Tables 2.1a and 2.1b summarize the major organic acids found in fruits and vegetables, of which a test of authenticity may be carried out for verification.

Table 2.1a Major natural organic acids found in fruits.

Source: http://www.hawkinswatts.com/documents/Natural%20Acids%20of%20Fruits%20and%20Vegetables.pd accessed 20 September, 2014.

	Abundant organic acid	**Fruit**	**Notes**
1	D-malic acid L-malic acid malic acid	apple, passion fruit, peach, nectarine, pear, cherry, grape, lemon, orange, banana, apricot, pineapple, cranberry	• Malic acid is found in minor quantities in figs, plums, strawberries and lemons • Malic and citric acids are found as major organic acids in many fruits • Some fruits contain both malic and citric acids as major organic acid
2	citric acid	fig, grapefruit, blueberry, kiwifruit, strawberry	• Citric acid is found in minor quantities in apples, cherries, grapes, peaches, pears, tangerines
3	tartaric acid	avocado	• Tartaric acid is found as major organic acid in very few fruits

			• Found in minor quantities in bananas, lemons and pears
4	isocitric acid	blackberry	• Isocitric acid is found as major organic acid in very few fruits • Found in minor quantities in oranges • Completely missing in lemons (thus a point to differentiate between oranges and lemons)
Other organic acids that are found in fruits in minor quantities			
	pyruvic acid fumaric acid lactic acid ketoglutaric acid	apple	• These organic acids are found in minor quantities, mostly in apples. This can be of high importance
	quinic acid	Apples, cherries, blackberries	

	succinic acid	Apples, cherries, strawberries	
	lactoisocitric acid	Blackberries	• This organic acid is found in minor quantities, mainly in this fruit
	glycolic acid glyceric acid	Cherries, strawberries	• These two organic acids are found mainly in these two fruits in minor quantities
	shikimic acid	Strawberries, blackberries, cherries	
	benzoic acid	Cranberries	• This organic acid is found in minor quantities, mainly in cranberries
	acetic acid	Figs	• This organic acid is found in minor quantities, mainly in figs
	aspartic acid	Strawberries	• This organic acid is found in minor quantities, mainly in strawberries

			quantities, mainly in strawberries
	HO, OH, O, O oxalic acid	Grapefruits, grapes, pears, plums, oranges, limes, lemons	• This organic acid is found in minor quantities in many different types of fruits
			• Apples, cherries and strawberries contain many different types of organic acids that are present in minor quantities • Blueberries and kiwifruits contain mainly citric acid as major organic acid and almost nothing of other organic acids that are in minor quantities

Table 2.1b Major natural organic acids found in vegetables.

Source: http://www.hawkinswatts.com/documents/Natural%20Acids%20of%20Fruits%20and%20Vegetables.pd accessed 20 September, 2014.

	Organic acid	Vegetable	Notes
1	D-malic acid L-malic acid malic acid	peas broccoli	• Malic and citric acid are found in abundance in many beans, carrots, potatoes and tomatoes; although in broccoli, citric

			and tomatoes, although in broccoli, citric acid is found in minor quantities
2	citric acid	beans carrots potatoes tomatoes	• In peas, only malic acid is found in abundance and no citric acid
3	isocitric acid	carrots	Isocitric acid is a characteristic organic acid in carrots
4	lactarimic acid	mushrooms	Lactarimic acid is found exclusively in mushrooms as the dominant organic acid
Other organic acids that are found in vegetables minor quantities			
	succinic acid	Beans, broccoli, tomatoes	
	fumaric acid	Beans, carrots, mushrooms, tomatoes	Found in mushrooms in minor quantities

	oxalic acid	Broccoli, potatoes	
	allantoic acid ketostearic acid	Mushrooms	• Minor quantities of these organic acids are found exclusively in mushrooms • These minor acids, together with lactarimic acid, are characteristic to mushrooms
	phosphoric acid	Potatoes, tomatoes	This organic acid is mostly found in potatoes and tomatoes
	pyroglutamic acid	Potatoes	This organic acid is mostly found in potatoes

The analytical methods of choice for organic acids include RP-HPLC or ion exchange HPLC equipped with UV-Vis-DAD, where derivatization of organic sugars may be desirable. Other detectors that are used with no need for derivatization are refractive index (RI), electrochemical detectors, conductimetric detectors, and mass spectrometers.

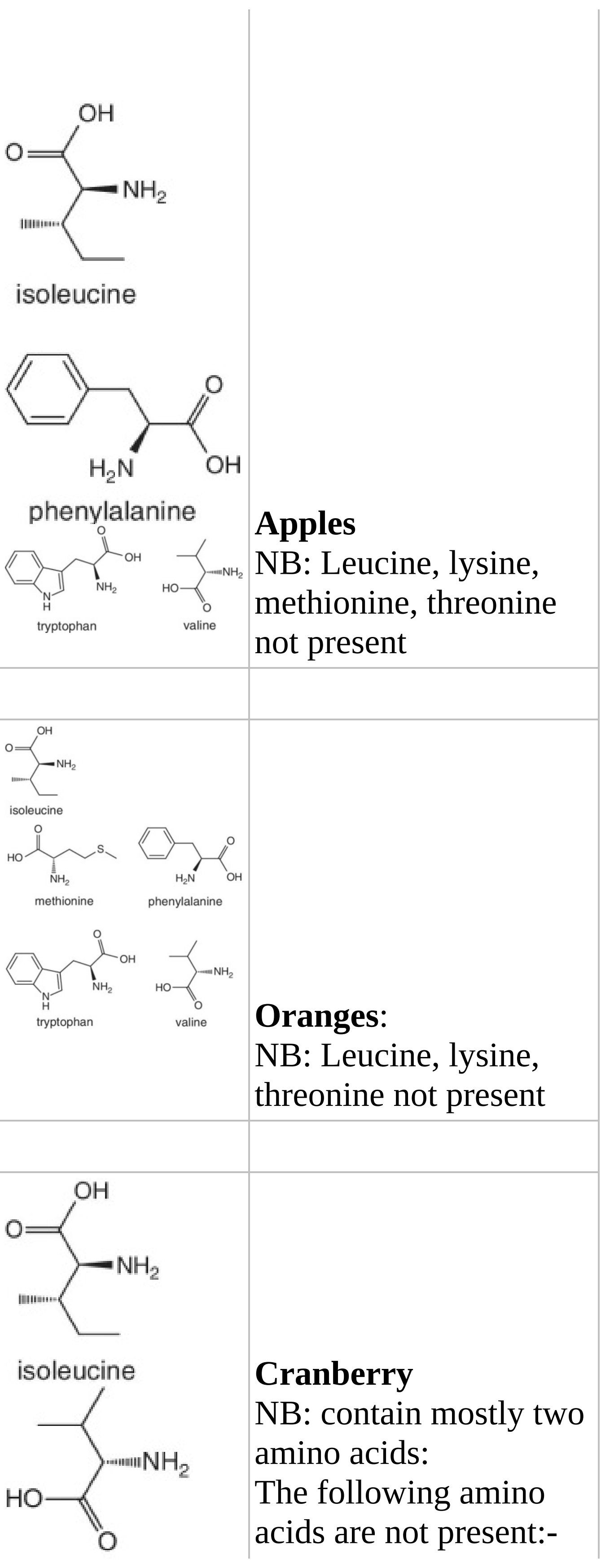
Amino Acids

Unlike foods derived from animal origin, plant-based foods such as fruits lack all the eight essential amino acids, though they are present in trace amounts. The presence of an appreciable amount of essential amino acids will indicate adulteration. Moreover, the distribution of essential amino acids differs in various fruits (as shown in Table 2.2).

Table 2.2 Essential amino acid profiles in fruits.

Essential amino acid	Fruits	Notes
isoleucine, leucine, lysine, methionine, phenylalanine		

<table>
<tr><td>threonine tryptophan valine</td><td>Bananas, apricots, peaches, avocados, persimmons</td><td rowspan="7">Trends of Isoleucine abundance in fruits are in the following order:- Bananas > apricots > peaches > avocados > persimmons > apricots > dates > kiwi > apples > oranges > cranberries > blueberries > plantains
Trends of Leucine abundance in fruits is in the following order:- Bananas > apricots > peaches > guavas > avocados > figs >persimmons > raisins > pears > dates > apples > kiwi > olives > blueberries
Trends of Lysine abundance in fruits are in the following order:- Amaranth > apricots > bananas > tamarinds > avocados > peaches > guavas > dates > oranges > pears > plantains > plums > watermelon
Trends of Methionine abundance in fruits are in the following order:- Peaches > avocados > figs > oranges > kiwi > pears > grapes > raisins > apricots > plantains > guavas > plums > blueberries > cantaloupe > ripe olives > persimmons
Trends of Phenylalanine abundance in fruits is in the following order:- Avocados > apricots > bananas > raisins > peaches > plums > figs > persimmons > oranges > dates > pears > grapefruit > elderberries > apples > star fruit > ripe olives
Trends of Threonine abundance in fruits are in the following order:- Peaches > apricots > bananas > guavas > figs > avocados > raisins > pears > dates</td></tr>
<tr><td>isoleucine leucine methionine phenylalanine tryptophan valine</td><td>Persimmons
NB: Lysine and threonine not present</td></tr>
<tr><td>isoleucine leucine lysine phenylalanine threonine tryptophan valine</td><td>Dates
NB: Methionine not present</td></tr>
<tr><td></td><td></td></tr>
<tr><td>isoleucine leucine methionine tryptophan valine</td><td>Kiwi
NB: Lysine, phenylalanine, threonine not present</td></tr>
<tr><td></td><td></td></tr>
<tr><td></td><td></td></tr>
</table>

	**Apples** NB: Leucine, lysine, methionine, threonine not present	Trends of **Tryptophan** abundance in fruits is in the following order:- Apricots > raisins > avocados > apples > plums > persimmons > guavas > figs > kiwi > dates > oranges > peaches Trends of **Valine** abundance in fruits is in the following order:- Bananas > peaches > figs > apricots > avocado > guavas > raisins > dates > pears > apples > persimmons > kiwi > cranberries > ripe olives > blueberries > oranges
	Oranges: NB: Leucine, lysine, threonine not present	
	Cranberry NB: contain mostly two amino acids: The following amino acids are not present:-	

valine	Leucine, lysine, methionine, phenylalanine, threonine, tryptophan	
isoleucine leucine methionine valine	**Blueberries** Missing the following amino acids: Lysine, phenylalanine, threonine, tryptophan	
leucine methionine phenylalanine threonine tryptophan valine	**Raisins and Figs:** NB: Isoleucine and lysine not present	
isoleucine lysine methionine	**Plantains:** NB: Missing leucine	

	NB: Missing leucine, phenylalanine, threonine, tryptophan, valine	
leucine lysine methionine phenylalanine threonine valine	**Pears** NB: Missing isoleucine, tryptophan	
leucine methionine phenylalanine valine	**Olives** NB: Missing isoleucine, lysine, threonine, tryptophan	
leucine lysine methionine threonine tryptophan valine	**Guavas** NB: Missing isoleucine, phenylalanine	

lysine, methionine, phenylalanine, tryptophan	**Plums** NB: Isoleucine, leucine, threonine, valine not present	
lysine	**Watermelon** NB: Mostly lysine present	
phenylalanine	**Elderberries and grapefruit** NB: Mostly phenylalanine present	
methionine	**Grapes** NB: Mostly methionine present	

Authentication of Alcoholic Beverages

An alcoholic product or alcoholic beverage refers to a drink containing ethanol content and includes wines, beers, and spirits. With regards to alcoholic beverages, normally the parameters that define the product are highly controlled to give unique properties in terms of alcoholic strength (ethanol concentration), color, flavor, odor, *etc.* These characteristics define a genuine product that may in many instances come from one manufacturer/industry/company. Any counterfeit/adulterated product will in most cases fail to meet these qualities, as they involve highly specific raw materials, fermenting agents, and production processes/procedures.

Generally, industries and brewing companies that produce alcoholic beverages use specific sources of products for fermentation. For example, some industries may prefer cereals such as corn, barley, etc.; others may use fruits such as grapes and others molasses, *etc.* Each of these sources will certainly result in specific fermentation products that will impart characteristic flavor, color, type, and strength of alcohol as well as alcohol ratios and compositions.

The specificity of various alcoholic beverages opens a way to detect adulteration and verification of authenticity. For example, in cases where a measure of methanol is lower than normal or where ethanol is higher than the expected concentration, adulteration may be suspected. The presence of methanol in an abnormal concentration from what is expected may be suspected to be due to the use of inferior or neutral raw materials. On the other hand, higher ethanol concentration than what may be expected may well be an indicator that the raw materials used are different and/or not genuine, something that may cause health problems to consumers. Moreover, some industries do allow the incorporation of additives such as specific sweeteners, colorants, or flavorings, while some do not. The various types of additives, amounts, ratios, *etc.* that are allowed by some industries, where they are added in the processing or in the case of those that do not allow the incorporation of additives, all of these scenarios provide ways to scientifically identify cases of adulteration or lack of authenticity. The test will certainly involve the analyses of test samples suspected of containing adulterations to the certified genuine (authentic) samples, where the discrepancy in terms of chemical composition will prove the presence and type of adulteration.

Instrumental analysis may also give evidence of adulteration. For example, if the counterfeit or test sample displays a strange UV/visible spectral pattern, it may be an indication of adulteration, which may be due to the presence of some foreign components in the product. Instrumental analysis may also give a

comparison in terms of composition (quality and quantity) of the test sample and the certified genuine product. Any discrepancy will point to the possibility of adulteration.

For example, many whiskies and whisky brands display a very specific profile of chemical composition (fingerprint) and therefore this makes it possible and easy to link to geographical locations of production as well as give a test for authenticity of the product where adulteration is suspected. Other alcoholic beverages, such as vodka brands produced from various geographical locations, display similar characteristics in terms of their chemical profiles and composition (with minor differences). The case for vodka brands may be attributed to the nature and pattern of the processes involved in production rather than geographical factors. Nevertheless, the composition of all alcohol brands are well documented, such that to identify cases of adulterations scientifically is possible through comparisons with certified genuine products.

The methods and techniques used for the verification of authenticity of alcoholic beverages and their respective brands involve the analysis of volatile components of both the test/counterfeit samples and comparison with a measure found in the certified genuine sample. The methods of choice include the use of gas chromatography, UV/visible, pyrolysis mass spectrometry, ^{2}H and ^{18}O stable isotopic analysis, carbon isotope ratio (for the detection of the addition of neutral alcohols), infrared, and near infrared spectroscopy, as well as copper and other metals.

Chemometric Tools and Methods for Food Authentication and Fingerprinting

In the discussion above, we saw that the authenticity of food samples needs to be ascertained using different types of analytical instruments. The measurement of food samples generates large volumes of data that need to be worked out in order to decode the information contained in it. To be able to work out data in a professional way, a number of chemometric tools and methods have been designed to manipulate the data by employing multivariate analysis of these data and also chemometrics takes care of the aspects of experimental design. In other words, chemometrics deals with both “how to obtain information (with the aid of some validated mathematical model concepts) from and about the sample” and also “how to generate the experimental data (which takes into account the noise as well),” and the results are normally presented graphically for a better reflection of the information. The limitations of mathematical models that are

reflection of the information. The limitations of mathematical models that are designed for chemometric applications are that they are approximations, which are useful within the specified intervals, thus falling short of narrating the details of the chemical environment where the system of the experiment is centred. Moreover, chemometrics is only valid in cases where the experimental design ensures that the variables are treated in the same way as in a simultaneous mode, in the sense that it has to be in a MULTIvariate style and not UNI-variate, otherwise the in-depth information about the sample will not be uncovered.

The chemometric experimental design optimizes the system and is vital, as it minimizes the number of the planned experiments (e.g. optimization experiments) and on the other hand, it ensures the acquiring of maximum information from and about the sample. The experimental design also creates the possibility to investigate how various factors interact, what kinds of responses are obtained, or what kinds of experimental domains are involved. By definition, “a factor” refers to those variables (quantitative or qualitative) normally plotted in the abscissa, *x*-axis of the graph, and varied at known intervals depending on the experimental design for the intended information. The measurement regime of factors is continuous or taken in a discrete fashion, differentiated between qualitative and quantitative factors, with the former known as discrete measurements and the latter as continuous. Examples of qualitative factors include the type of solvents used as eluting phases in chromatography, *etc.* and examples of quantitative factors include concentration, temperature, pH, *etc.* On the other hand, responses as far as chemometrics are concerned refer to the observations (or the results) obtained from a particular experiment.

In chemometrics, the data involved may be categorized in three main ways, such that it may either be one way, two way, or three way. The way in which data is presented determines the choice of chemometric technique to be used for data treatment. For instance, if the spirulina tablet (food supplement) is suspected to contain microcystins (toxins) and the analysis of microcystin is conducted using HPLC-DAD at 238 nm to give an absorbance unit magnitude of 2.74 mAU, this will constitute data containing just a single figure. However, if the absorbance measurements are considered together with other measurements, such as the retention time of microcystins at a fixed wavelength of 238 nm, then this will give a one-way set of data measurement. If the absorbance measurements are generated at a range of wavelengths, for example 238–254 nm collected at an interval of 1 nm giving 17 absorbance measurements, this will be a two-way kind of data (absorbance + retention time). In the case where the number of samples is taken into account, then it will give a three-way kind of data

(absorbance + retention time + number of samples with a known run time, *e.g.* 15 minutes and the spectrum being collected at a particular frequency, *e.g.* one spectrum per second).

This automatically points to the fact that the minimum condition for the multivariate chemometric technique to be considered is where the data has more than one dimension. Moreover, even where the data exist in more than one dimension, the choice of multivariate methods still depends on a number of factors. For example, principal component analysis (PCA) or partial least squares regression (PLS) can only be considered where a first-order data set of a number of samples is tabulated. In cases where multi-way sets of data are tabulated in two or more categories of variables, then other multivariate chemometric methods may be adopted, and include n-way PLS, Parallel Factor Analysis (PARAFAC), *etc.*

When the analysis of the sample/samples has been performed, the results obtained (observations) can be employed to evaluate linear/quadratic/polynomial approximations of the regression model that relates the factors (plotted as *x*-axis in the graph) to the respective responses (plotted as *y*-axis in the graph) representing the following mathematical expression:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + E \quad (2.3)$$

where β_0 = intercept; β_1 = regression coefficient of the first factor (x_1); β_2 = regression coefficient of the second factor (x_2); β_{12} = regression coefficient of the interaction term between x_1 and x_2; and E denotes the error/residual.

In cases where the experiment is conducted for screening purposes, a linear model may be appropriate and where the experiment is meant for optimization purposes, then a quadratic model can be adopted, and cases that involve several dependent factors or responses in an experimental set, are best suited for a PLS model.

Conclusions

The need for proving provenance or fingerprinting of food products and ensuring the authenticity of food products is of paramount importance. This is due to a number of fraud cases that have involved food products. In general, consumers have been at risk due to fraudulence of food products. A good report is that food forensics has been able to develop methods to verify the authenticity of nearly

all food products with great accuracy, such that culprits can easily be caught when and where they transgress the regulations and guidelines.

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3
Food Forensics Cases Related to Food Allergenomic Sabotage and Food Intolerance: Allergens and Allergenicity of Processed Foods

There have been numerous cases related to deliberate food sabotage, where various innocuous substances, which are highly offensive and have even caused fatalities, have been purposely added to foods. There have also been cases of fraudulent substitution of ingredients with allergy-causing ingredients along the supply chain. The presence of these unscrupulous substances in foods has caused serious detriment to public health and tampered with the quality of life of the victims who suffer from food allergies and put them at the risk of anaphylaxis (a serious and fatal allergic reaction that is rapid in onset), apart from causing heavy economic and management burdens to food industries. Generally, scientific studies have suggested that food allergies affect between 2 and 4% of the adult population and 6 and 8% of children. When the associated food allergy has been diagnosed, victims are then advised to refrain from consuming the food that contains that particular allergen. And for preventive measures, labeling legislation regarding the presence of all ingredients for each food product or item has been enforced, which all food manufacturers, producers and distributors must comply and adhere to.

From the molecular biology and biochemistry point of view, a food allergy refers to an immune system-mediated adverse reaction to food proteins. There are two main classes of food allergens, class 1 and class 2. Food allergens that may be found in foods in class 1 include those that are present in peanuts, egg white and cow’s milk. They are known to be both heat-and acid-stable glycoproteins that have a tendency to induce allergic stimulation through the gastrointestinal tract to induce systemic reactions. Class 2 food allergens are mainly proteins in nature and they develop as a result of respiratory sensitization. Like class 1, they are very heat-labile, but only responsible for induction of reactions that are limited to oral types of allergy symptoms.

Food Allergy and Food Intolerance, What is the Difference?

In many instances, food allergy is confused with food intolerance, but the two are not the same. The following examples can help explain the difference.

Milk Sugar (Lactose) Intolerance

Lactose is a milk sugar which is digested by an enzyme known as lactase, which is found in the gut lining. When this enzyme is in short supply, lactose will be digested by the microbial community present in the gut and the result of the action of these bacteria on lactose produces gases which cause symptoms of bloating, abdominal pain and sometimes diarrhea. This is not an allergy but rather food/lactose intolerance.

Food Intolerance

Certain types of food additives are associated with some types of food hyper reactions, which are actually not a food allergy. These hyper reactions are termed as food intolerance and they refer to adverse hyper reactions to a food or food component by certain individuals, but they do not involve the body's immune system.

The ingredients listed in Table 3.1 must be listed on the product label to notify individuals who may be sensitive to them.

Table 3.1 Food ingredients and food allergy or food intolerance.

Ingredient	Use	Allergy	Intolerance	Reference
Food additives	Maintain product consistency; improve or maintain nutritional value; maintain palatability and wholesomeness; provide leavening; control acidity and alkalinity; enhance flavor; impart desired color		Cause food intolerance	Taylor *et al.*, 2000
D&C Yellow No. 5	Coloring agent for beverages, candy	Causes hives		Murdoch *et al.*, 1987

(tartrazine) FD&C Yellow No. 5 (tartrazine). Provokes asthma attacks (?); individuals who react to aspirin have a cross-sensitivity to FD&C Yellow No. 5 (tartrazine) (?)	and other foods			Stevenson *et al.*, 1986
Monosodium glutamate (MSG)	Flavor enhancer	Thought to cause or exacerbate asthma, but scientific results have disapproved the claim. But may cause mild and short-lived transitory symptoms when large quantities of MSG have been consumed. Large consumption of MSG may also cause flushing, sensations		Woessner *et al.*, 2000;Woods *et al.*, 1998

		sensations of warmth, headache, chest discomfort		
Sulfites/Sulfur dioxide	Preserve food color in dried fruits such as raisins, dried apricots and prunes and vegetables; also inhibit microbial growth in fermented beverages such as wine and beer	May cause some people to develop shortness of breath or fatal shock shortly after consuming foodstuffs or beverages that contain it Can provoke severe asthma attacks in individuals who are especially sulfite-sensitive asthmatics		Simon, 1989Bush *et al*., 1986
Soya	Often found in bean curd, edamame beans, miso paste, textured soya protein, soya flour or tofu; soya is a staple ingredient in oriental food. It can also be found in desserts, ice cream, meat products, sauces and			

	vegetarian products			
Leguminous peanuts (e.g. groundnuts)	Used as ingredients in biscuits, cakes, curries, desserts, sauces (such as satay sauce), as well as in groundnut oil and peanut flour			
Nuts	Unlike peanuts (which are types of legumes and grow underground) nuts used here refer to the ones that grow on trees, such as cashew nuts, almonds and hazelnuts. Used in breads, biscuits, crackers, desserts, nut powders, in Asian curries, stir-fried dishes, ice cream, marzipan (almond paste), nut oils and sauces			
Sesame seeds	These seeds can often be found in bread (e.g. sprinkled on hamburger buns), breadsticks, houmous, sesame oil and tahini. They are sometimes toasted and used in			

	salads			
Molluscs (e.g. mussels, land snails, squid and whelks)	Found in oyster sauce or as an ingredient in fish stews			
Lupin	Used in some types of bread, pastries and even in pasta. Found in flour			
Mustard (e.g. liquid mustard, mustard powder and mustard seeds)	Found in breads, curries, marinades, meat products, salad dressings, sauces and soups			
Milk	Common ingredient in butter, cheese, cream, milk powders and yoghurt. Also found in foods brushed or glazed with milk, and in powdered soups and sauces			
Eggs	Ingredient in cakes, some meat products, mayonnaise, mousses, pasta, quiche, sauces and pastries or foods brushed or glazed with egg			
Cereals (wheat, rye, barley) containing gluten Wheat (such as			Gluten intolerance is associated with **celiac disease**, also	

Wheat (such as spelt and Khorasan wheat/Kamut), rye, barley and oats are often found in foods containing flour, such as some types of baking powder, batter, breadcrumbs, bread, cakes, couscous, meat products, pasta, pastry, sauces, soups and fried foods which are dusted with flour			**disease,** also called gluten-sensitive enteropathy. This disease develops when the immune system responds abnormally to gluten. This abnormal response does not involve the IgE antibody and is not considered a food allergy	
Crustaceans (e.g. crabs, lobster, prawns and scampi. Shrimp paste)	Used in Thai and Southeast Asian curries or salads			
Fish	Made in various designs such as fish sauces, pizzas, relishes, salad dressings, stock cubes and Worcestershire sauce **Histamine toxicity** Fish, such as tuna and mackerel that are not refrigerated			

	properly and become contaminated by bacteria, may contain very high levels of histamine. A person who eats such fish may show symptoms that are similar to a food allergy. However, this reaction is not a true allergic reaction. Instead, the reaction is called **histamine toxicity** or scombroid food poisoning			
Celery (e.g. celery stalks, leaves, seeds and the root called celeriac)	Used in celery salt, salads, some meat products, soups and stock cubes			
Milk	Cow's milk can cause symptoms related to IgE antibodies to milk, including abdominal pain, hives and eczema in children. Allergy to cow's milk can lead to other symptoms associated with immune responses that are not related		Cow's milk can cause lactose intolerance in children	

	to IgE antibody, such as complications that can give rise to colic and sleeplessness, as well as blood in the stool and poor growth			

Labeling of Foods that Contain Allergens

Foods that are known or suspected of containing food allergens as ingredients must comply with the labeling requirements as per the regulatory authorities, in order to protect the health status of consumers, especially those who may find themselves suffering from allergies. The compliance of labeling and the ingredients in different types of foods is verified using a number of methods and techniques, mainly proteomics-based methods, immunochemical methods and molecular biology methods, hyphenation of chromatography and mass spectrometry methods.

Generally there are methods that are used for specific target food allergen parameters that need to be investigated. For example, when the isoforms or sequencing information of allergens is to be determined, then N-terminal sequencing techniques as well as mass spectrometric-based techniques and their hyphenations such as Q-TOF-MS/LC-Q-TOF-MS; MALDI-TOF-MS are the techniques of choice. Circular dichroism (CD), nuclear magnetic resonance techniques and Fourier transform infrared techniques will be suitable for the investigation of the folding pattern and folding tendency as well as the structural studies. Molecular weight and aggregation studies of the allergens can be probed using gel permeation chromatography (GPC), while the purity, proteolysis and glycosylation can be determined using size exclusion chromatography (SEC), electrospray mass spectrometry and silver stain sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The IgE reactivity and allergenicity activity can be investigated using the EAST method, RAST method, immunoblotting approaches with human sera and histamine release based assays. The ability of inhibition using extracts from natural sources can be probed using techniques such as CAP inhibition, EAST and ELISA.

Diagnosis of Food Allergy Reactions

Food allergy diagnosis is a crucial step that enables physicians to prescribe appropriate treatment and also alert the victim to the types of foods or food ingredients to avoid as a prevention measure. The food allergy diagnosis can be performed using *in vivo* tests and *in vitro* tests. The *in vivo* tests for food allergy involve the application of either skin prick test (SPT), oral food challenge and suspicious product elimination diet (Condemi, 2000; Smith and Munoz-Furlong, 2000). Of the *in vivo* diagnosis tests, SPT has been regarded as more attractive as a safer option as compared to oral food challenge, which comes with risks of causing anaphylaxis and/or other adverse side effects to the victims (Bock, 1982; Condemi, 2000; Smith and Munoz-Furlong, 2000). The *in vitro* food allergy tests on the other hand, can be performed in two ways:

1. they may involve IgE quantification;
2. the other several variants, including the test that involves the *in vitro* activation of the blood cells that release histamine or other substances causing allergic symptoms (basophil), which consists of the flow cytometry analysis of the victim's basophils. The activation is triggered by the binding process of either the allergen extracts or the recombinant allergens to IgE antibodies (Sampson and Scanlon, 1989).

IgE quantification-based methods on the other hand rely on the quantification of either total or specific IgE antibodies, which are universal biomarkers of the food allergic sensitization.

There are other approaches that can be used for food allergy, mainly the immunoassay based techniques such as the enzyme-linked immunosorbent assay (ELISA), which have proven to be useful in the assessment of the victim's level of both the total and specific IgE antibodies. Apart from the ELISA, there are other imaging based immunoassay based techniques, such as surface plasmon resonance imaging immunoassays, that may be used for food allergy diagnosis and for the probing of the antibody avidity.

Other possibilities of food allergy diagnosis, which do not involve the use of antihuman IgE antibodies, make use of artificial nucleic acid (DNA or RNA) oligonucleotides that are characterized with a high binding affinity towards specific molecules. These nucleic acid oligonucleotides are known as aptamers and play roles similar to the one played by antibodies in the affinity-based assays.

Other assays that have been used for the diagnosis of food allergy include the

Other assays that have been used for the diagnosis of food allergy include the fast lateral flow assay or lateral flow paper microarray equipped with a colorimetric detector, together with gold nanoparticles labeled antihuman IgE antibodies as well as horseradish peroxide. Another assay is the immunoaffinity capillary electrophoresis (IACE) equipped with laser-induced fluorescence (LIF) detection, which takes advantage of the immunoaffinity assays and that of electrophoresis separation.

Detection of Allergenic Species in Foods

There are several techniques that are available for the determination of allergenic species that are present in foods. These include proteomics based techniques, molecular biology based methods, immunological/immunochemical based methods and chromatography-mass spectrometric based methods.

Proteomics Based Methods for Food Allergens

Proteomic based techniques in combination with immunoblotting methods have been used almost routinely in the determination of IgE-protein binding and interactions. These techniques are highly useful in the determination of allergens in foods, simply because they can be used as sample preparation methods that enable the generation of pure and authentic allergen samples with known properties of the allergen’s proteins. Once good-quality and authentic food allergens have been obtained, they can serve many purposes, including as possible vaccines for immunotherapy and also as certified reference materials that can be used for calibration in quantitative studies of allergens. These methods (proteomic) are powerful techniques in which the knowledge regarding the contribution of both genetical and environmental factors can be deduced and they can thus be used for genetic and phenotypic variability in crops/animals which form the source of food. Moreover, proteomic methods and techniques can enable the determination of the allergenic species as well as their isoforms in foods. Moreover, advanced proteomic techniques have the flexibility to provide information about the pattern of the protein or peptide forms, the allergen determination as well as the possible biomarkers (quantitative and qualitative measurements).

Immunochemical Identification and Detection Methods for Food Allergens

Antibody-based immunochemical methods are very useful for the analyses of allergenic protein molecules that are present in foods. These methods are advantageous in that they are capable of analyzing directly the presence of protein types of allergens regardless of the type of allergic reaction involved. Some limitations that may be associated with immunological methods are that they are associated with the possibilities of cross-reaction and also their inability to detect denatured proteins.

Molecular Biology Based Methods for the Determination of Food Allergens

Of the molecular biology methods, the application of DNA techniques which involve the use of ds-cDNA (double strand complementary DNA, which is normally synthesized from a messenger RNA template in a process that is catalyzed by the reverse transcriptase enzyme), as well as polymerase chain reaction techniques for the amplification of the DNA, is very promising due to the fact that the technique can be used to identify specific DNA sequences associated with the presence of unwanted foods. In these techniques, the allergenic species is targeted and amplified for detection and quantization. Unlike immunochemical methods, molecular biology methods that employ DNA technology are attractive in that the DNA is stable to heat/temperature that is normally used during food processing, also stable to hydrolysis reactions, pH changes, and are not associated with the risk of cross-reactivity tendencies. The limitation of this approach (use of DNA techniques) is that it cannot provide information as to whether the allergenic causing protein is present or not, and also DNA techniques are not sensitive enough to detect very low levels of allergenic food species.

Determination of Food Allergens Using Chromatography and Mass Spectrometry Based Methods

It is possible to use chromatographic techniques such as chromatography or capillary electrophoresis with their traditional detectors such as UV-Vis, fluorescence, etc., but such approaches are associated with false positive results due to co-elution of matrix molecules that elute at the same retention time with allergenic molecules. However, when mass spectrometers are used as chromatographic or capillary electrophoresis detectors, this limitation is

chromatographic or capillary electrophoresis detectors, this limitation is eliminated because mass spectrometry is capable of detecting the actual allergenic species directly. The use of mass spectrometers for the detection is advantageous because it offers the possibility of direct identification, quantification and the possibility of the allergen structural characterization and identification of new allergenic species with high sensitivity, accuracy and specificity.

It is worthy to note that sample preparation prior to LC-MS is crucial, as it determines methods to the samples using appropriate solvents, buffers *etc.* under the optimized experimental conditions.

Conclusion

The problem due to the presence of allergenic species in foods is a serious one and therefore food manufacturers and distributors should adhere to the proper labeling of food products. In addition, efforts should be made to probe and identify new food allergenic molecules (allergenomics).

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4
Food Forensics Cases Related to Food Bioterrorism/Food Bio-Weapons and Food Poisoning Agents: Agrochemical Food Poisoning Agents

> *Food bioterrorism refers to any deliberate/intentional practice or actions that may lead to the presence of harmful substances such as poisons in food that may affect the consumer negatively by causing illness, disease, sickness, physical body deformation, or death. Substances that may poison foods may be in the form of chemical substances such as agrochemicals/pesticides/insecticides, antibiotics, metals (e.g. cadmium, arsenic, lead, mercury, radionuclides, etc.), they may also be in the form of biological agents such as biological substances caused by naturally occurring toxins that are present in food such as algal toxins, red kidney beans, or green potatoes, and they may be microbially derived poisons caused by pathogenic microorganisms deliberately introduced into foods. In this category, there may be microbial toxic secondary metabolites (e.g. mycotoxins, cyanotoxins, botulinum, tetanus, anthrax, H5N1 virus poison, H1N1 virus poison, etc.) in foods, or microbes or their spores introduced into foods.*

Introduction

Conflicts within and between societies have been characteristic of our planet since time immemorial. Political, social, and economic factors have been at the center of many if not all of the major frictions that have led to the loss of peace, and in many cases loss of life. One of the easy target weapons for inflicting damage or even eliminating those who are on the opposite side of the conflict, has been in the area of food and water (bioterrorism), through our basic needs such as for food that is required by all human beings on a daily basis. The deliberate or intentional unlawful act of inflicting damage to or annihilation of human life and well-being or livestock, crops, social, or economic installations, etc., that involves the use of biological agents such as microorganisms or secondary metabolites derived from microbes, is known as bioterrorism (Centers

for Disease Control and Prevention (2008); Frerichs *et al*., 2004; Wein and Liu, 2005).

There are several food production, processing, or distribution stages that could be a target in food bioterrorism. They include the whole production and supply chain (from farm-to-table food continuum), for example crops that are still in the field, livestock, food products that have entered the processing stage, supply and distribution chain, wholesale/retail shops, storage facilities, transportation, food laboratories, and agriculture research infrastructure (Mackby, 2006). Bioterrorism agents can inflict serious health problems, illness, sickness, and disease ranging from severe acute respiratory syndrome (SARS), foot-and-mouth disease, botulism, tetanus, mad cow disease, central nervous system collapse, monkey pox, pulmonary related diseases, avian influenza, *etc*.

Chemical Food Poisoning Agents: Agrochemicals in Foods

The main fact about agrochemical toxicity is that all agrochemicals are usually, but not always, poisonous to humans. Although they are termed agrochemicals, pesticides, insecticides, fungicides, herbicides, rodenticides, algicides, bactericides, etc., they are used for both agricultural-related applications and also non-agricultural-related application. The main applications of these chemicals in agriculture involve the prevention, destruction, repulsion, and mitigation of pests, such as insects, rodents, or other destructive animals, weeds and harmful microbes including fungi, bacteria, and viruses, that can cause or act as disease vectors (Laws and Hayes, 1991). Non-agricultural use of agrochemicals includes their application in the control and reduction of foodborne and vector-borne diseases, such as mosquitoes (Centers for Disease Control and Prevention (Disease Information), 2002; Gubler, 1998).

Agrochemicals and their residues can be transferred to humans in several possible ways, including drinking water that is contaminated with agrochemicals, eating meat from animals that have previously been fed on feed contaminated with agrochemicals, or animals previously subjected to medicated premixes, consumption of eggs, milk, honey, vegetables, and fruits.

The agrochemical classes and their respective metabolites that have been reported in human biological matrices for forensic poisoning-related cases include organophosphate agrochemicals and their respective metabolites (Hardt and Angerer, 2000; Lacassie *et al*., 2001; Pitarch *et al*., 2003); organochlorine

agrochemicals (Barr *et al*., 2003; Burke *et al*., 2003; Burse *et al*., 2000; Campoy *et al*., 2001; Conka *et al*., 2005; Hong *et al*., 2002; Lacassie *et al*., 2001; Sundberg *et al*., 2006); synthetic pyrethroid-based insecticides (Arrebola *et al*., 1999; Lacassie *et al*., 2001; Leng and Gries, 2005; Ramesh and Ravi, 2004; Schettgen *et al*., 2002); triazines (Barr *et al*., 2002; Lacassie *et al*., 2001; Norrgran *et al*., 2006); chloroacetanilides (Bradman *et al*., 2003; Whyatt *et al*., 2003); phenoxyacetic acid herbicides (Hill *et al*. 1995); chlorophenols (Bravo *et al*., 2005); and neurotoxic carbamates (Meeker *et al*., 2004).

Sampling of Target Specimens

Human specimens that are normally collected for the analysis of agrochemical poisoning include hair, fluids (urine, sweat, saliva, blood, intestinal fluids, milk, human hair, finger nails, amniotic fluids, semen, etc.). Of the fluid specimens, urine is the most commonly used, due to the fact that it is easily obtained in abundance. However, there are some drawbacks that are associated with urine sampling, which are due to temporal variability of the volume, and also the variability of the levels of endogenous and exogenous compositions. Some reports have suggested that urine sampling should be done first thing in the morning to ensure the stability of the acceptable representative as opposed to sampling at other times of the day (Kapka-Skrzypczak *et al*., 2011). Moreover, it is required that normalization using specific standards such as creatine should be included to account for cases of either over-dilution or over-concentration of the samples that have been collected on the spot (Arcury *et al*., 2009).

Milk is known to be a very complex matrix due to the presence of numerous biomolecules such as fats and lipids, proteins, carbohydrates, etc., which may have strong affinity to the agrochemical residues that may be present, making it difficult to extract and quantify them properly.

Saliva sampling is easy but the analyst must be knowledgeable enough to be able to present acceptably reliable measurements related to an internal dose from a saliva specimen that has been instantly collected (spot saliva sample) and must have a thorough knowledge of the stability (half-life), pharmacokinetics data of the agrochemical molecules being investigated, and the distribution ratio of the same agrochemical between the saliva and blood (Barr *et al*., 2006; Timchalk *et al*., 2004). Some researchers have suggested that the distribution ratio of agrochemicals in saliva is outweighed by that found in the blood and this ratio is governed by the type and chemistry of the protein binding to the agrochemical species (Kapka-Skrzypczak *et al*., 2011). Sweat has also been reported to be a

possible specimen for the analysis of pesticide poisoning (Rosenberg *et al*., 1985).

Hair specimens have been used as an indicator of long-term agrochemical exposure/poisoning because the drugs remain longer in the hair. Hair strands from the suspected victims have to be decontaminated from cosmetics and facial secretions using appropriate aqueous-organic reagents (e.g. surfactants such Triton-X 100, deionized water, acetone), cut into small pieces and incubated at temperatures between 45 and 75 °C (depending on the type of suspected agrochemicals). Then hair samples are dissolved in methanol to extract agrochemical residues before injecting into an analytical instrument such as GC-FID/NPD/FPD/MS for detection and quantification (Cirimele *et al*., 1999; Oluremi *et al*., 2011a,b).

However, despite the fact that a hair specimen gives a relatively good biological measure of exposure or poisoning due to many persistent organic pollutants (POPs), including some of the agrochemicals, there are some limitations that are associated with the difficulties in distinguishing between exogenous and endogenous contamination. Another bottleneck related to the use of hair specimens in the analysis of agrochemicals and other POPs is lack of availability of standardized methods for hair sampling, decontamination (washing), and analysis, as well as the inadequacy of useful information that is available in the literature regarding the correlation and distribution ratio between concentrations of the POPs, such as agrochemicals in hair and other body matrices such as blood, adipose tissue, *etc*. (Altshu *et al*., 2004).

Finger nails are also used as specimens in food poisoning cases, as they can retain residues of metabolites for a considerable period of time (Dablool, 2014).

Blood samples are also useful in providing evidence in cases of agrochemical poisoning. For example, for poisoning with organophosphates or carbamates, blood samples can provide a good measure of the depression associated with plasma butyrylcholinesterase (pseudocholinesterase) as well as that of red blood cell (RBC) AChE levels as caused by the absorption of organophosphate in the blood (Eddleston *et al*., 2008).

Other specimen that are normally collected to examine microbial food poisoning include throat swabs, nasal swabs, nail swabs, and stool samples and after collection the culturing of the microbes follows and they can be identified using molecular biological techniques.

The umbilical cord has been mentioned as a suitable sampling organ for analysis

of agrochemical poisoning (Burse *et al*., 2000).

Agrochemical Poisoning Metabolite Indicators and Markers: Indicator Molecules and Markers Found in Urine Samples After Poisoning

In most cases, when there is poisoning due to chemicals, it is difficult to track parent molecules in any of the body matrices for several reasons, including the fact that they become metabolized in the body system and change their chemistry altogether.

In general, the trend and series of events that follow just after the poisonous or toxic molecule comes into contact with and/or if it gets into the body's system, a toxicokinetic process commences, which involves absorption processes, distribution, metabolism, and elimination through excretion routes (**NB**: the rate and time it takes for agrochemicals or their metabolites to be eliminated differs within and between agrochemicals from various matrices, urine, blood/blood components, etc., with some being rapid while others have slow elimination rates depending on their respective half-life (Angerer *et al*., 2007)). These processes trigger and result in the body metabolic pathways producing certain specific molecules (biomarkers/indicators) in response to the presence of these toxic molecules. Each of the poisonous/toxic molecules results in a particular set of markers/indicator molecules, which can be identified and measured from each of the processes, whether it is during absorption, distribution, metabolism, or elimination. Therefore toxicity due to poisoning molecules takes into account the detection and measurement of these specific biomarkers or indicator molecules that may include parent molecules (in case they are present in the matrix chosen for analysis), the specific metabolites in any of the processes or the reaction products present in the sampled specimen's matrix such as urine, hair, tissues, blood, and/or blood-derived components, nails, exhaled air, *etc*. (Barr *et al*., 2006; Ngo *et al*., 2010). In some cases, such as the phenomena that happen with organophosphorus agrochemicals that are characterized with having common urinary metabolites but which have a tendency to mask the signals of their respective parent molecules in the victim's body system, it is thus expedient to deal with the measurements of mainly metabolites rather than the parent molecules (Albertini *et al*., 2006; Barr *et al*., 2006; Kissel *et al*., 2005).

Analysis of Agrochemical Residues and their

Metabolites in Biologic Samples in Individuals Affected by Food Poisoning

There are three major classes of organophosphorus agrochemicals including: i) phosphoroamidothiolates; ii) phosphorodithioates; and iii) phosphothionates (Chambers, 1992). Within these three classes there are numerous individual organophosphate compounds. The analytical strategy for the analysis includes the measurement of both the intact agrochemical molecules and/or their metabolites in various biologic matrices such as serum, breast milk, stool, meconium (the earliest stool of an infant), urine, *etc.* The analytical procedures for the agrochemical parent molecules and their respective metabolites in all of these biological matrices involve the application of effective sample extraction and sample purification procedures, as well as very sensitive and selective detection methods.

Biomarkers in Agrochemical Poisoning

There are different types of biomarkers that can be used to provide indication of the presence of a foreign substance inside living systems, which triggers the body's system of an organism to respond by either upregulating or downregulating certain biomolecules (biomarkers) specific to that particular foreign substance (WHO, 1993). The upregulation and/or downregulation of these biomolecules shows that biomarkers are genetically controlled and are also useful in predicting parameters or species that will either increase or decrease as a result of poisoning (Hernandez *et al*., 2003). Biomarkers are useful in forensic cases because their detection provides evidence of not only poisoning but which poisons were actually used (WHO, 1993).

Specific biomarkers in agrochemical poisoning cases arise during the metabolism of the agrochemical once ingested, which then proceeds through bioactivation steps before the detoxification procedures begin. It should be noted that the bioactivation and detoxification mechanisms can be influenced by genetic polymorphisms through the catalytic actions of a number of enzymes that are coded by various genetic factors. For this reason, all these biotransformation pathways and processes may significantly influence the toxicity of different agrochemicals to humans (Costa *et al*., 2005; Rose *et al*., 2005).

These specific responses by the body after poisoning, which result in the

formation of different types of biomarkers, are caused by several polymorphic principal enzymes that may belong to a number of enzyme classes and in some cases a synergetic combination of these enzymes from different classes, especially in the processes involved in bioactivation (Hernandez *et al*., 2003). On the other hand, the process of elimination of agrochemical intact molecules and their metabolites from the body is genetically controlled, such that some individuals with weaker versions of polymorphic genes may experience elevated levels of bioactivation that may be associated with slow detoxification of poisoning agents and these individuals are likely to suffer severe poisoning consequences (Bolognesi, 2003). The metabolic process of elimination of poisons emanating from agrochemical poisoning involves glutathione S-transferase enzymes, cytochrome P450 enzymes, and also esterase enzymes. The glutathione S-transferase enzymes are useful in identifying genetic polymorphisms that cause glutathione conjugation due to agrochemical or other chemical poisoning (Bolognesi, 2003; Schroeder, 2005). These enzymatic controlled polymorphisms play different roles, for example, glutathione S-transferase polymorphism 1 (GSTP1) has been reported to play a role in influencing both substrate selectivity and stability, also the rate at which metabolites are formed or the rate at which metabolites and other toxic species are eliminated (Liu *et al*., 2006).

The enzymes that belong to the family of cytochrome P450 (CYP450) are important in the metabolism of agrochemicals, such as carbamates, nicotinoid class of agrochemicals, etc., as they are responsible for catalyzing various metabolic reactions and processes and their variation profiles in the body may be a useful indication of agrochemical poisoning (Hodgson, 2003; Mutch and Williams, 2006). The catalytic mechanism of action of CYP450 enzymes (e.g. CYP2C8, CYP3A4, CYP3A5, CYP2D6, CYP1A2) involves oxidative desulfuration of the agrochemical molecules (mainly the anticholinerase ones such as carbamates and organothiophosphates to the oxon derivatives of the respective agrochemicals). These enzymes (e.g. CYP1A2, CYP2, and CYP3) are also important in catalyzing both the bioactivation and elimination processes of anticholinerase agrochemicals (Costa *et al*., 2005; Nebert, 2005).

Another class of enzyme that is important in catalyzing bioactivation and detoxification of agrochemicals and their metabolites is esterase enzymes, which include the serine hydrolase class of enzymes (mainly carboxylesterase enzymes), paraoxonase enzymes, and cholinesterase enzymes.

The carboxylase enzymes (produced in the liver) are mainly responsible for the

hydrolysis of ester-containing agrochemicals, as well as the detoxification of anticholinesterase agrochemicals (Ross *et al*., 2006; Wu *et al*., 2007; Zhou *et al*., 2007). In the case of paraoxonase (PON), it also includes several classes such as classes 1, 2, and 3, such that there are PON1, PON2, and PON3, mainly responsible for the metabolism of oxygen containing agrochemicals (Hernandez *et al*., 2004). The esterases normally target the anticholinesterase agrochemicals, mainly the organophosphates.

Organophosphate and Carbamate Agrochemicals: Mode of Action, Toxicity, Metabolism, and Biomarkers

The organophosphate class of agrochemicals includes a number of other sub-classes (with nerve poisoning moiety), as shown in Figure 4.1a to g.

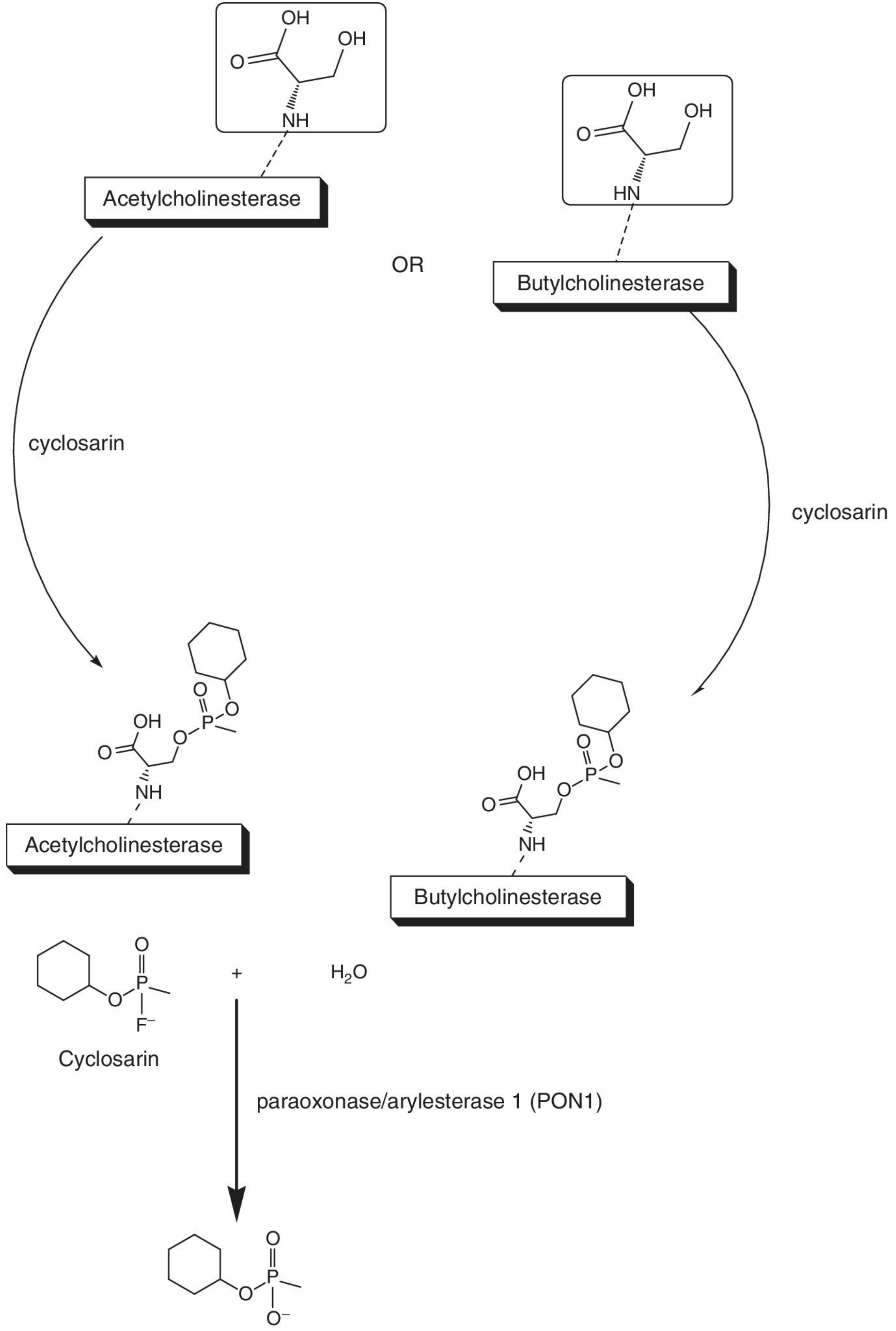
Figure 4.1 General chemical structures of organophosphate agrochemical sub-classes: (a) phosphorothioate; (b) sodium-di-sec-butyl-phosphorodithioate; (c) phosphorothioic acid, methyl, 0-ethyl; (d) phosphates; (e) phosphonates; (f) phosphoramidite; and (g) O,S-dimethyl phosphoramidothioate.

The raw materials that are normally used in the preparation of organophosphate agrochemicals, are mainly phosphorylating compounds that originate from various sources such as diisopropylfluorophosphate, sarin, *etc.* These substances are well known for their powerful effects as nerve agents. In cases of poisoning, organophosphates normally tend to phosphorylate specific targets in the body of the victim, mainly an enzyme acetylcholinesterase that plays an important role in facilitating the hydrolysis of acetylcholine (a neurotransmitter), found in the synaptic membrane in the central nervous system to form choline and acetic acid. Other targets include proteins and enzymes such as neuropathy target

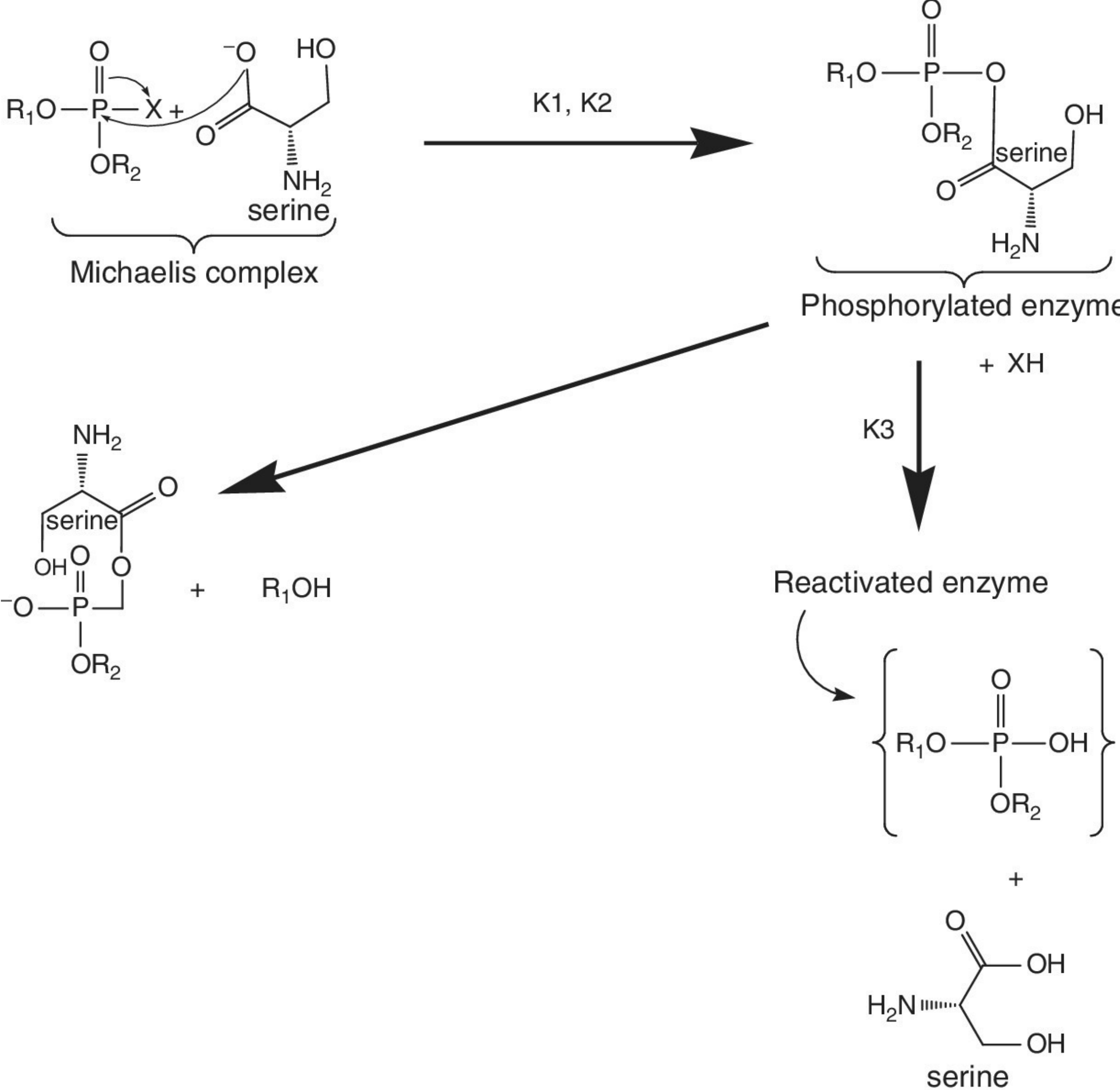
esterases (NTE), nicotinic acid, and muscarinic acetylcholine receptors (Eldefrawi *et al*., 1992).

The action of organophosphates in the body system prevents the hydrolysis of acetylcholine, thus causing failure in the binding of the ion channel of the nicotinic acetylcholine. Due to this, these ion channels will remain open permanently and this will allow sodium ions to pass through, continuously causing a number of fatal events including membrane depolarization, convulsions which may be fatal to the victim, and also non-stop neuronal firing that may also be fatal. Cases where organophosphates bind NTE will lead to disorders related to progressive neuronal degeneration syndrome, also known as organophosphate-induced delayed neuropathy (OIDN), which results in symptoms such as ataxia.

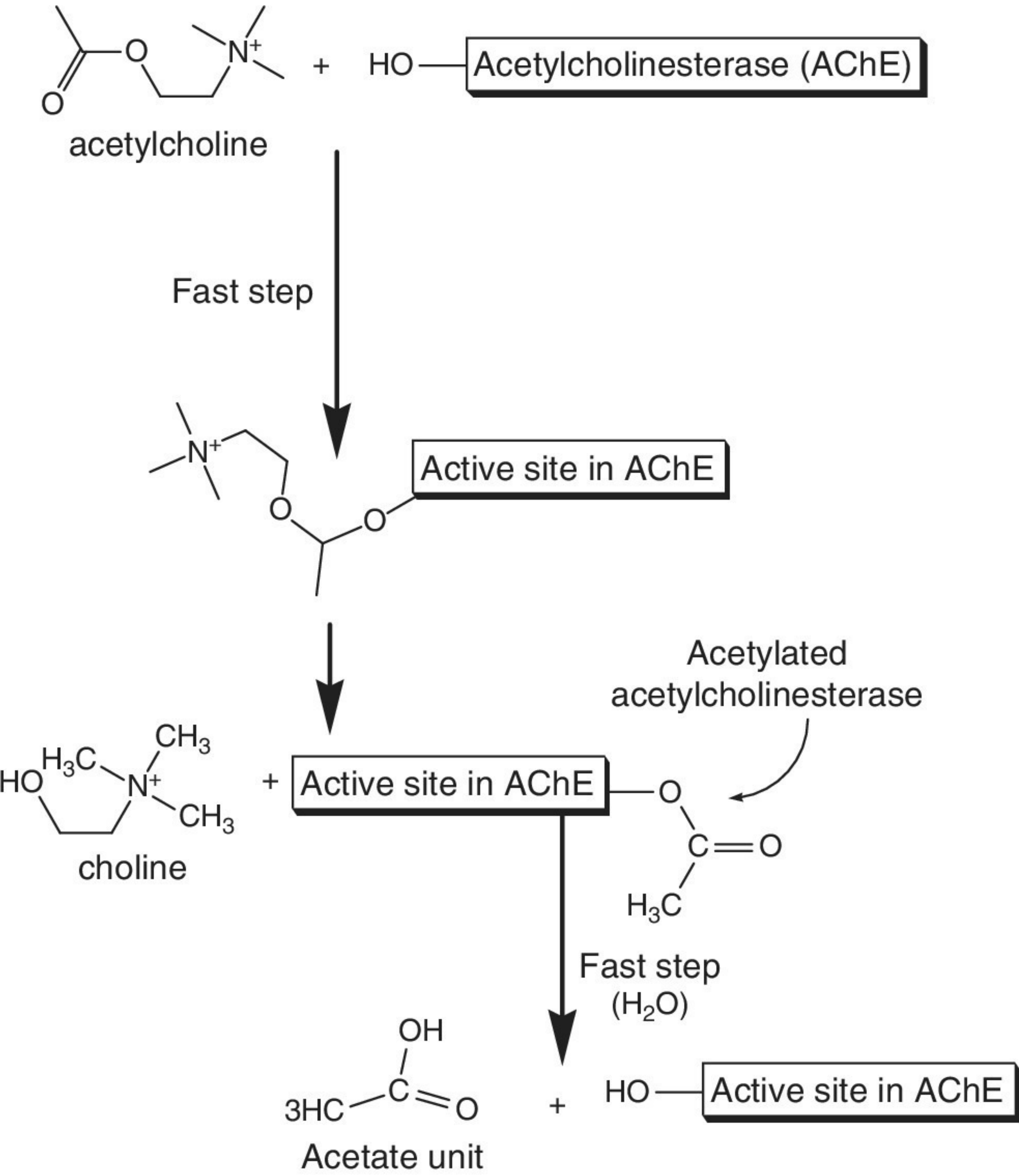
The reaction of organophosphates with acetylcholinesterase proceeds via mechanisms that involve nucleophilic reactions of the serine hydroxyl group, which is the active site on the phosphorus atoms that belong to phosphate ester functionality (Schemes 4.1–4.3). The nucleophilic attack is thus responsible for the cleavage of bonds such as P–O and P–S. After these processes follows a step that involves phosphorylation of the amino acid serine followed by the release of the leaving group. Serine may also undergo acetylation using acetylcholine as the process of acetylcholine hydrolysis proceeds.


OH
OH
O
NH
Acetylcholinesterase
OR
OH
OH
O
HN
Butylcholinesterase
cyclosarin
cyclosarin
Acetylcholinesterase
Butylcholinesterase
Cyclosarin
+
H2O
paraoxonase/arylesterase 1 (PON1)

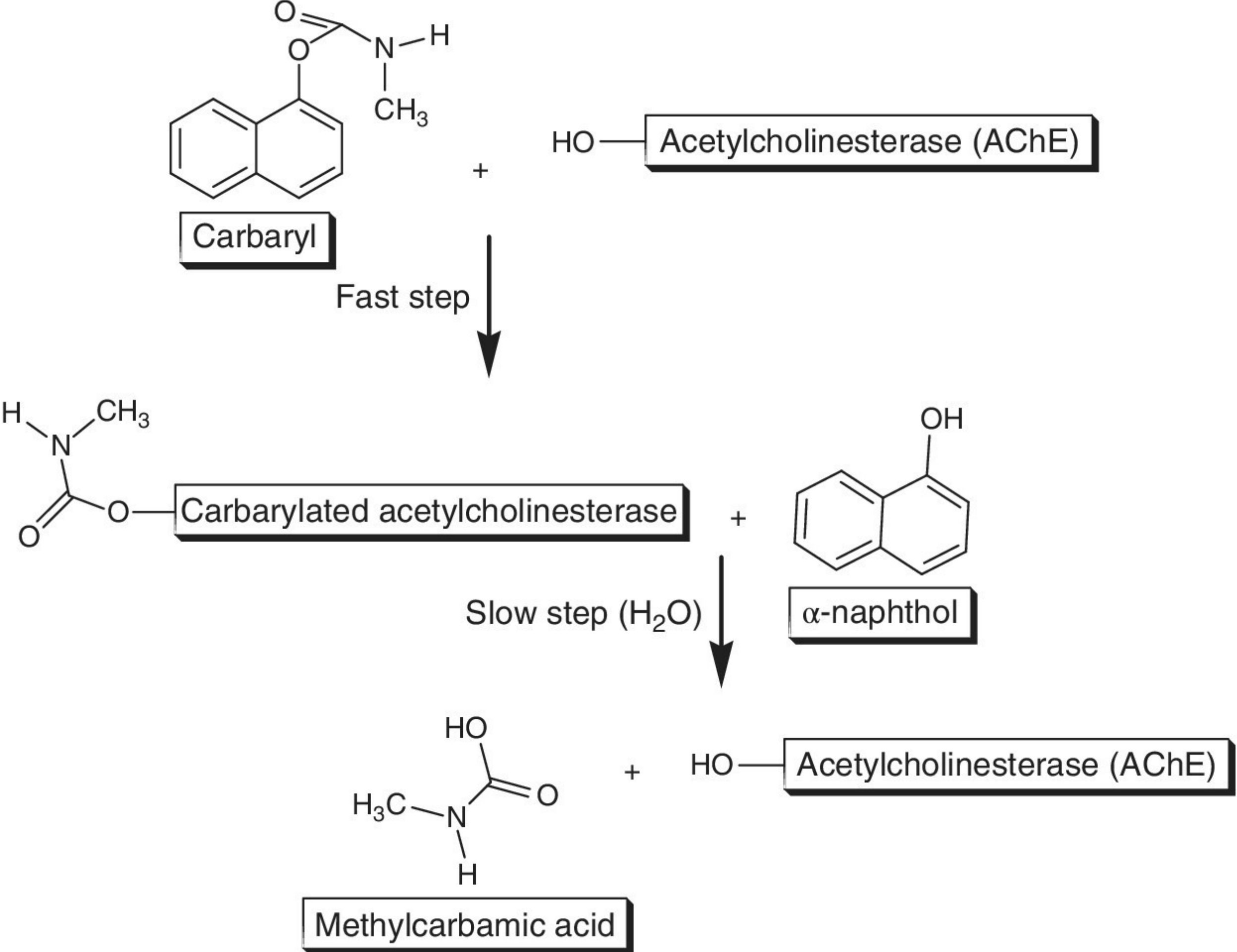
Scheme 4.1 Proposed reactions of organophosphates and acetylcholinesterase (Raushel, 2011).



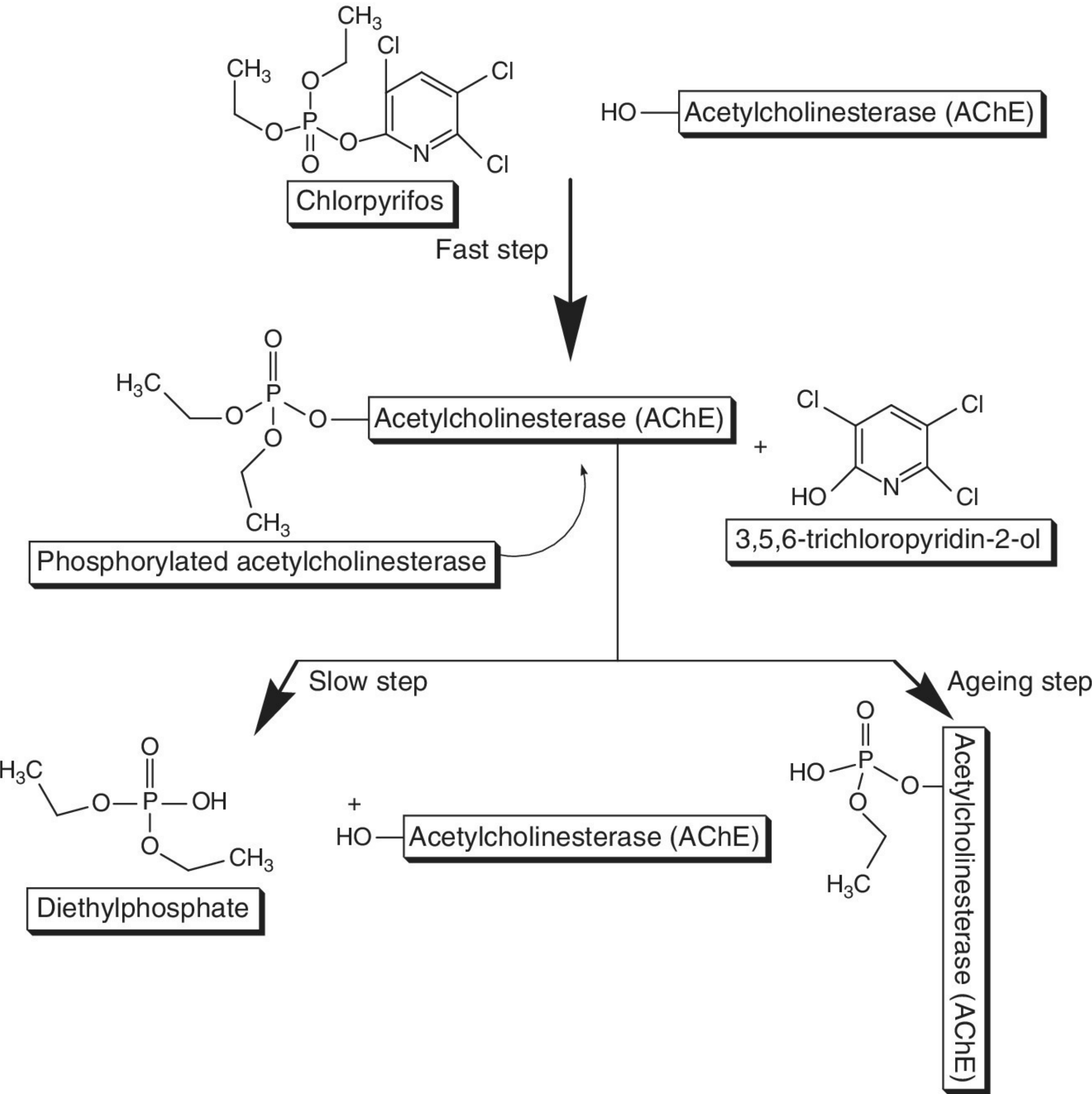
Scheme 4.2 Proposed acetylcholinesterase mechanism of action.



Scheme 4.3a Proposed interaction of acetylcholine with acetyl cholinesterase.



Scheme 4.3b Proposed interaction of acetylcholine with carbaryl.



Scheme 4.3c Proposed interaction of acetylcholine with chlorpyrifos.

Organophosphate Agrochemicals: Malathion

Malathion (carbophos) ($C_{10}H_{19}O_6PS_2$; molar mass: 330.358 g/mol; density: 1.23 g/mL; chemical name diethyl-2-[(dimethoxyphorothothionyl) sulfanyl butanedionate) is an organophosphate pesticide with a water solubility value of 145 mg/L (at 25 °C) and $\log K_{OW}$ value of 2.75. The chemical structure for malathion is depicted in Figure 4.2.

Figure 4.2 Chemical structure of malathion.

Malathion is known to produce a number of metabolic compounds in metabolically active plant tissues, fish, animals, and soils (Mostaf *et al*., 1974).

The metabolites of malathion formed in fish are mainly monoacids and dicarboxylic acids (Cook and Moore, 1976). In humans, the principal metabolites include malathion monoacids, malathion dicarboxylic acids, and malaoxon, which is among several minor metabolites. Other metabolites of malathion include *O*,*O*-dimethylphosphorodithionate, *O*,*O*-dimethylphosphorothionate, dimethylphosphate, and monomethyl phosphate. Detailed metabolic pathways for malathion are presented in Figure 4.3a–d.

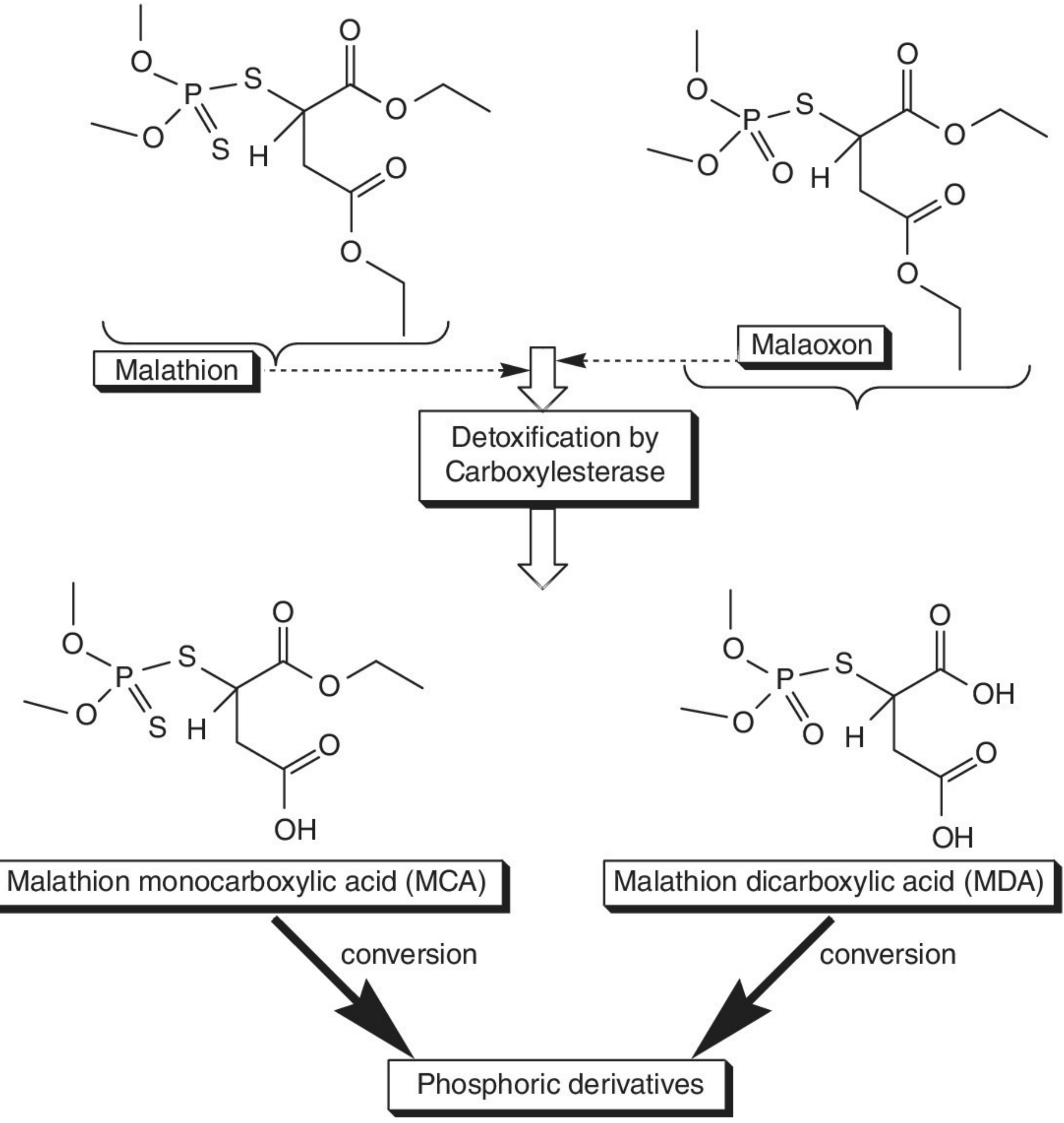


Figure 4.3a(i) Proposed metabolic pathways of malathion in humans: Formation of specific malathion/malaoxon biomarkers in urine (MCA and MDA).

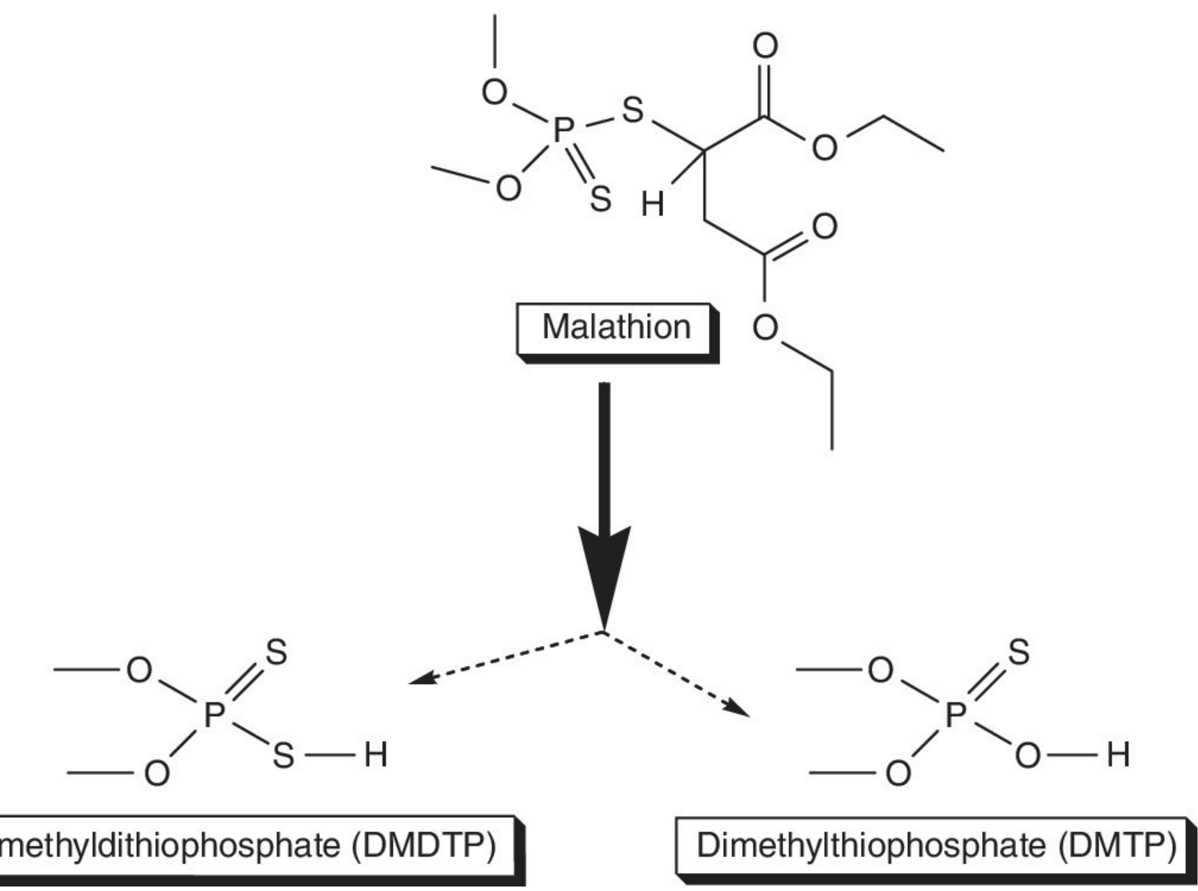


Figure 4.3a(ii) Proposed metabolic pathways of malathion in humans: Formation of non-specific malathion biomarkers in urine (DMDTP and DMTP).

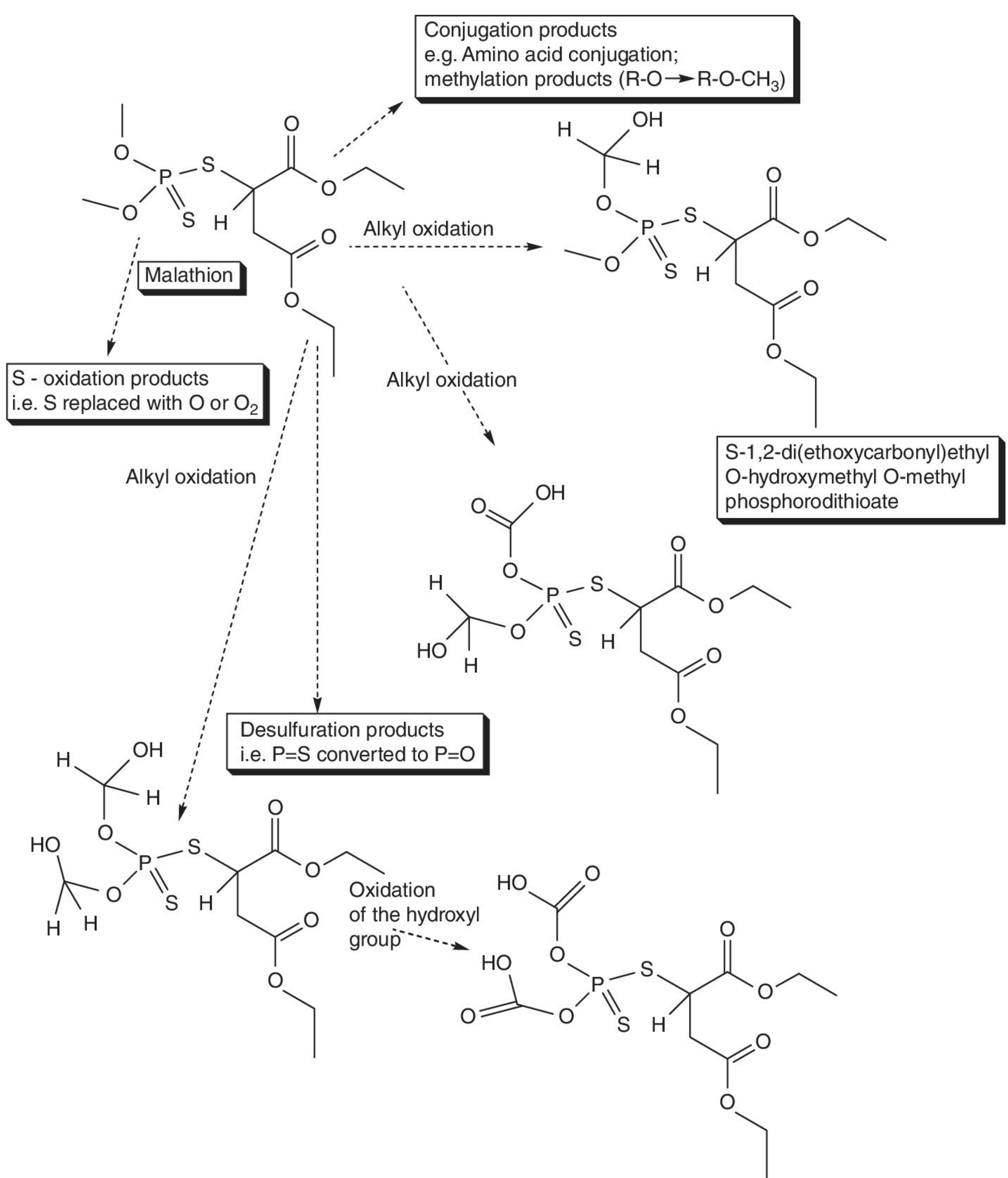


Figure 4.3b Proposed metabolism of malathion in fish/aquatic organisms.

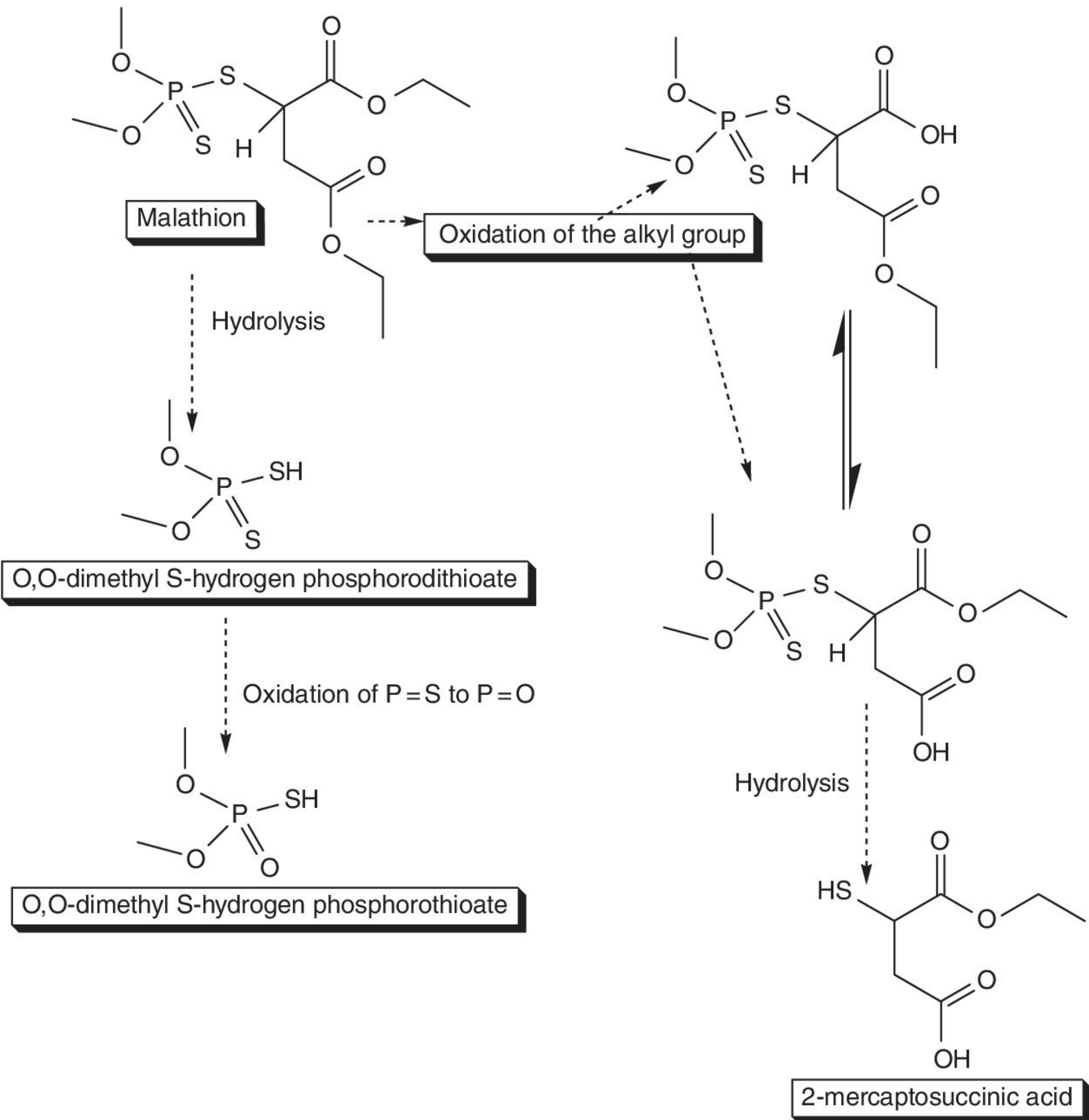


Figure 4.3c Proposed formation mechanism of malathion metabolic biomarkers in plants (e.g. plant vegetables, crop plants).

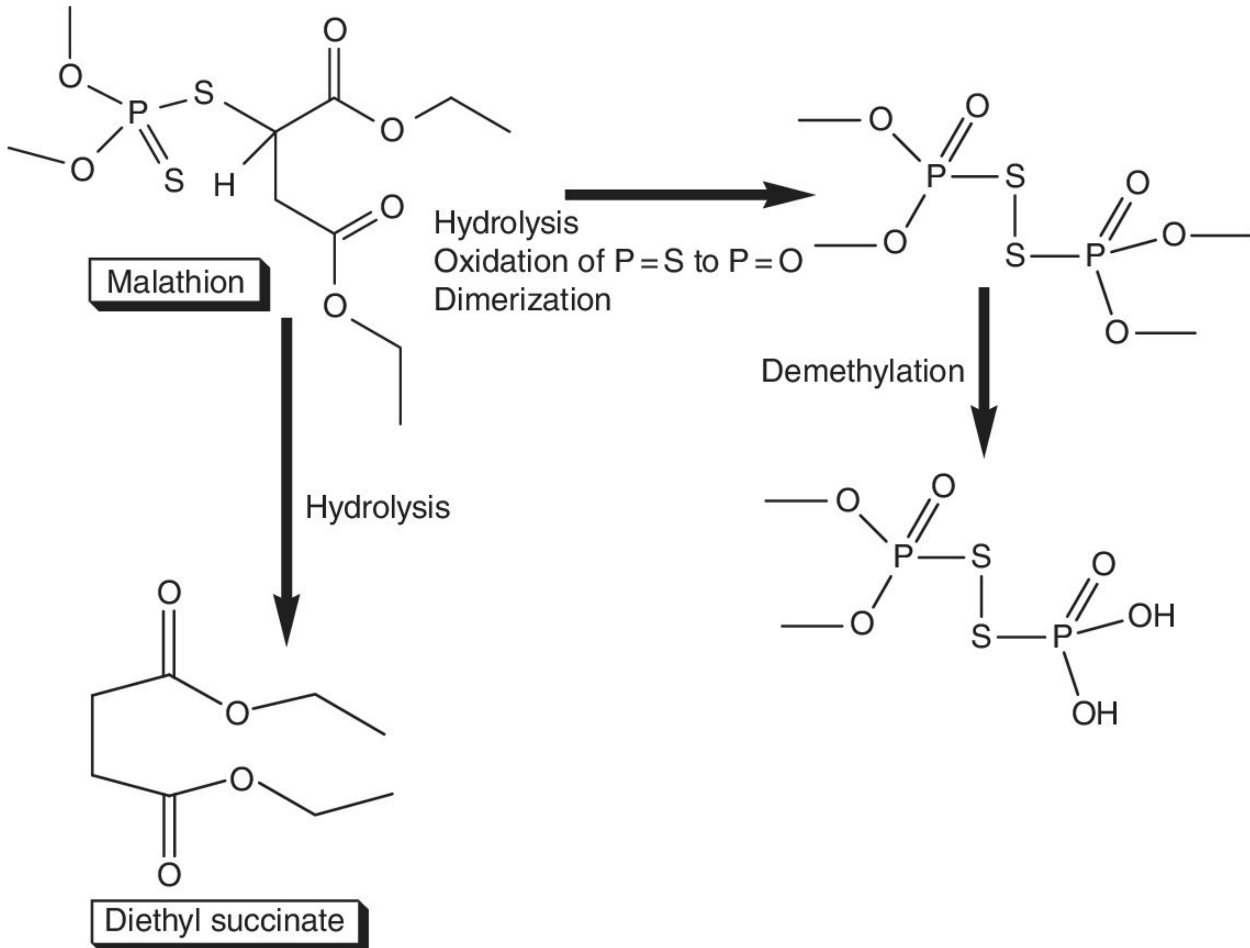


Figure 4.3d Proposed metabolites of malathion in water.

Organophosphate Agrochemicals: Parathion and Methyl Parathion

Parathion (folidol) (*O*,*O*-Diethyl *O*-(4-nitrophenyl) phosphorothioate) has a water solubility of 11 mg/L (at 20 °C), $\log K_{OW}$ value of 3.83, chemical formula: $C_{10}H_{14}NO_5PS$, and molar mass: 291.26 g · mol^{-1}. The chemical structure of parathion is depicted in Figure 4.4.

Figure 4.4 Chemical structure of parathion.

In humans and other animals, the metabolism of parathion proceeds via the route of the de-esterification process that generates 4-nitrophenol and de-ethlation, and oxidation that produces paraoxon products. In the case of orally ingested pathion, the detoxification process proceeds via dearylation, which generates 4-nitrophenol and *O*,*O*-diethylphosphorothionate. The dearylation is an enzymatic mediated reaction where NADPH-dependent microsomal oxidases occur as well as glutathione-S-aryl transferases (Hollingworth *et al*., 1973).

Anticholinesterase paraoxon as well as sulfates are other metabolites generated through the oxidative desulfuration process, which is mediated by cytochrome P450 enzymes. Paraoxon metabolites also undergo hydrolytic biotransformation, which catalyzes esterases to generate 4-nitrophenol and diethyl phosphate. Another biotransformation reaction of paraoxon produces desethyl paraoxon, a product which is excreted in urine, and is catalyzed by glutathione–S-alkyl transferases. Other parathion metabolites that are excreted in urine include monoethyl phosphate, which is itself a by-product of desethyl paraoxon hydrolysis and also an inorganic phosphate.

By-products of 4-nitrophenol metabolism proceed via the route of glucuronide conjugation and form major metabolites in urine. In some cases, only glucuronide conjugation products without other unconjugated metabolites are the ones excreted in urine. Parathion, paraoxon, and ethyl paraoxon (EPN) can also be reduced by *in vitro* enzymes such as NADPH and NAD to form several products, including amino paraoxon and amino ethyl paraoxon, 4-aminophenol and these are excreted in urine as one of the major metabolite products. The detailed metabolic pathways for parathion are depicted in Figure 4.5a–f.

(a)

O-4-aminophenyl *O*,*O*-diethyl phosphorothioate

diethyl hydrogen phosphate

O-ethyl *O*-methyl *O*-4-nitrophenyl phosphorothioate

diethyl 4-nitrophenyl phosphate

O,*O*-diethyl *O*-hydrogen phosphorothioate

diethyl hydrogen phosphate

(b)

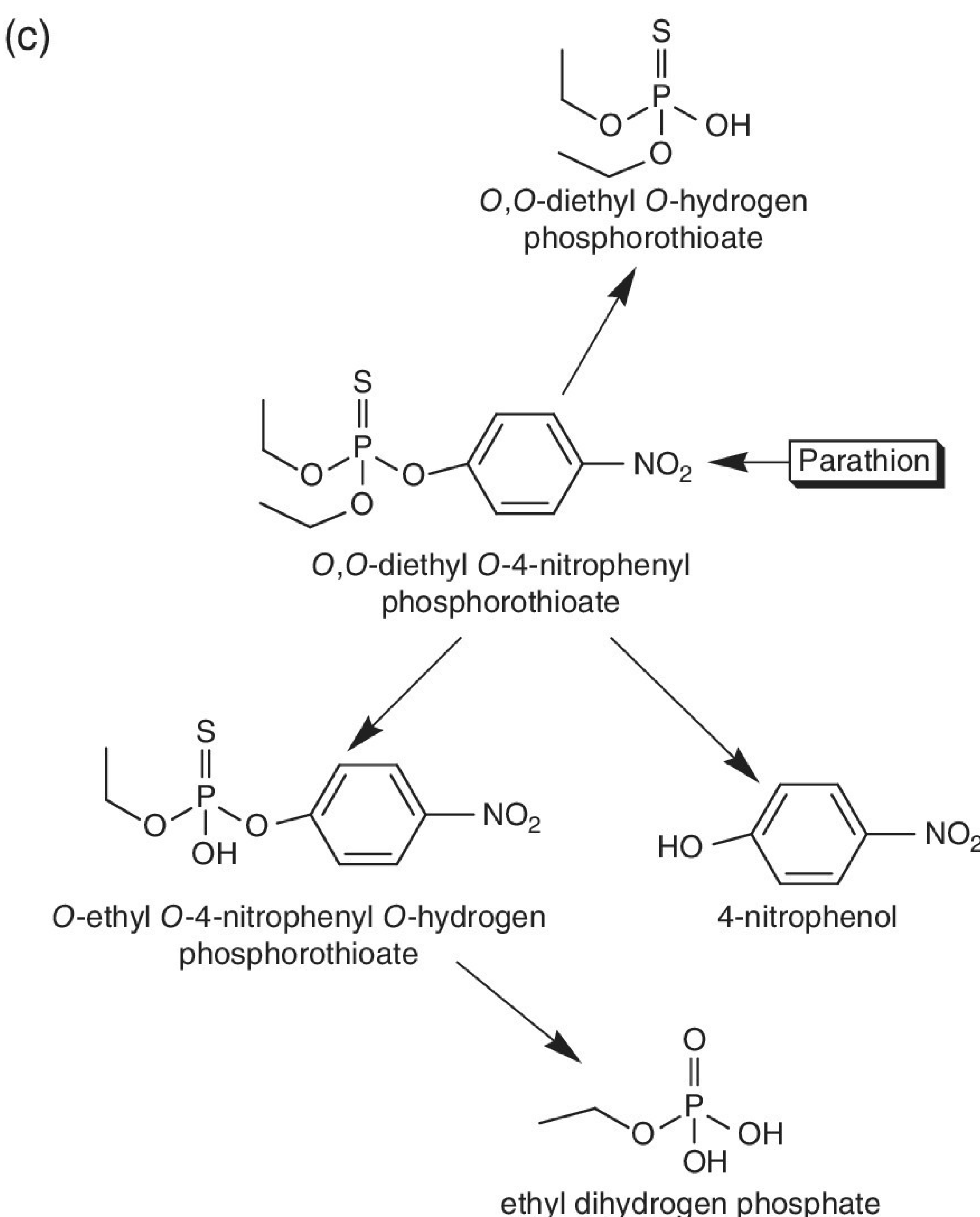
diethyl hydrogen phosphate

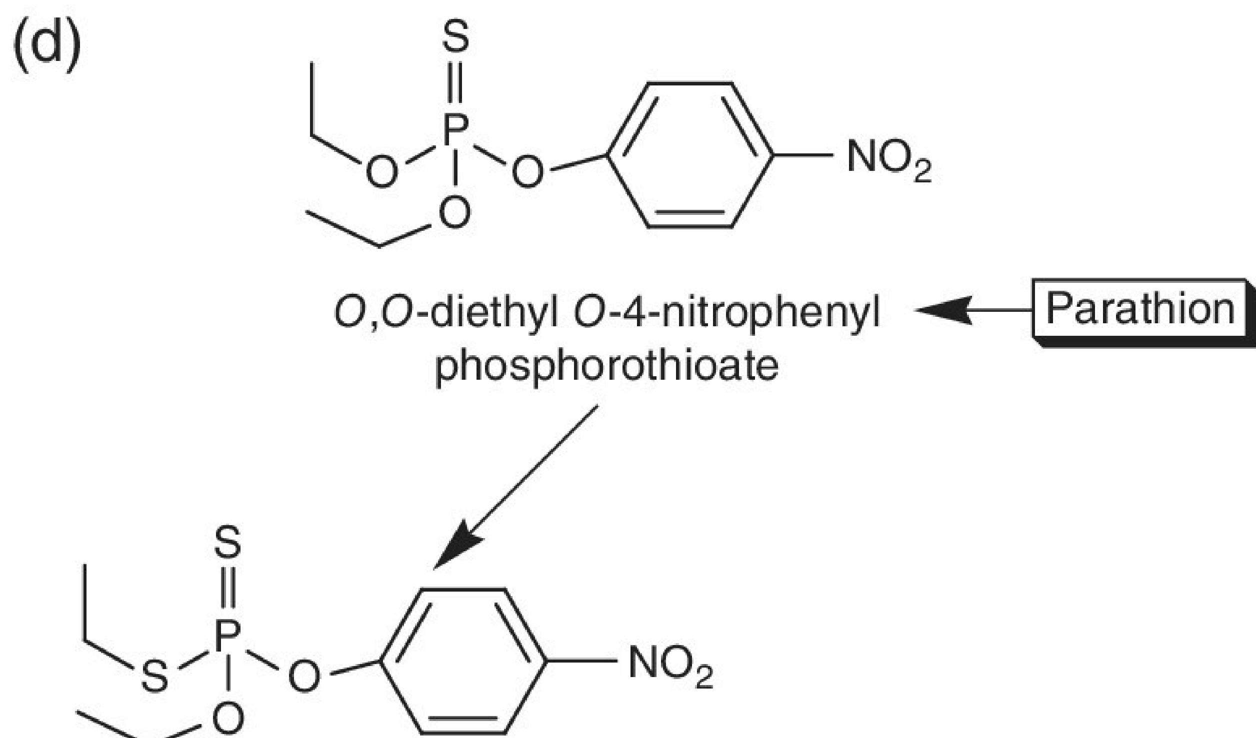
Parathion

O,*O*-diethyl *O*-4-nitrophenyl phosphorothioate

diethyl 4-nitrophenyl phosphate

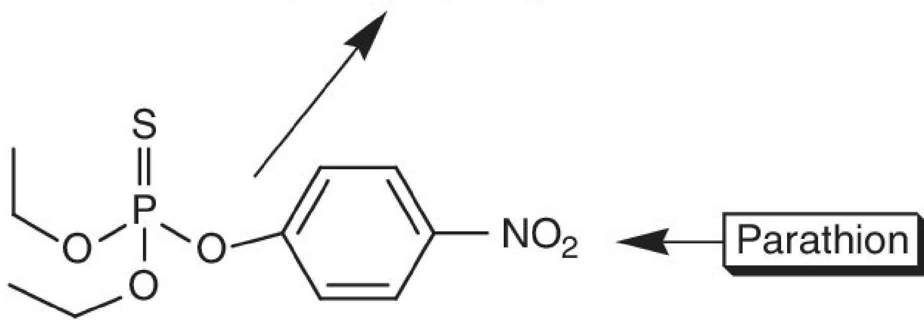
4-nitrophenol


(c)
S
P
O
OH
O
O,O-diethyl O-hydrogen
phosphorothioate
S
P
O
O
O
NO2
Parathion
O,O-diethyl O-4-nitrophenyl
phosphorothioate
S
P
O
O
OH
NO2
HO
NO2
O-ethyl O-4-nitrophenyl O-hydrogen
phosphorothioate
4-nitrophenol
O
P
O
OH
OH
ethyl dihydrogen phosphate

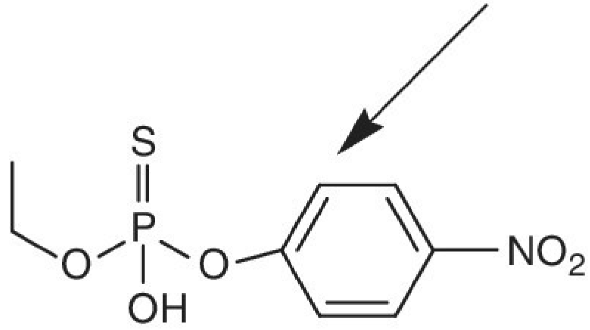

(d)
S
P
O
O
O
NO_2
O,*O*-diethyl *O*-4-nitrophenyl
phosphorothioate
Parathion
S
P
S
O
O
NO_2
O,*S*-diethyl *O*-4-nitrophenyl
phosphorodithioate

(e)

4-nitrophenyl hydrogen sulfite



O,*O*-diethyl *O*-4-nitrophenyl phosphorothioate



O-ethyl *O*-4-nitrophenyl *O*-hydrogen phosphorothioate

(f)

Parathion

O,O-diethyl O-4-nitrophenyl phosphorothioate

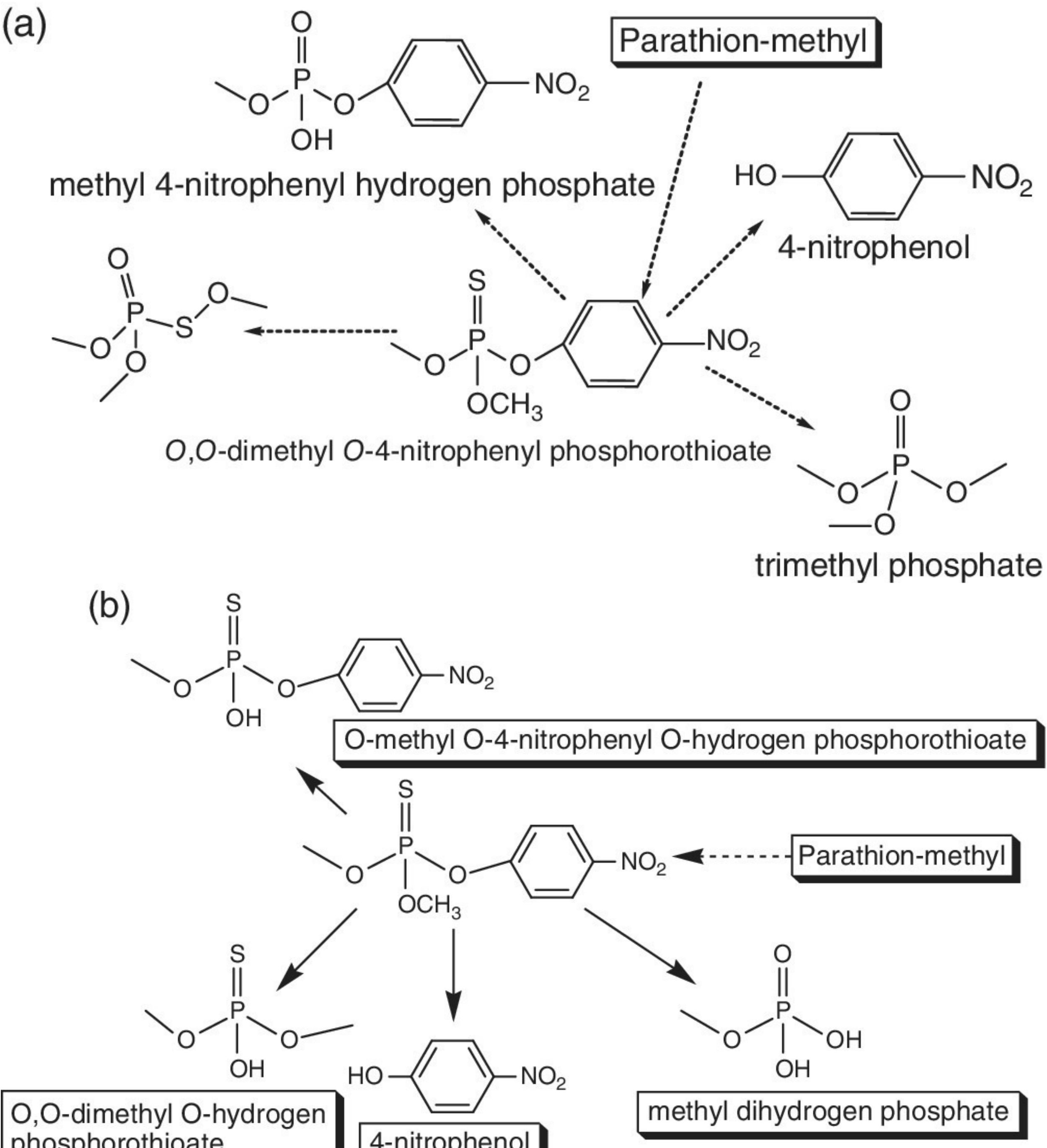
4-nitrophenol

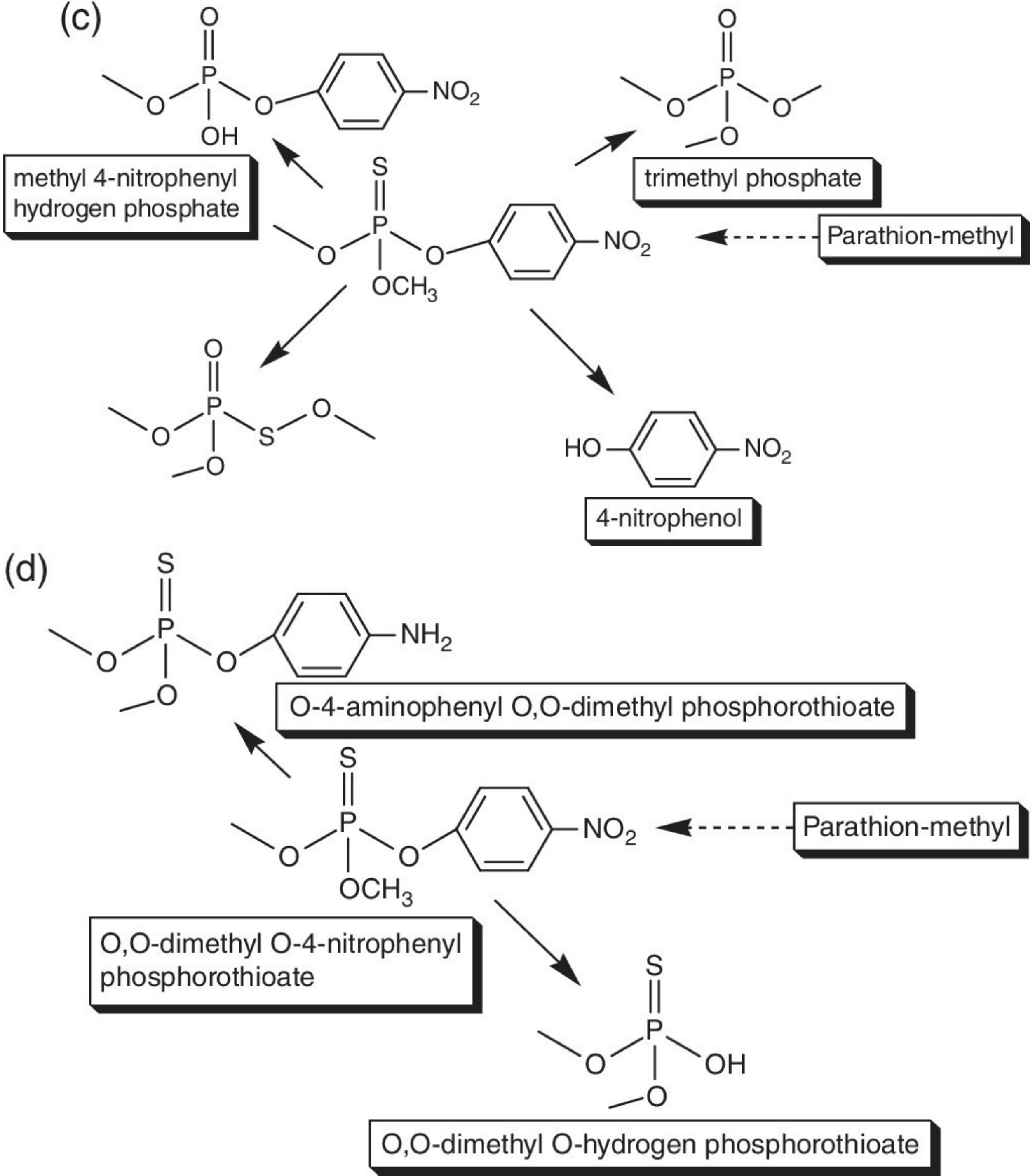
4-nitro-glucuronide derivative

Figure 4.5 (a) Proposed metabolic pathways for parathion in animals; (b) Proposed photolysis metabolic pathways for parathion.

Parathion-methyl, on the other hand, has a $\log K_{OW}$ value of 3.0 (thus relatively less hydrophobic as compared to parathion) and water solubility of 55 mg/L (20 °C) (thus more soluble in water as compared to parathion.

The major biotransformation pathways for parathion-methyl proceed via desulfuration, a process that leads to the generation of paraoxon methyl (an oxon derivative). Another biotransformation route for parathion methyl proceeds via hydrolysis, which results in the generation of products such as dimethyl phosphate, dimethylphosphorothionate, and 4-nitrophenol. Other metabolites include those which are obtained via the reduction of the nitro functional group and also via desmethylation to produce the desmethyl-paraoxon methyl. The process of desmethylation takes place in the liver and is catalyzed by glutathione-S-methyl transferase. The metabolic pathways in phase two metabolism proceed mainly via conjugation of 4-nitrophenol and then these conjugated metabolites are eliminated through excretion. Figure 4.6a–f depicts the detailed metabolic pathway of parathion methyl.


(a)
Parathion-methyl
NO_2
OH
methyl 4-nitrophenyl hydrogen phosphate
HO
NO_2
4-nitrophenol
S
OCH_3
O,O-dimethyl O-4-nitrophenyl phosphorothioate
trimethyl phosphate
(b)
O-methyl O-4-nitrophenyl O-hydrogen phosphorothioate
Parathion-methyl
O,O-dimethyl O-hydrogen phosphorothioate
4-nitrophenol
methyl dihydrogen phosphate



(c)
methyl 4-nitrophenyl hydrogen phosphate
trimethyl phosphate
Parathion-methyl
4-nitrophenol
(d)
O-4-aminophenyl O,O-dimethyl phosphorothioate
Parathion-methyl
O,O-dimethyl O-4-nitrophenyl phosphorothioate
O,O-dimethyl O-hydrogen phosphorothioate

Figure 4.6 (a) Proposed metabolic pathway for parathion-methyl; (b) Proposed acidic hydrolysis metabolic pathway for parathion-methyl; (c) Proposed alkaline hydrolysis metabolic pathway for parathion-methyl; (d) Proposed metabolic pathway for parathion-methyl in goats.

Organophosphate Agrochemicals: Pirimiphos

Organophosphate Agrochemicals: Pirimiphos-methyl

Pirimiphos-methyl (Actellic) (*O*-[2-(diethylamino)-6-methylpyrimidin-4-yl] *O*,*O*-dimethyl phosphorothioate), has a molecular formula of $C_{11}H_{20}N_3O_3PS$ (Mwt 305.334); is an organophosphate pesticide with log K_{OW} value of 4.2; and water solubility of 9.9 mg/L (at 30 °C and pH 5.2). Its chemical structure is presented in Figure 4.7.

Figure 4.7 Chemical structure of pirimiphos-methyl.

The metabolism of pirimiphos-methyl begins with its hydrolysis to produce 2-diethylamino-6-methyl-pyrimidin-4-ol and the hydrolytic by-product may either undergo conjugation or become *N*-de-ethylated. The hydrolysis can either be acidic or alkaline, which generates pyrimidinol as the major metabolic product (Figure 4.8a,b).

Glucuronide derivative

1-(*N*-ethyl-*N*-(pyrimidin-5-yl)amino)ethanol

Pirimiphos-methyl

O-2-(diethylamino)-6-methylpyrimidin-4-yl *O*,*O*-dimethyl phosphorothioate

2-(ethylamino)-6-methylpyrimidin-4-ol

2-(diethylamino)-6-methylpyrimidin-4-ol

2-amino-6-methylpyrimidin-4-ol

Figure 4.8a Proposed metabolic pathways of pirimiphos-methyl in animals.

Figure 4.8b Proposed metabolic pathways of pirimiphos-methyl in plants.

Organophosphate Agrochemicals: Diazinon

Diazinon (Figure 4.9) (*O*,*O*-diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate; chemical formula $C_{12}H_{21}N_2O_3PS$; and Mwt = 304.34 g ·

mol^{-1}), is another organophosphate agrochemical. It has a logK_{OW} value of 3.3 and water solubility of 60 mg/L (20 °C). The chemical structure is depicted in Figure 4.9.

Figure 4.9 Chemical structure of diazinon.

The metabolic pathways for diazinon proceed via the cleavage of the P-*O*-pyrimidine linkage to form 3-isopropyl-4-methyl-6-hydroxypyrimidine. Other metabolic products such as diazoxon, in which the pyrimidinyl group has been lost, are obtained through the process that involves the oxidative desulfuration of the thiono functional group. This process is mediated by microsomal mixed function oxidases. The diazoxon then becomes hydrolysed by the action of either A-esterases or enzymatic oxidative processes mediated by mixed function oxidases to generate diethyl phosphorothioate. In addition to these, other metabolites are formed through hydroxylation processes of the alkyl groups, mainly the methyl and isopropyl groups present on the pyrimidine ring. Figure 4.10a–d depicts the metabolic pathways of diazinon.

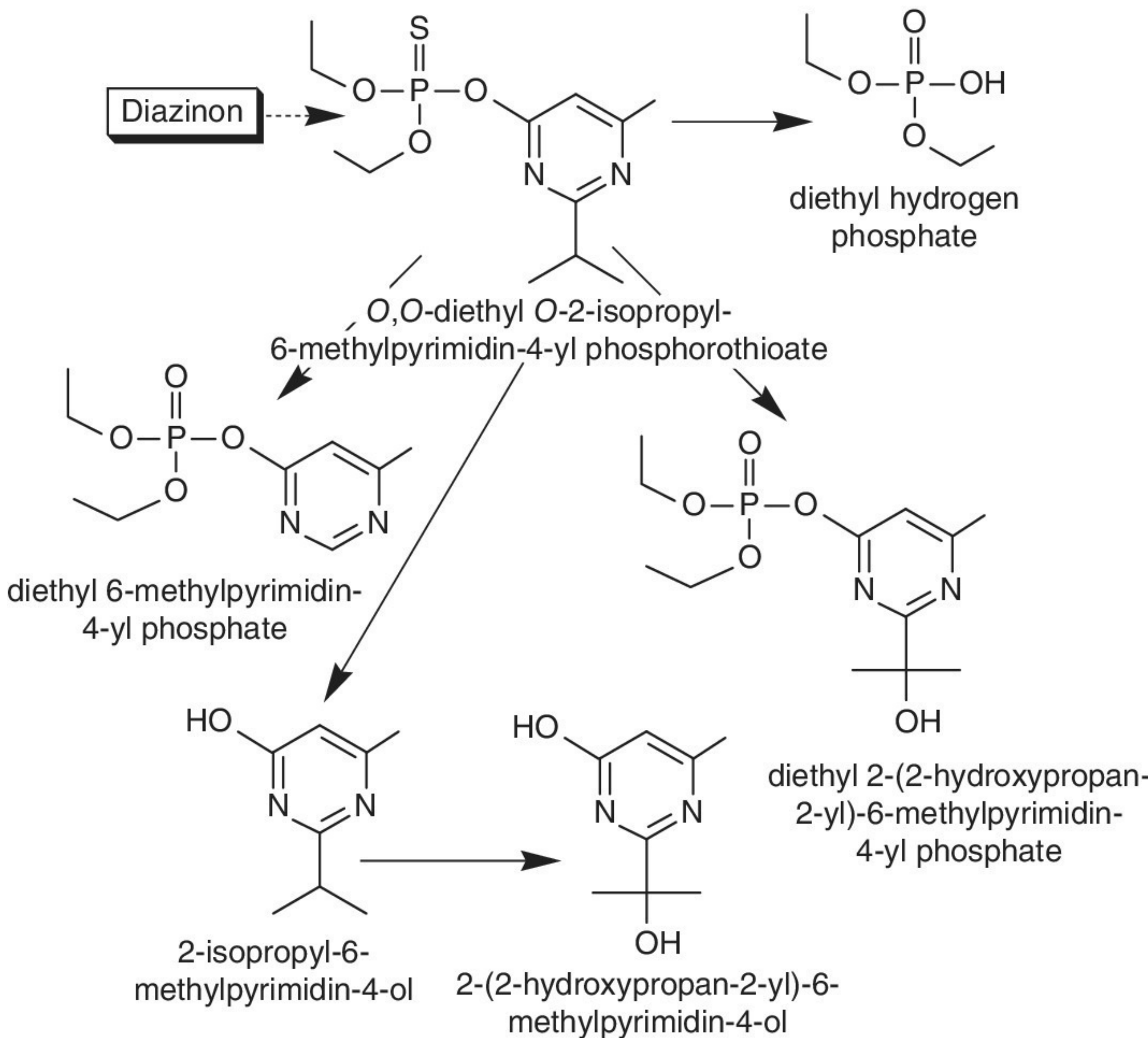


Figure 4.10a Proposed metabolic pathways for diazinon in plants.

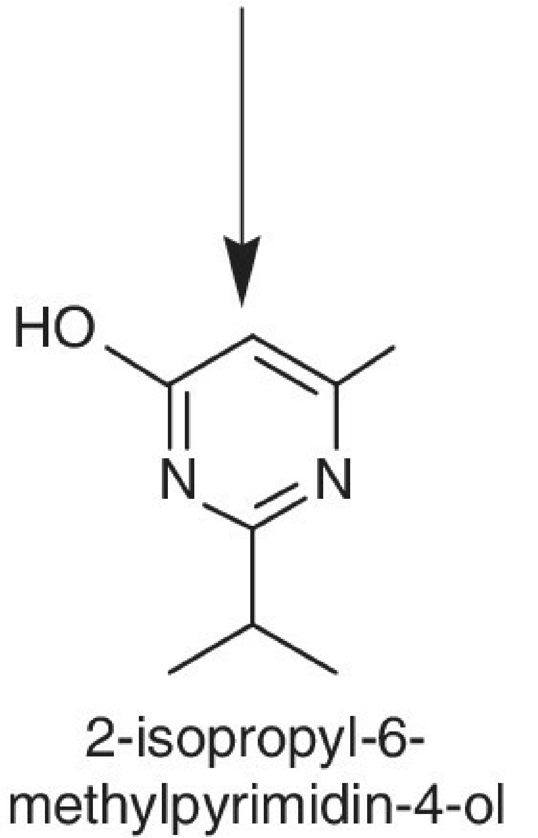
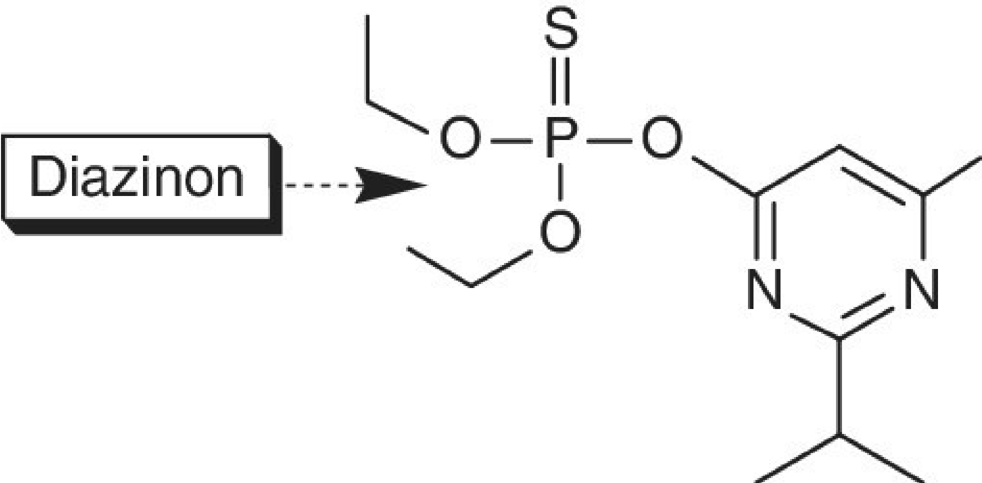


Figure 4.10b Proposed hydrolysis metabolic pathways for diazinon.



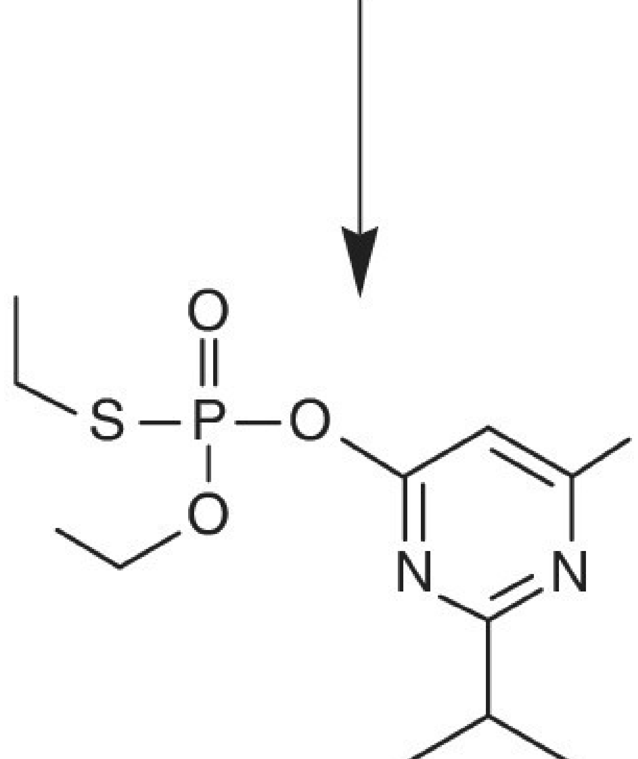


Figure 4.10c Proposed photolysis metabolic pathways for diazinon.

Diazinon

O,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate

O,*O*-diethyl *O*-6-(hydroxymethyl)-2-isopropylpyrimidin-4-yl phosphorothioate

O,*S*-diethyl *O*-2-(2-hydroxypropan-2-yl)-6-methylpyrimidin-4-yl phosphorothioate

diethyl 2-isopropyl-6-methylpyrimidin-4-yl phosphate

2-isopropyl-6-methylpyrimidin-4-ol

2-(1-hydroxyethyl)-6-methylpyrimidin-4-ol

2-(1-hydroxy-2-methylpropan-2-yl)-6-methylpyrimidin-4-ol

O,*O*-diethyl *O*-6-(hydroxymethyl)-2-(prop-1-en-2-yl)pyrimidin-4-yl phosphorothioate

Figure 4.10d Proposed metabolic pathways for diazinon in animals.

Organophosphate Agrochemicals: Dichlorvos

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate, DDVP; molar mass: 220.98 g/mol; $\log K_{OW}$ value of 1.9; and water solubility of 18.0 mg/L (25 °C)), is an organophosphate with the chemical structure shown in Figure 4.11.

Figure 4.11 Chemical structure of dichlorvos.

The biotransformation of dichlorvos proceeds via enzymatically catalyzed hydrolytic processes, which then generate dimethyl phosphate and dichloroacetaldehyde as initial products. Dichloroacetaldehyde can then proceed through more steps of metabolic reactions to yield 2,2-dichloroethanol, dechlorination of dichloroacetaldehyde, to form glycolic acid. Other metabolites that are of importance include methyl glutathione and desmethyldichlorvos, which are produced via the demethylation processes that are mediated by glutathione-S-methyl transferases.

Organophosphate Agrochemicals: Coumaphos

Coumaphos organophosphate (*O*,*O*-diethyl *O*-3-chloro-4-methyl-2-oxo-2H-chromen-7-yl phosphorothioate) has a molecular formula $C_{14}H_{16}C_{l}O_{5}PS$; Mwt 362.77 g/mol; $\log K_{OW}$ value of 4.13; and water solubility of 1.3 mg/L (20 °C). Its chemical structure is depicted in Figure 4.12.

Figure 4.12 Chemical structure of coumaphos.

The metabolites related to coumaphos are normally produced through a number of processes such as hydrolysis, oxidation, and oxidative desulfuration, which generate coumaphos-oxon prior to hydrolysis. Other metabolic products are formed through dechlorination, as well as those formed after the lactone group of the coumarin ring has been opened. The metabolic pathways for coumaphos are depicted in Figure 4.13a–c.

Coumaphos

O-3-chloro-4-methyl-2-oxo-
2*H*-chromen-7-yl *O*,*O*-diethyl phosphorothioate

3-chloro-7-hydroxy-4-methyl-
2*H*-chromen-2-one

Figure 4.13a Proposed metabolic pathways for coumaphos in animals.

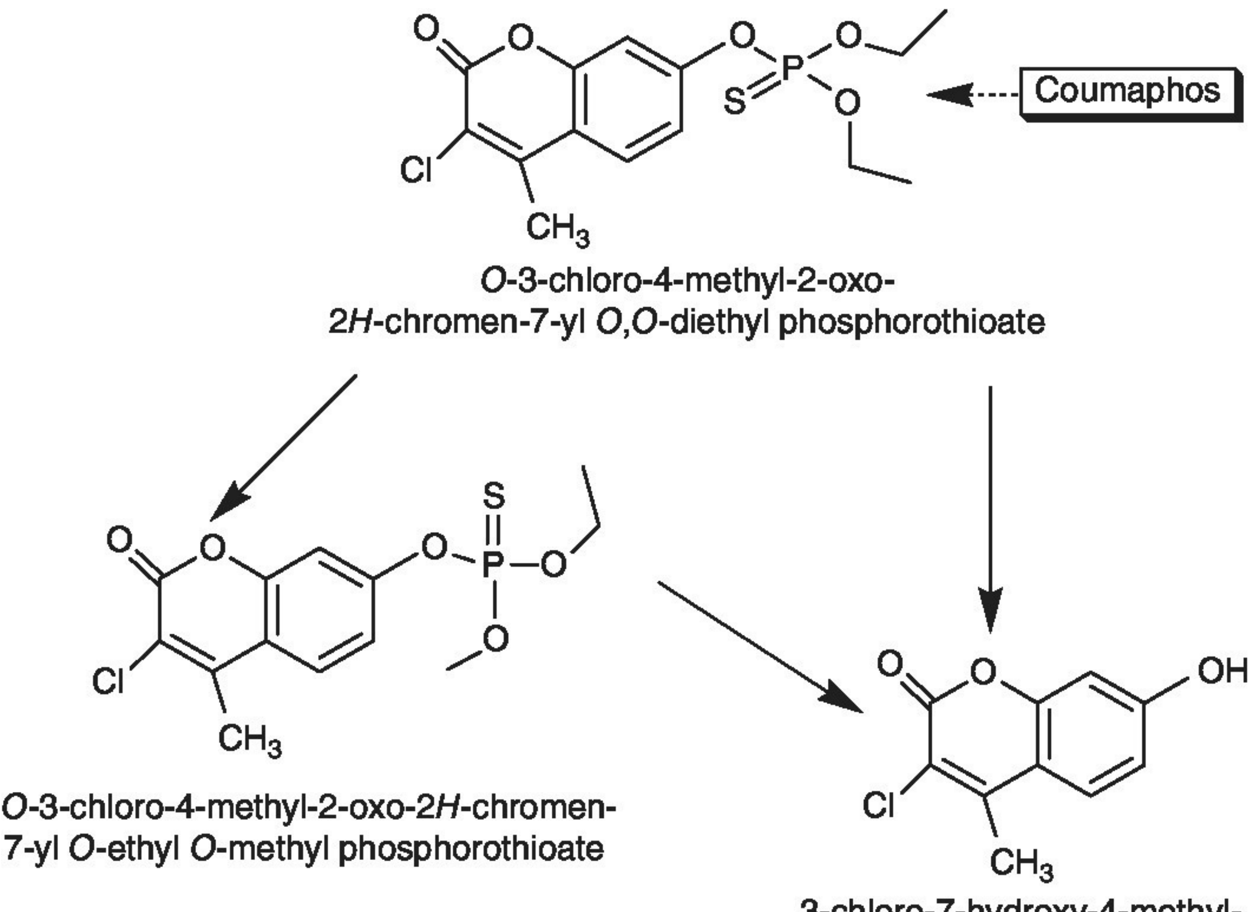


Figure 4.13b Proposed hydrolysis metabolic pathways for coumaphos.

Coumaphos

O-3-chloro-4-methyl-2-oxo-
2*H*-chromen-7-yl *O*,*O*-diethyl phosphorothioate

A DIMER OF
O-3-chloro-4-methyl-2-oxo-2*H*-chromen-
7-yl *O*-ethyl *O*-methyl phosphorothioate

A DIMER OF
3-chloro-4-methyl-2-oxo-2*H*-chromen-
7-yl ethyl methyl phosphate

Figure 4.13c Proposed photolysis metabolic pathways for coumaphos.

Organophosphate Agrochemicals: Chlorpyrifos

Chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate) has a molecular formula $C_9H_{11}C_{l3}NO_3PS$; Mwt = 350.59 g/mol; $\log K_{OW}$ value of 4.7; and water solubility of 1.4 mg/L (25 °C). The structure of chlorpyrifos is depicted in Figure 4.14.

Figure 4.14 Chemical structure of chlorpyrifos.

The metabolites in cases of chlorpyrifos poisoning are generated through a number of metabolic pathways, including oxidative dealkylation or hydrolysis. The oxidation of chlorpyrifos normally proceeds via acidic, neutral, and alkaline media. The products of hydrolysis include diethyl phosphorothioate and 3,5,6-trichloro-2-pyridinol. The 3,5,6-trichloro-2-pyridinol is then conjugated to form either glycoside or glucuronide derivatives. Chlorpyrifos-oxon is another metabolite that is generated; however, it is rapidly hydrolysed, thus normally not detected. The metabolic pathways are shown in Figure 4.15a–d.

Chlorpyrifos

O-3,5,6-trichloropyridin-2-yl
O,*O*-diethyl phosphorothioate

O-3,6-dichloro-5-(methylthio)pyridin-2-yl
O,*O*-diethyl phosphorothioate

Figure 4.15a Proposed metabolic pathways for chlorpyrifos in humans.

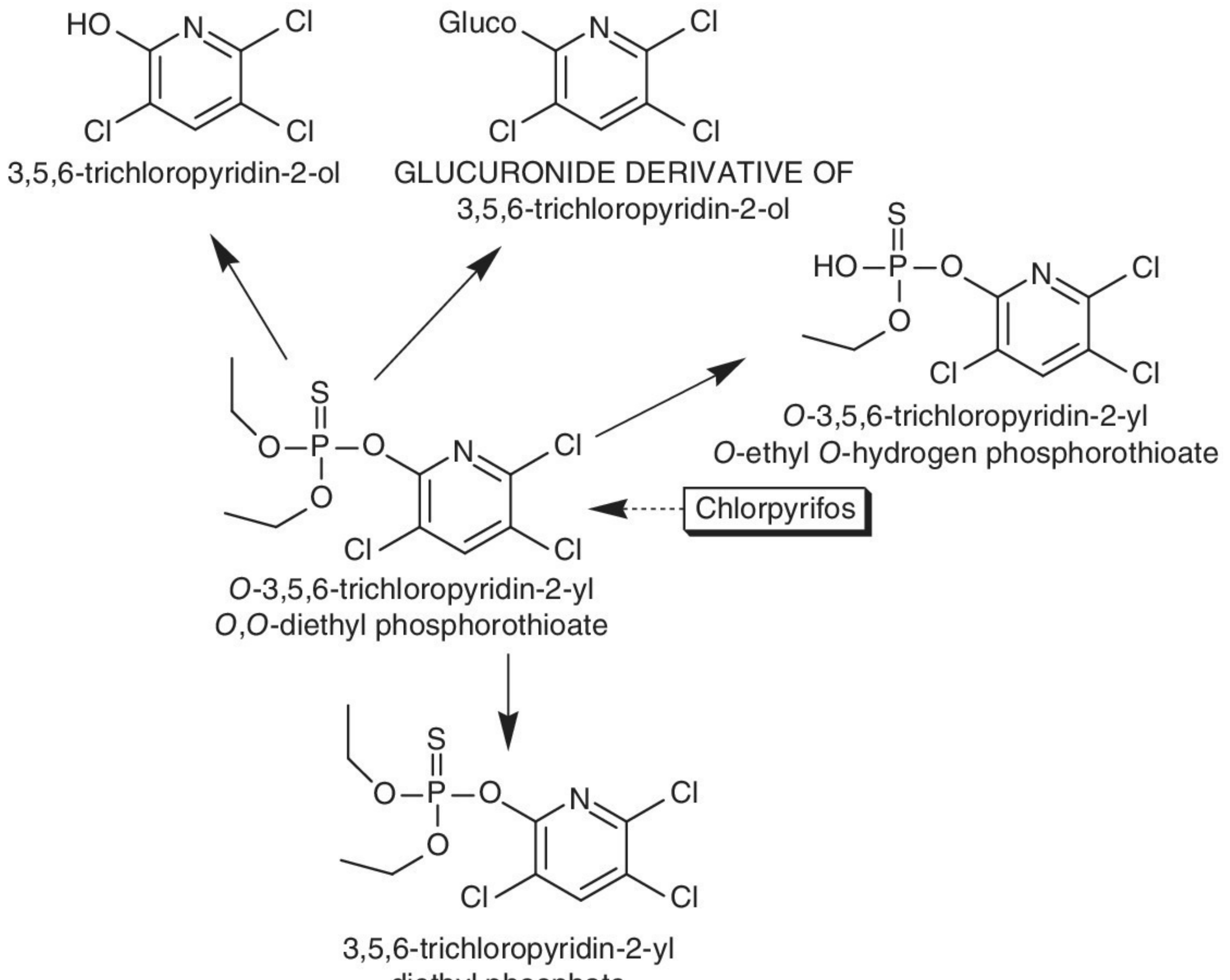


Figure 4.15b Proposed metabolic pathways for chlorpyrifos in plants.

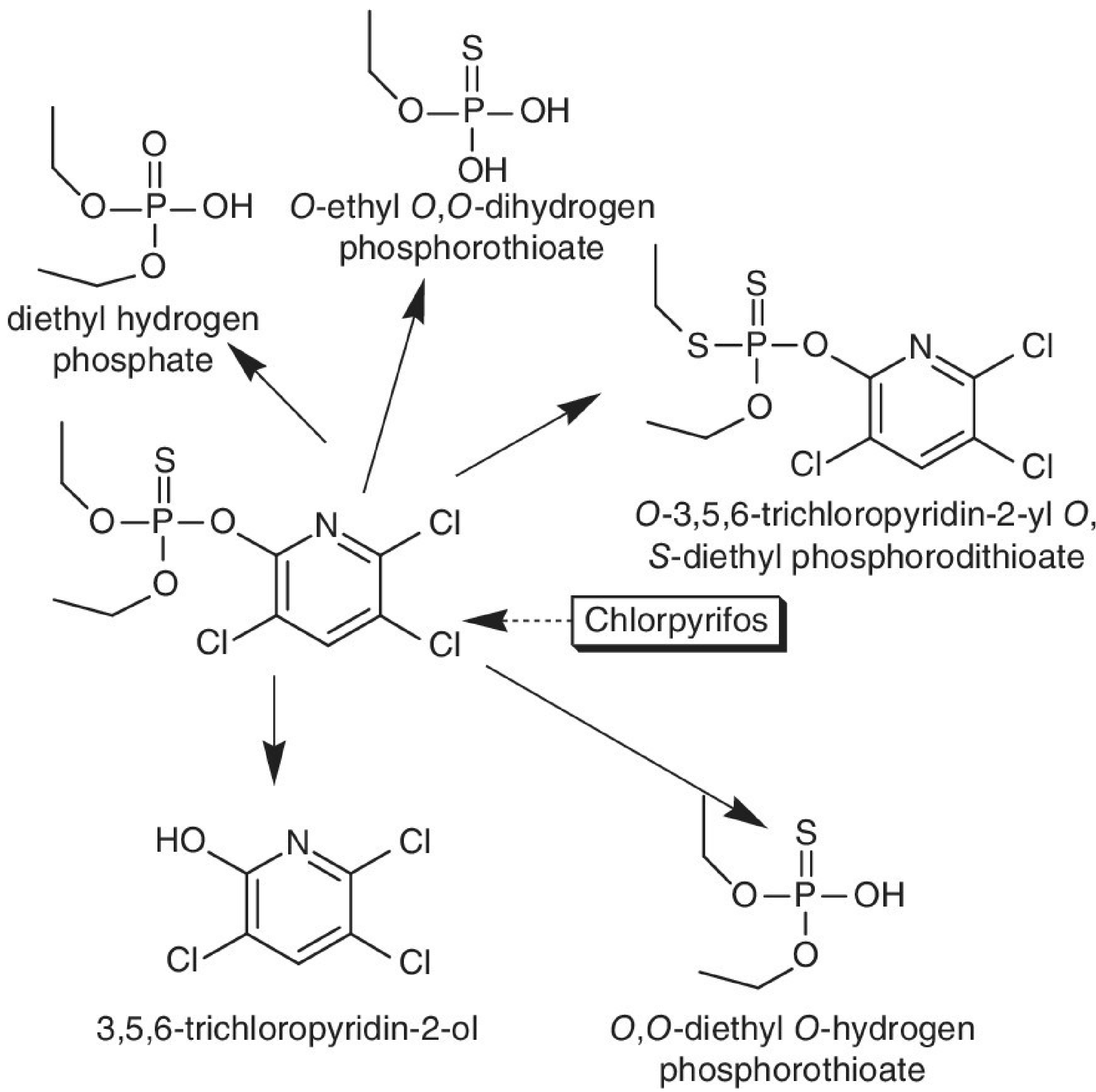


Figure 4.15c Proposed metabolic pathways for chlorpyrifos in goats.

H_3CO N Cl

Cl Cl

2,3,5-trichloro-6-methoxypyridine

S

O—P—O N Cl

O

Cl Cl

Chlorpyrifos

O-3,5,6-trichloropyridin-2-yl
O,*O*-diethyl phosphorothioate

HO N Cl

Cl Cl

3,5,6-trichloropyridin-2-ol

GlucO N Cl

Cl Cl

GLUCURONIDE DERIVATIVE OF
3,5,6-trichloropyridin-2-ol

Figure 4.15d Proposed metabolic pathways for chlorpyrifos in fish.

Organophosphate Agrochemicals: Chlorpyrifos-methyl

Chlorpyrifos-methyl (*O*,*O*-dimethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) with a molecular formula of $C_7H_7C_{l3}NO_3PS$; Mwt = 322.5 g/mol; water solubility of 2.6 mg/L (25 °C); and $logK_{OW}$ value of 4.26, is an organophosphosphate pesticide with the chemical structure as depicted in Figure 4.16.

Figure 4.16 Chemical structure of chlorpyrifos-methyl.

Unlike the metabolic pathways in chlorpyrifos, in chlopyrifos-methyl, the most important metabolic route for the degradation of the compound involves the demethylation process, which is catalyzed by glutathione-S-alkyl transferase to generate demethylchlorpyrifos-methyl. On the other hand, the major elimination route of chlorpyrifos-methyl metabolites from the body is mediated by the NADP-dependent oxidative dearylation process, which yields a conjugated product 3,5,6-trichloro-2-pyridinol. Like chlorpyrifos, the oxon derivative is rapidly hydrolysed and therefore difficult to detect. The metabolic pathways for chlorpyrifos-methyl are presented in Figure 4.17a–c.

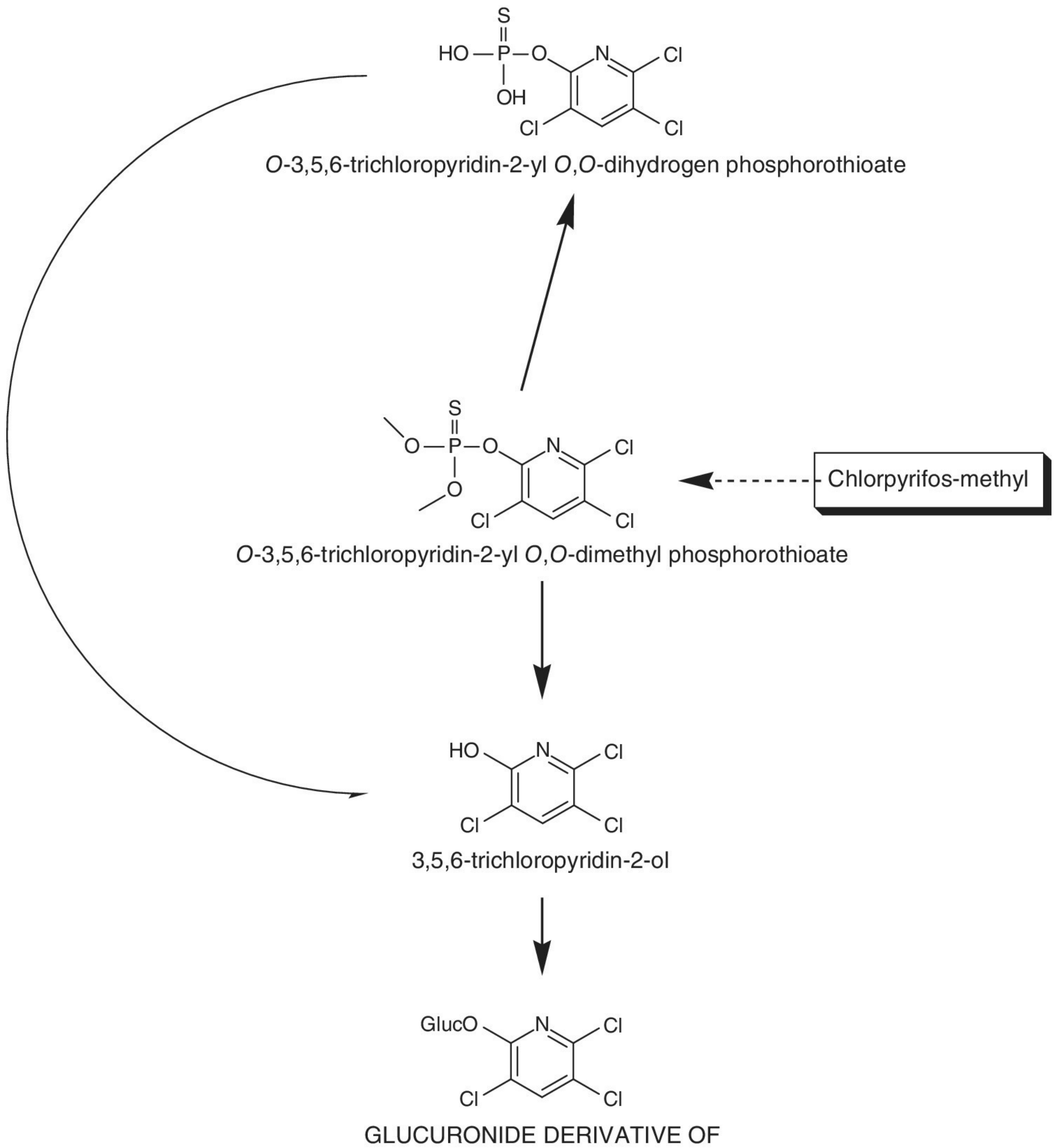


Figure 4.17a Proposed metabolic pathways for chlorpyrifos-methyl in animals.

S
O — P — O N Cl
O
Cl Cl

O-3,5,6-trichloropyridin-2-yl *O*,*O*-dimethyl phosphorothioate

Chlorpyrifos-methyl

HO N Cl
Cl Cl

3,5,6-trichloropyridin-2-ol

Figure 4.17b Proposed metabolic pathways for chlorpyrifos-methyl in plants.

Chlorpyrifos-methyl

O-3,5,6-trichloropyridin-2-yl *O*,*O*-dimethyl phosphorothioate

O-3,5,6-trichloropyridin-2-yl *O*-methyl *O*-hydrogen phosphorothioate

3,5,6-trichloropyridin-2-ol

3,5,6-trichloropyridin-2-yl dihydrogen phosphate

Figure 4.17c Proposed hydrolysis metabolic pathways for chlorpyrifos-methyl.

Organophosphate Agrochemicals: Azinphos-ethyl

Azinphos-ethyl (3-(diethoxyphosphinothioylsulfanylmethyl)-1,2,3-benzotriazin-4-one) with a chemical formula of $C_{12}H_{16}N_3O_3PS_2$, Mwt = 345.37 g/mol; $\log K_{OW}$ value of 3.18; and water solubility of 4.5 mg/L (20 °C), is an organophosphate agrochemical with the chemical structure depicted in Figure 4.18.

Figure 4.18 Chemical structure of azinphos-ethyl.

In poisoning incidences that involve azinphos-ethyl, metabolites are produced via both acidic and alkaline hydrolysis processes. Azinphos is known to be unstable under neutral conditions. The details of the metabolic pathway are depicted in Figure 4.19a,b.

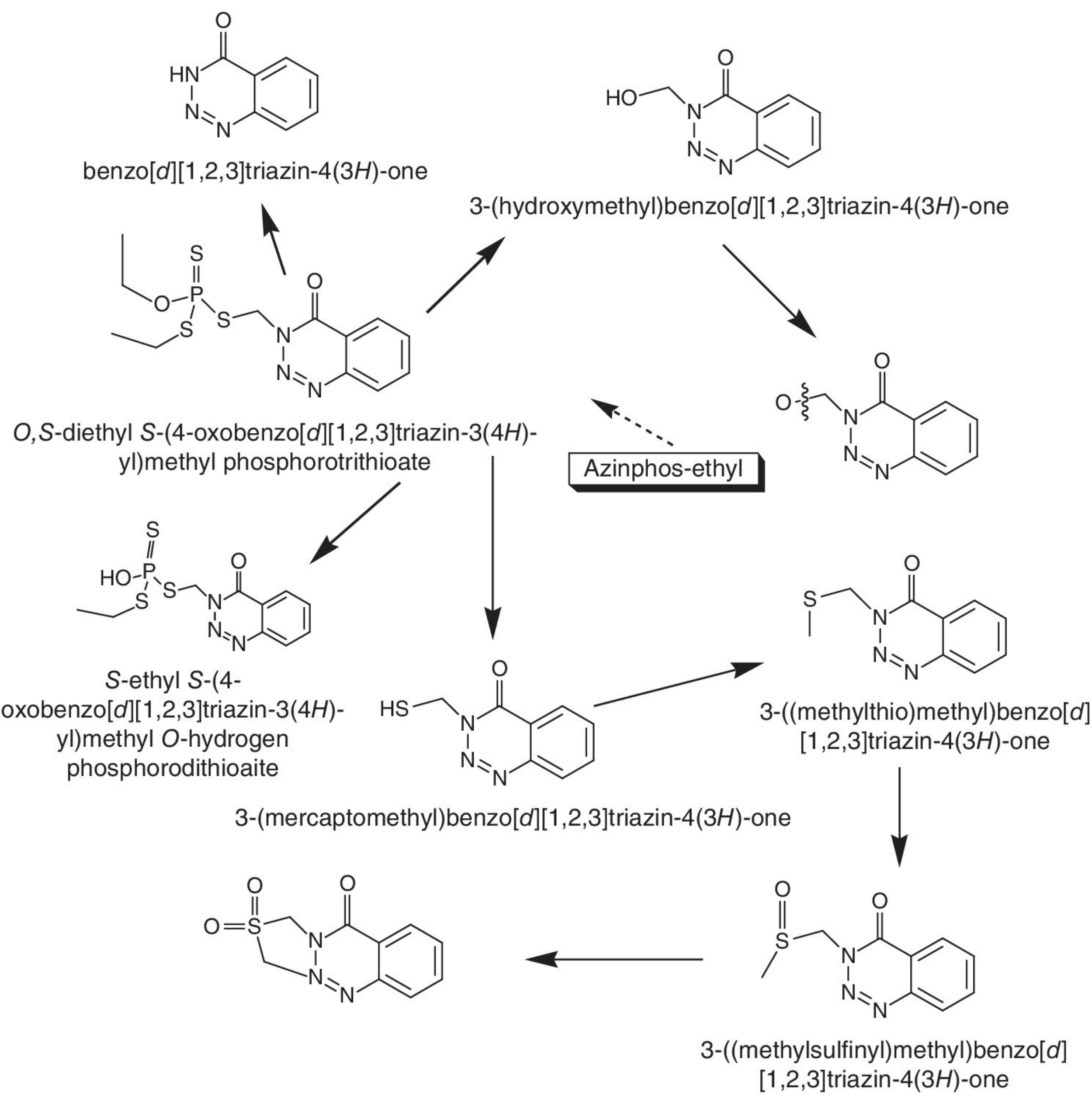


Figure 4.19a Proposed metabolic pathways for azinphos-ethyl in animals.

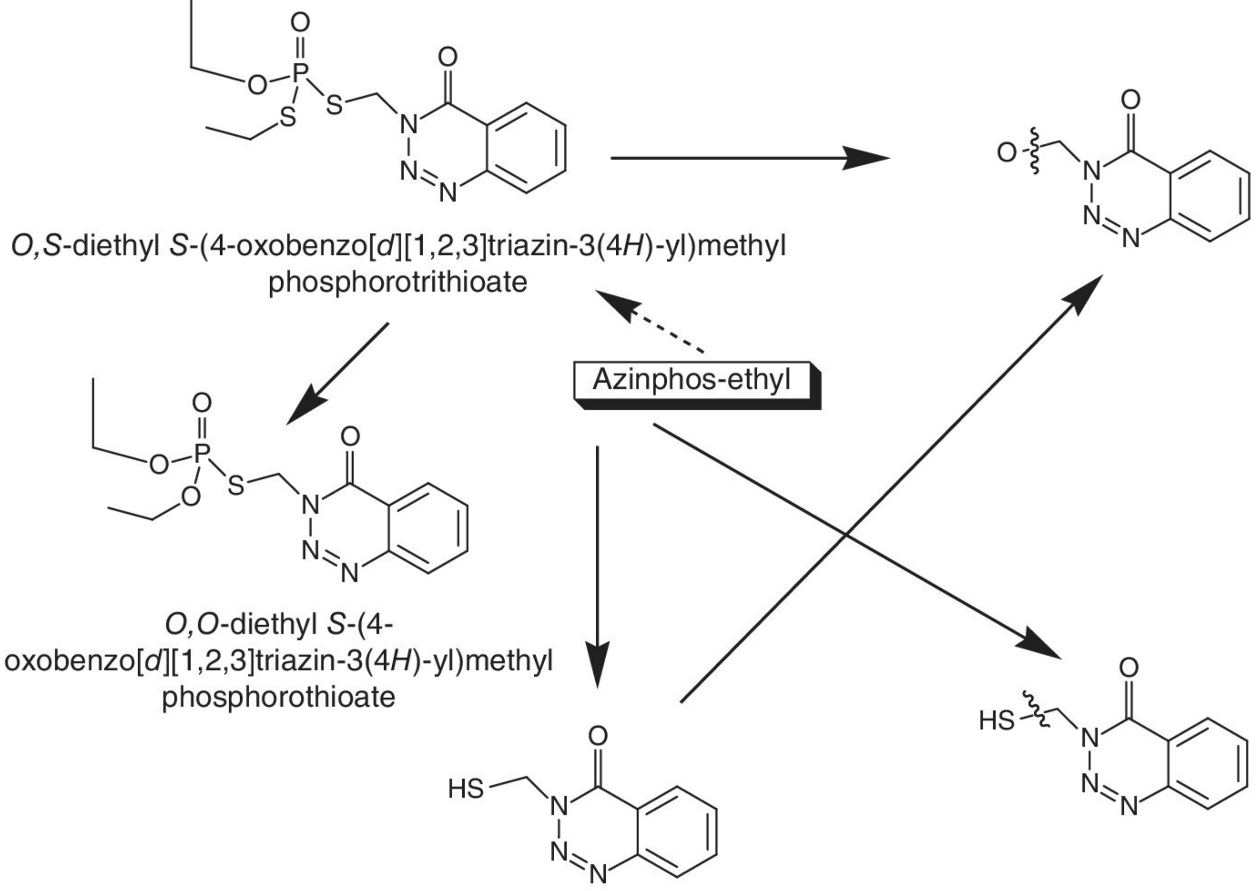


Figure 4.19b Proposed metabolic pathways for azinphos-ethyl in plants.

Organosphosphate Agrochemicals: Azinphos-methyl

Azinphos-methyl (*O*,*O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl]dithiophosphate) with molecular formula $C_{10}PN_3H_{12}S_2O_3$; Mwt of 317.324 g/mol; $logK_{OW}$ value of 2.96; water solubility of 28 mg/L (20 °C), has its chemical structure given in Figure 4.20.

Figure 4.20 Chemical structure of azinphos-methyl.

In azinphos-methyl poisoning incidences, the metabolites that are generated include 3-(thiomethyl)benzazimide, which is further biotransformed through dimerization processes on the one hand and also by another route, the metabolites may proceed either via the reduction of the thiol group or via 3-demethylation or ring opening that generates disulfide metabolites such as 3-methylbenzazimide, benzazimide, and anthranilic acid. Conjugated metabolites are also formed. Figure 4.21a–d details the metabolic pathways for azinphos-methyl.

H_3C S N O N N

3-((methylthio)methyl)benzo[*d*][1,2,3]triazin-4(3*H*)-one

S P O S S N O N N

O,S-dimethyl *S*-(4-oxobenzo[*d*][1,2,3]triazin-3(4*H*)-yl)methyl phosphorotrithioate

S N O N N

Azinphos-methyl

O P O O S N O N N

O,O-diethyl *S*-(4-oxobenzo[*d*][1,2,3]triazin-3(4*H*)-yl)methyl phosphorothioate

O HN N N

benzo[*d*][1,2,3]triazin-4(3*H*)-one

S Cys N O N N

Figure 4.21a Proposed photolysis metabolic pathways for azinphos-methyl.

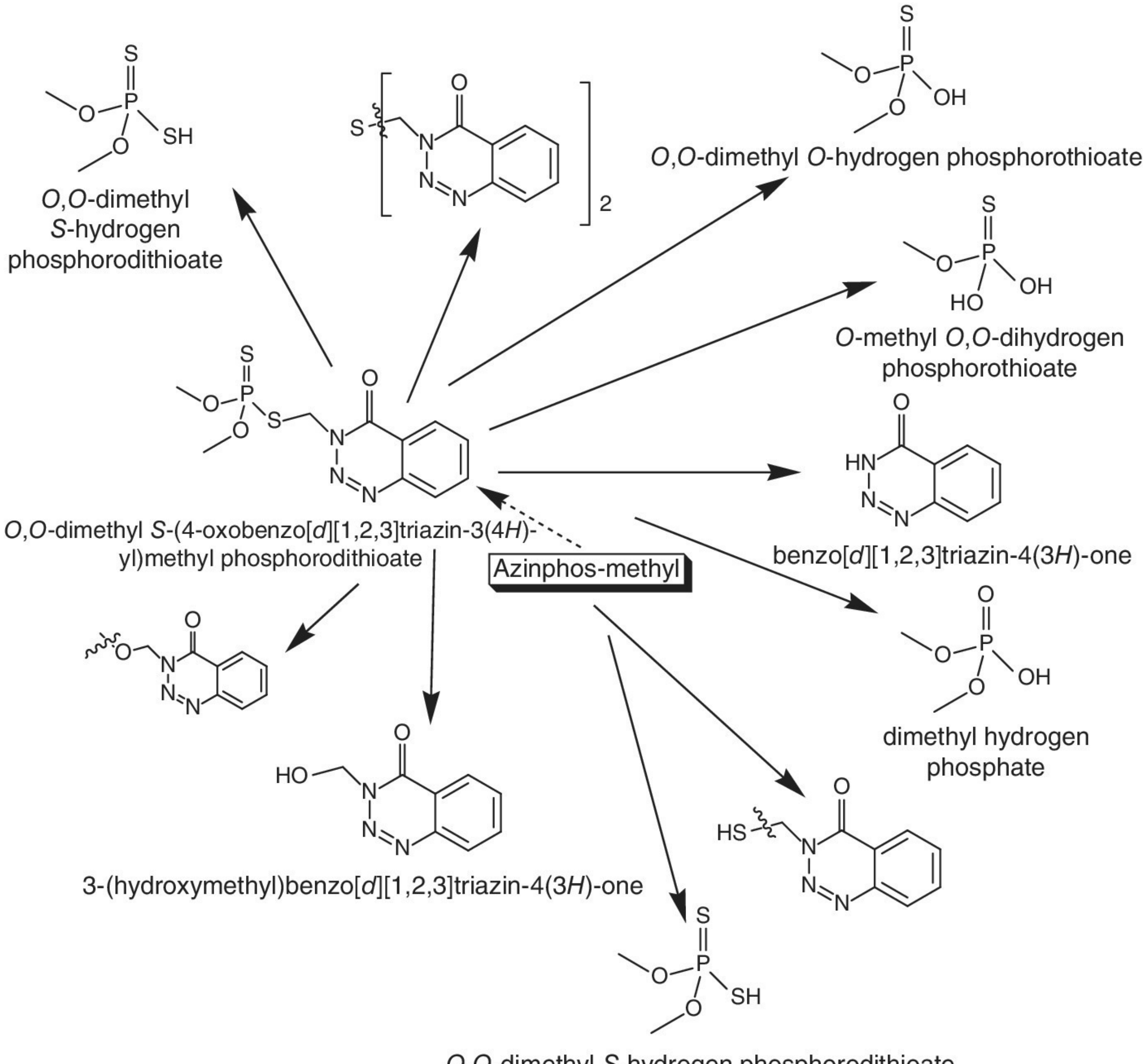


Figure 4.21b Proposed metabolic pathways for azinphos-methyl in animals.

O,*O*-dimethyl *S*-(4-oxobenzo[*d*][1,2,3]triazin-3(4*H*)-yl)methyl phosphorodithioate

benzo[*d*][1,2,3]triazin-4(3*H*)-one

Azinphos-methyl

benzamide

Figure 4.21c Proposed metabolic pathways for azinphos-methyl in plants.

Figure 4.21d Proposed thermal degradation metabolic pathways for azinphos-methyl.

Generally, different classes and individual organophosphates as well as carbamates tend to inhibit acetylcholinesterase (Costa *et al*., 2005).

However, there is a common factor in that all classes and individual organophosphates and carbamates poisonings result in toxic effects to the victims when they inhibit cholinesterase enzymes in the nervous system, because these agrochemicals cause cholinesterase enzymes to be phosphorylated, thus blocking their ability to break down acetylcholine into choline and acetic acid (Costa *et al*., 2005). The high levels of toxicity due to the organophosphates and their metabolites in the body can thus be indicated by a measure in the reduction

in the level of a number of enzymes, including that of cholinesterase enzyme activity in blood as worked out from the difference between the pre-dose and post-dose poisoning (He *et al*., 2002). The measure of cholinesterase to indicate poisoning due to organophosphates may become unreliable in cases of low level poisoning, due to lack of selectivity and sensitivity and it also requires that the experiment incorporates a control experiment for the sake of establishing the baseline activity (Margariti *et al*., 2007).

Apart from acetylcholinesterase (AChE), measurement of other blood cholinesterase enzymes such as butyrylcholinesterase (BChE) activities have also been used as primary biomarkers for poisoning cases related to organophosphate and/or carbamate agrochemicals (Simoniello *et al*., 2010; Stefanidou *et al*., 2009). It should be noted that acetylcholinesterase is an enzyme that performs the hydrolytic cleavage of acetylcholine, which is a mediator molecule that facilitates the physiological transmission of nerve action potential in the nervous system (Araoud, 2011).

Butyrylcholinesterase (BChE) is a plasmic pseudocholinesterase enzyme that is found in plasma (Costa *et al*., 2005). Unlike acetylcholinestearse, the decrease in the activity of BChE in the blood is not necessarily an indication of poisoning that is associated with anti-cholinergic substances such as carbamates or organophosphorus compounds, but BChE is normally used as a predictive biomarker for anticholinesterase agents such as organophosphates and carbamate possible poisoning cases (Ranjbar *et al*., 2002; Rastogi *et al*., 2008). However, according to some reports, the poisoning due to certain organophosphate compounds such as diazinon, malathion, dichlorvos, *etc.* can have plasma cholinesterase activity depression effects as a more sensitive indicator than it does for the other organophosphates (Costa *et al*., 2005). This can explain why the extent of inhibition of both AChE and BChE tends to vary significantly within different classes and individual compounds of organophosphates and carbamates, though the measurements of the depression of AChE activity has been universally accepted as a better indication of chronic poisoning exposure to organophosphorus compounds than BChE measurements (Kamel and Hoppin, 2004).

Apart from the toxicity that can be monitored by measuring the depression of cholinesterase and which causes cholinergenic symptoms associated with illness, organophosphorus poisoning is also known to cause another form of neurotoxicity, a central peripheral distal sensory-motor axonopathy, which is known as organophosphate-induced delayed-polyneuropathy (OPIDP). This

neurotoxicity disease is not associated with any depression of cholinesterase enzymes such as AChE, but with phosphorylation of an esterase enzyme known as neuropathy target esterase (NTE) that is present in the nervous system, blood lymphocytes, and platelets and liver (Maroni *et al*., 2000; Costa *et al*., 2005). The chain of biochemical reactions during organophosphorus compound poisoning begins with phosphorylation and this reaction is followed by the transformation of the phosphorylated target and is highly dependent on the nature and functional groups present in organophosphorus compounds, because it can only take place with phosphate, phosphonates, and phosphoramidates compounds. This transformation reaction cannot take place for sulfonates and carbamates (Maroni *et al*., 2000; Costa *et al*., 2005). Therefore measuring levels of NTE in lymphocytes may be a good biomonitoring indicative predictive marker, especially for the organophosphorus compounds that are associated with causing delayed polyneuropathy (Costa and Manzo, 1995).

Another useful biomarker for poisoning that is related to anticholinesterase agrochemicals is β-glucuronidase activity measurements (Ueyama *et al*., 2010). Poisoning due to either organophosphorus or carbamate agrochemicals is normally followed by the cleavage of an enzyme known as the egasyn-glucuronidase complex, which results in an increase in β-glucuronidase activity in the plasma, especially in cases of acute organophosphate poisoning (Hernandez *et al*., 2004).

Genotoxicity (DNA damage) effects caused by agrochemicals (e.g. organochlorines, herbicides, etc.) poisoning has been reported widely and is normally monitored by using cytogenetic markers that include the measurements of chromosomal aberrations (CA), sister chromatid exchange (SCE), and micronuclei (MN) (Bolognesi, 2003; Das *et al*., 2007). The techniques that are normally used to measure changes in the measurements for these markers are mainly either single cell gel electrophoresis (SCGE) or Comet assay, which are capable of determining the degree of DNA or chromosome damage in the victim’s blood cells, such as leukocytes, lymphocytes, *etc.* (Angerer *et al*., 2007).

Some agrochemical poisoning such as that due to paraquat is known to influence changes in the levels of an erythrocyte enzyme known as erythrocyte δ-aminolevulinic acid dehydratase (ALA-D) (Hernandez *et al*., 2005). Scientific reports have indicated that poisoning due to some agrochemicals causes significant depression of ALA-D due to a number of possible reasons, including (i) the generation of oxidative stress; (ii) non-competitive binding of

agrochemical molecules to the enzyme; and (iii) modification of the vicinal sulfhydryl of ALA-D after poisoning, which might trigger certain phenomena that may induce the inhibition of enzyme activity (Noriega *et al*., 2002; Hernandez *et al*., 2005). Variations in the mean corpuscular hemoglobin concentration (MCHC) and variation in the mean platelet volume (MPV) have been used as biomarkers and indicator parameters in cases involving poisoning due to agrochemicals such as organophosphates, organochlorines, and others (Parron *et al*., 1996). In some cases, there is abnormality in the functioning of some organs such as the liver (where there are unusual variations in liver enzyme activities, *e.g.* in serum alanine aminotransferase (ALT), as well as that of aspartate aminotransferase (AST) (Anwar, 1997)) or kidneys (e.g. nephrotic changes as exemplified by increased levels of serum creatine, as well as that of blood urea (Hernandez *et al*., 2006)).

In some cases, poisoning due to agrochemicals tends to disrupt the metabolism of certain amino acids such as tryptophan causing hyperglycemia, and also alters the activity of ALT, AST, gamma glutamyl transferase (GGT), and lactate dehydrogenase (LDH) enzymes (Tsatsakis *et al*., 2009). Therefore, levels of these enzymes can be used as biomarkers of agrochemical poisoning. Moreover, enhancement of the concentration of some molecules such as triglycerides, GGT activity, inorganic phosphorus, and creatine kinase (CK), can be used as an indication of poisoning due to agrochemicals that possess anticholinesterase properties (Parron *et al*., 1996).

However, the measurement of parent molecules of organophosphate agrochemicals in the biological matrices is in most cases not possible, because they are rapidly metabolized by the body system and may not appear in blood or urine. Immediately when they enter the body, they become converted to oxo-phosphate metabolites by the action of enzymes and these oxo-phosphates are then subjected to a reaction with cholinesterase enzymes (Barr *et al*., 2004). Further reactions of oxo-phosphates may take place and such reactions include enzyme/spontaneous hydrolysis, which may result in the generation of specific metabolites and non-specific metabolites, mainly dialkyl phosphates (DAP), which are common metabolites of organophosphate agrochemicals.

Examples of specific organophosphate metabolites include para-nitrophenol (PNP), which is associated with poisoning due mainly to methyl parathion, but also other similar compounds such as ethyl parathion and nitrobenzene (Barr and Needham, 2002; Esteban *et al*., 1996). Other specific organophosphate metabolites include 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPY), which

is a specific metabolite for diazinon poisoning; malathion dicarboxylic acid (MDA), a specific metabolite for malathion poisoning; para-nitrophenol (PNP), a specific metabolite for parathion and methyl parathion poisoning; 3,5,6-trichloro-2-pyridinol (TCPY), a specific metabolite for chlorpyrifos; and chlorpyrifos methyl poisoning (Margariti *et al*., 2007).

In some cases, it is possible to have a situation where organophosphates do not react to generate oxo-phosphate metabolites and the unconverted organophosphates become hydrolysed to generate their respective specific metabolites as well as dialkylthionate metabolites, such as dialkylthiophosphate, glucono-dialkylthiophosphate, sulpho-dialkylthiophosphate metabolites, and/or dialkyldithiophosphate, glucono-dialkyldithiophosphate, and sulpho-dialkyldithiophosphate metabolites, which are normally excreted through urine and can thus be regarded as useful biomarkers for poisoning by organophosphate agrochemicals (Barr *et al*., 2004).

The most useful urinary dialkyl phosphate metabolites that can be used as biomarkers or indicators of organophosphate poisoning include dimethylphosphate (which is an indication of poisoning by azinphos-methyl, dichlorvos, dicrotophos, dimethoate, fenitrothion, fenthion, malathion, methyl parathion, trichlorfon); diethylphosphate (indicator for poisoning due to chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate); dimethylthiophosphate (poisoning due to azinphos-methyl, dimethoate, fenchlorphos, fenitrothion, fenthion, malathion, methyl parathion); dimethyldithiophosphate (poisoning due to azinphos-methyl, dimethoate, malathion); diethylthiophosphate (poisoning due to chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate); and diethyldithiophosphate (poisoning due to disulfoton, phorate) (Margariti *et al*., 2007).

Although these dialkyl phosphate metabolites can be detected in urine, they will only serve as an indication of organophosphate poisoning and not of a specific organophosphate compound. These dialkyphosphates are normally excreted in urine in the form of sodium or potassium salts.

The potency and affinity of organophosphate compounds or their metabolites differ from one compound to another, or may differ between a parent compound and its metabolite. For example, chlorpyrifos, a phosphorothionate organophosphorus compound, may exhibit weak inhibition for acetylcholinesterase as a parent compound, but the metabolic reactions through desulfation activation result in an oxygenated oxo moiety derivative known as chlorpyrifos-oxon, which becomes more potent as compared to the parent

compound, due to the fact that the chlorpyrifos-oxon displays a stronger affinity as well as potency in terms of phosphorylating the hydroxyl groups of the amino acid serine present on the active sites of the enzyme acetylcholinesterase (Timchalk, 2010).

The toxic potency of organophosphorus agrochemicals and their metabolites is governed by a number of factors, including the amount of the organophosphate ingested or of the metabolite formed, and the kinetics of their bioactivation/bioelimination (Calabrese, 1990). The mediation of the metabolic bioactivation of all these organophosphorus compounds is catalyzed by cytochrome P450 oxidases and the reactions take place mainly in the liver and in some cases in other organs such as the brain (Chambers and Chambers, 1989).

Carbamate Agrochemicals: Mode of Action, Toxicity, and Metabolism

Carbamates are derivatives of carbamic acid, NH_2COOH, which share a similar mode of action as organophosphates, whereby they both exert an anticholinesterase action on the nervous system. Unlike organophosphates, the inhibitory effect to carbon cholinesterase due to carbamate poisoning is short-lived and so is the reverse of the effect of carbamate levels on the cholinesterase enzyme in the blood, such that in most cases the level may even appear normal. Apart from their agricultural applications to control plant and crop disease, they are also used in household fumigation in the forms of spray or baits (control of pests). Examples of carbamate agrochemicals include carbaryl, propoxur, bendiocarb, methomyl aldicarb, carbofuran, methiocarb, and mexacarbate.

In cases of carbamate poisoning, the level of cholinesterase activity is normally measured in blood samples and because the reduction in cholinesterase is short-lived, it is imperative that the sampling of whole blood specimens be done immediately after the poisoning incident. Since the rapidly metabolized carbamates, together with their metabolites are excreted through urine, then urine can be another suitable biological matrix where carbamates and their metabolites can be measured.

The Biotransformation of Carbamate Agrochemicals

There are several chemical reactions that facilitate the biotransformation of

carbamates once poisoning has taken place and include oxidative reactions, ester hydrolysis reactions, and also conjugate reactions (Ecobichon, 1994, 2001). The degree of metabolism for different carbamate compounds is governed by the chemistry of the carbamate molecules themselves, mainly the nature of substituent groups as well as their positions where they attach on either side of oxygen atom or nitrogen atom (Ecobichon, 2001; Timchalk, 2010).

The pathways of bioelimination reactions of carbamates and their respective metabolites normally involve hydrolysis processes, which are generally catalyzed by non-specific esterases. These hydrolytic processes result in a number of products such as alpha-naphthol and methyl-carbamic acid. The formed methyl-carbamic acid is very unstable, as it rapidly decomposes into carbon dioxide and monomethylamine. The alpha-naphthol tends to undergo conjugation reactions with other compounds such as glucuronide or some sulfate compounds, which are excreted together in urine (Chin *et al*., 1979a). There are other non-enzymic mediated metabolites that are associated with carbamate poisoning, which include oxidative metabolites that contain ring structures or ring structures with side chain hydroxylations that are also excreted in urine (Chin *et al*., 1979b).

Poisoning Biomarkers/Indicator Compounds, Metabolites, and Monitoring Matrices

Both carbamate parent compounds and their respective metabolites are normally monitored in poisoning cases in target biomatrices. For example, aldicarb-sulfone is monitored in urine in aldicarb-related poisoning cases, while carbaryl is monitored in blood, and its metabolite (1-naphthol) is monitored in urine. Methonyl is monitored as a parent compound in blood, and pyrimicarb’s metabolites, mainly 2-dimethyl-4-hydroxy-5,6-dimethylpyrimidine and 2-methyl-4-hydroxy-5,6-dimethylpyrimidine, are normally monitored in urine. Propoxur carbamate is monitored as a parent compound in blood, while its metabolite (2-isopropoxyphenol) is monitored in urine. Poisoning due to carbofuran and carbosulfan is monitored in appropriate biomatrices using their metabolites, mainly carbofuranphenol and carbosulfanphenol.

Organohalogen Agrochemicals Poisoning: Indicator Compounds and Monitoring Biomatrices

Biomatrices

Agrochemicals that belong to the class of organohalogens that include polychlorinated biphenyls (PCBs) and pesticides such as dichlorodiphenyl tetrachloroethane (DDT), metabolites of DDT, such as DDD and DDE, hexachlorocyclohexane (HCH), dieldrin, hexachlorobenzene (HCB), chlordanes, endosulfan, dioxins, polybrominated diphenyl ethers (PBDE), *etc.* are known to have high lipophilicity property, which enables them to cross the lipophilic cell membranes of animal cells by the process of diffusion. These molecules have been found to be contaminating foodstuffs such as fruits, vegetables, honey, cow's milk, human milk, fat tissues of animals, *etc*. The consumption of food items that are contaminated with these persistent molecules can lead to poisoning. The poisoning effects are aggravated by the fact that these molecules tend to bioaccumulate and bioconcentrate in the body system. There have been many deliberate poisoning cases that have resulted in health problems of varying magnitude to the victims, due to the fact that their fate when they are in an individual's body system varies significantly, depending on the nature of either degradation taking place by metabolism or by the processes in various tissues such as lipid tissue where these molecules bioaccumulate (Letcher *et al*., 2000).

Organohalogen agrochemicals and their metabolites are known for their persistence as well as their potential adverse long-term effects to human health in cases of poisoning. This makes it plausible to monitor their respective parent/intact compounds in addition to their metabolites in various matrices, mainly serum, breast milk, meconium, and umbilical cord (Margariti *et al*., 2007). For example, dichlorodiphenyltrichloroethane (p,p'-DDT) is normally monitored both as an intact molecule and also in its metabolite forms such as dichlorodiphenyldichloroethylene (p,p'-DDE). Due to their hydrophobicity, monitoring of the intact molecules and/or their respective metabolites in these biological matrices requires the experimental design to employ effective sample extraction and sample purification methods and very sensitive detection methods (Margariti *et al*., 2007).

Metabolism of Organohalogen Agrochemicals and their Respective Metabolites

Metabolism of organohalogens in the human/animal body system is known to be dependent on the presence and action of a number of microsomal enzymes. The bioactivity and induction of these microsomal enzymes can be indicated by the

presence of 6-β-hydroxycortisol and D-glucaric acid excretion in biomatrices such as urine from the victims. The challenge of using such enzymatic induction biomarkers as an indication of poisoning due to organochlorine lies in the fact that poisoning due to some other chemicals such as tranquilizers (e.g. barbiturates), alcohol, *etc.* are also known to result in the same induction biomarkers. For this reason, identification of intact organohalogens and/or their metabolites in biomatrices such as blood and its components or urine is always highly recommended.

Each intact organohalogen molecule is associated with certain metabolites that can be traced in specific biometrics. For example, hexachlorobenzene poisoning in humans is associated with the porphyria metabolite biomarker that is eliminated from the body via urine (Maroni *et al*., 2000).

Other organohalogen compounds, such as dichlorodiphenyl trichloroethane (DDT), has mainly 4.4’-dichlorodiphenyldichloroethane as the main metabolite biomarker, which together with its parent/intact molecule (DDT), are analyzed in either urine, breast milk, blood, or adipose tissues. Heptachlor-epoxide is a biomarker metabolite associated with heptachlor poisoning and both parent and metabolites of this compound are normally analyzed in milk, urine, serum, and adipose tissues. Endrin and its metabolite biomarker (anti-12-hydroxy-endrine) are normally analyzed in the urine matrix, while aldrin poisoning yields dieldrin as a metabolite biomarker and both the metabolite and parent compound are analyzed in biomatrices such as serum, milk, and fat tissues. Organohalogen agrochemicals such as α-and β-endosulfan (analyzed in serum), lindane, vinclozolin, and β-HCH are analyzed in serum and breast milk. Metolachlor and its metabolite metolachlor mercapturate are also analyzed in the same biomatrices.

Generally, all other toxic organohalogens such as dichlorobenzene (with its metabolite 2,4-dichlorophenol); p-dichlorobenzene (with its metabolite 2,5-dichlorophenol); chlorinated benzene (with its metabolites 2,4,5-trichlorophenol and 2,4,6-trichlorophenol); dioxins; polybrominated diphenyl ethers; and brominated flame retardants etc., are analyzed as both intact molecules and their respective metabolites in biomatrices such as urine, serum, and milk.

Pyrethroid Agrochemicals

Research has shown that in mammals, pyrethroid insecticides are normally rapidly metabolized into their respective carboxylic acids through a mechanism

that involves hydrolytic cleavage of their ester bonds, then by oxidation processes such as glucuronidization (Figure 4.22). They are normally excreted through urine as conjugate metabolites (Dorman and Beasley, 1991; Soderlund *et al.*, 2002). Other biological matrices such as blood and its components (i.e. serum) are normally not preferred as important biomatrices where pyrethroid metabolites can be found simply because the metabolic process is rapid metabolism and therefore levels of either intact molecules or their respective metabolites are at any time low as compared to that which may be found in urine (Margariti *et al.*, 2007).

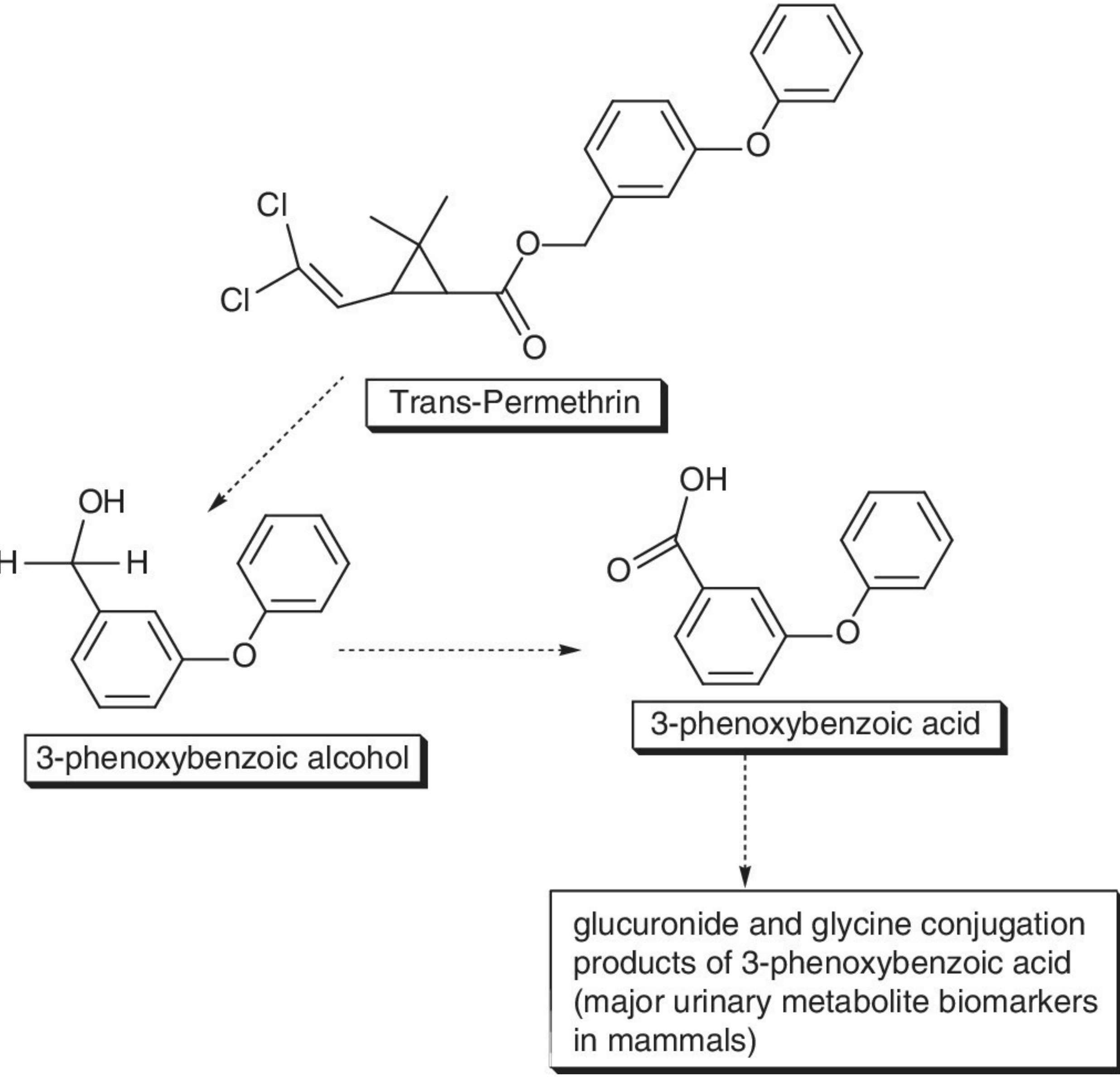


Figure 4.22 Proposed urinary metabolites of permethrin in mammals (Crawford *et al.*, 1981; Miyamoto *et al.*, 1988).

Pyrethroid insecticide intact compounds such as cyfluthrin, cypermethrin, and permethrin are normally monitored in their metabolite forms, which include cis- and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acids (cis-/trans-DCCA), while other pyrethroid herbicides such as deltamethrin are monitored as cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DBCA) metabolites. Cyfluthrin is monitored as 4-fluoro-3-phenoxybenzoic acid (4F3PBA), while a number of synthetic pyrethroids such as cypermethrin, deltamethrin, and permethrin are usually monitored in urine or milk in the form of their common metabolite 3-phenoxybenzoic acid (3PBA) (Margariti *et al*., 2007).

Herbicides Poisoning

Scientific findings have revealed that phenoxy herbicides hardly undergo biotransformation in mammals and therefore monitoring of these compounds in cases of poisoning involves mainly parent/unmodified compounds, mainly in urine (Garry *et al*., 2001). Chloroacetanilide herbicides such as acetochlor are monitored in the form of the metabolites, mainly acetochlor mercapturate, while alachlor is monitored as alachlor mercapturate and metolachlor as mercapturate metolachlor. Atrazine herbicides are normally monitored in their atrazine mercapturate (AM) forms (Margariti *et al*., 2007).

Detection Methods for Food Forensic Specimens Suspected of Contamination with Agrochemical Molecules and/or their Metabolites in Various Biomatrices

From the above discussion, it follows that the most likely specimens that may be presented as evidence in forensic investigation due to food poisoning by agrochemical molecules or others will be biological fluids (urine, breast milk, blood and its components such as serum, saliva, and vomit), hair, nails, meconium, stool, *etc*.

The majority of these biomatrices are complex and interact with the analytes in various ways and to a various degree of bonding strengths. This therefore requires very selective sample preparation methods to isolate analytes of interest before subjecting them to analytical instruments for determination. A number of sample preparation methods, separation, and detection techniques have been

reported for agrochemical molecules and their respective residues in various biomatrices, as outlined below.

Analytical Methods of Organophosphate Agrochemicals and their Residues in Human Urine Specimens

The urine matrix is one of the most used specimens in the determination of organophosphate agrochemical molecules and their metabolites. Different sample preparation approaches with different separation and detection techniques have been reported. For example, Barr *et al*. (2002) determined methyl parathion and its metabolite p-nitrophenol where they reported sample preparation methods that involved enzyme hydrolysis followed by solvent extraction and then concentrated the sample by drying using anhydrous sodium sulfate (Barr *et al*., 2002). Enzyme hydrolysis in combination with solid phase extraction was also reported by Olsson *et al*. (2003) in the analysis of certain organophosphate biomarkers in urine before detection using high resolution LC-MS/MS (Olsson *et al*., 2003).

Liquid–liquid extraction (solvent extraction) has been used alone and also in combination with solid phase extraction (florisil/PSA cartridge sorbent) as a sample preparation for dialkylphosphates and their metabolites (mainly dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate diethylthiophosphate, and diethyldithiophosphate) in urine samples (Dulaurent *et al*., 2006; Hardt and Angerer, 2000; Ueyama *et al*., 2006). Where the detection method involved GC-EI-MS, the derivatization step was also involved to ensure that the derivative was volatile enough for GC-MS analysis.

In certain other reports, organophosphates (dimethylthiophosphate, diethylphosphate, diethylthiophosphate, and diethyldithiophosphate) have been analyzed using direct injection onto high resolution LC-MS/MS (Hernandez *et al*., 2002, 2004). Mainly dimethylphosphate and dimethylthiophosphate, and other compounds were analyzed. In other reports, azeotropic distillation, chloropropylation, and concentration of dialkyl organophosphates and their metabolites (mainly dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate diethylthiophosphate, and diethyldithiophosphate) were used as sample preparation methods before determination using high resolution GC-MS/MS, where the use of isotopic

internal standards were employed in quantitation steps (Bravo *et al.*, 2002). Lyophilization in combination with solvent extraction using methyl cyanide (acetonitrile) and diethyl ether, followed by chloropropylation concentration was the method for sample preparation before detecting dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate diethylthiophosphate, and diethyldithiophosphate, using high resolution GC-MS/MS, which was operated in the positive chemical ionization mode (Bravo *et al.*, 2004).

Analytical Methods of Organophosphate Agrochemicals and their Residues in Human Serum Specimens

Protein precipitation has been reported as a sample preparation method in the analysis of chlorpyrifos and its major metabolite (3,5,6-trichloro-2-pyridinol) in human serum and urine, where high resolution LC-MS/MS was used for separation and detection of these molecules (Sancho *et al.*, 2000). Solid phase extraction has been used as a sample preparation in the multiresidue analysis that involved organochlorine and organophosphates in human serum, where GC-MS was used for separation and detection purposes (Pitarch *et al.*, 2003).

Analytical Methods of Organophosphate Agrochemicals and their Residues in other Biomatrices Specimens

Other biomatrices where organophosphates (dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate diethylthiophosphate, and diethyldithiophosphate) are analyzed, include meconium and amniotic fluid. For meconium, the sample preparation method involving a combination of lyophilization, and solvent extraction using methanol and chloropropylation was reported by Whyatt and Barr (2001), where they measured metabolites of certain organophosphates in postpartum meconium. The use of amniotic fluid in the analysis of biomarkers associated with organophosphorus agrochemical poisoning was reported by Bradman *et al.* (2003), where the sample preparation method of choice for dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylphosphate, diethylthiophosphate, and diethyldithiophosphate involved azeotropic distillation

followed by chloropropylation and concentration prior to detection using high resolution GC-MS/MS that was operated in the positive chemical ionization mode.

Analytical Methods of Organochlorine Agrochemicals and their Residues in Human-related Biomatrices

The majority (if not all) of the organochlorine agrochemicals and their respective metabolites are very hydrophobic and characterized with long half-lives in the biomatrices. This suggests that some of the biomatrices such as urine will be of little importance when considering specimen collection for analyses intended to find evidence of poisoning. The method of organochlorine detection has mainly been either GC-MS or GC-ECD.

Analytical Methods of Organochlorine Agrochemicals and their Residues in Human Milk Specimens

The sample preparation approaches for organochlorine and their respective metabolites (mainly dichlorodiphenyldichloroethylene (p,p-DDE), o,p-dichlorodiphenyltrichloroethane (o,p-DDT), dichlorodiphenyltrichloroethane (p,p-DDT), mirex, and dieldrin in milk) involve a combination of solvent extraction (using appropriate solvents for appropriate compounds) and solid phase extraction (mainly florisil sorbent) (Burke *et al*., 2003; Campoy *et al*., 2001).

Analytical Methods of Organochlorine Agrochemicals and their Residues in Human Serum Specimens

A sample preparation method utilizing a combination of solid phase extraction and solvent extraction (methanol:dichloromethane) and clean-up using silica and gel permeation chromatography for a multiresidue analysis of a mixture of PCBs and chlorinated pesticide molecules (mainly dichlorodiphenyldichloroethylene (p,p-DDE); o,p-dichlorodiphenyltrichloroethane (o,p-DDT);

dichlorodiphenyltrichloroethane (p,p-DDT); mirex; dieldrin; heptachlor epoxide; oxychlor; trans-nonachlor; hexachlorobenzene (HCB); gamma-hexachlorocyclohexane (lindane, γ-HCH); and beta-hexachlorocyclohexane (β-HCH)) in serum has been reported by Barr *et al*. (2003). A combination of lyophilization and acceleration solvent extraction with clean-up using gel permeation for PCBs and agrochemicals and their metabolites (dichlorodiphenyldichloroethylene (p,p-DDE); o,p-dichlorodiphenyltrichloroethane (o,p-DDT); dichlorodiphenyltrichloroethane (p,p-DDT); mirex; dieldrin; heptachlor epoxide; oxychlor; trans-nonachlor; hexachlorobenzene (HCB); gamma-hexachlorocyclohexane (lindane, γ-HCH); and beta-hexachlorocyclohexane (β-HCH)) in serum was reported by Barr *et al*. (2003).

Čonka *et al*. (2005) used solid phase extraction (SPE) and liquid–liquid extraction (dichloromethane:hexane) and clean-up with florisil/silica gel column as sample preparation methods before GC-ECD detection of a mixture of PCBs and organochlorines (mainly dichlorodiphenyldichloroethylene (p,p-DDE); dichlorodiphenyltrichloroethane (p,p-DDT); hexachlorobenzene (HCB); gamma-hexachlorocyclohexane (lindane, γ-HCH); beta-hexachlorocyclohexane (β-HCH); and alpha-hexachlorocyclohexane (α-HCH)) (Čonka *et al*. 2005).

Lacassie *et al*. (2001) employed solid phase extraction for serum specimen and eluted the sorbed PCBs and organochlorines (dichlorodiphenyldichloroethylene (p,p-DDE); dichlorodiphenyltrichloroethane (p,p-DDT); dieldrin; hexachlorobenzene (HCB); gamma-hexachlorocyclohexane (lindane, g-HCH); a-endosulfan; b-endosulfan; and aldrin) using ethyl acetate prior to GC-EI-MS, while Sundberg *et al*. (2006) eluted the same classes of chlorinated compounds (mainly dichlorodiphenyldichloroethylene (p,p-DDE); hexachlorobenzene (HCB); aldrin; chlordane; endrin; and dichlorodiphenyldichloroethane (p,p-DDD)) using methylene chloride prior to GC-ECD detection.

Analytical Methods of Organochlorine Agrochemicals and their Residues in Meconium Specimens

A method involving solvent extraction of the organochlorine metabolite, dichlorodiphenyldichloroethylene (p,p-DDE), followed by filtration and concentration prior to GC-EI-MS has been reported by Hong *et al*. (2002).

Analytical Methods of Multiresidue Analysis of Miscellaneous Agrochemicals and their Metabolites in Various Biomatrices

Methods for the analysis of different classes of herbicides, insecticides, fungicides, molluscicides, rodenticides, *etc.* in various biomatrices have been developed. For example, Baker *et al*. (2000) and Bradman *et al*. (2003) reported the analysis of pyrethroid metabolites (2,4-dichlorophenoxyacetic acid (2,4-D); atrazine mercapturate (AM); malathion dicarboxylic acid (MDA, malathion metabolite); and 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPY, diazinon metabolite)) in urine and amniotic fluid biomatrices respectively. In both reports, the sample preparation method involved enzyme hydrolysis and solvent extraction, while the detection was by LC-APCI-MS/MS.

Corrion *et al*. (2005) and Bielawski *et al*. (2005) reported the analysis of pyrethroid metabolites (pyrethroids, 3,5,6-trichloro-2-pyridinol (TCPY) and methyl/ethyl chlorpyrifos metabolite), organophosphates, carbamates and/or metabolites, chloroacetanilides, organochlorine pesticides, and malathion monocarboxylic acid (MMA, malathion metabolite)) in blood and meconium biomatrices respectively. In the report by Corrion *et al*. (2005), the sample preparation method involved the use of solvent extraction and derivation prior to GC-EI-MS, while Bielawski *et al*. (2005) used the solid phase extraction method and the sample was analyzed using GC-MS. In another report by Barr *et al*. (2002), a multiresidue mixture comprised of pyrethroids (*N*,*N*-diethyl-m-toluamide (DEET), atrazine, organophosphates, carbamates and/or metabolites, and chloroacetanilides in serum and plasma biomatrices). Solid phase extraction sample preparation method used methyl chloride as the elution solvent prior to detection using high resolution GC-MS.

The analysis of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), acetochlor mercapturate, alachlor mercapturate, metolachlor mercapturate, and atrazine mercapturate (AM) in urine was reported by Norrgram *et al*. (2006), where solid phase extraction with methanol elution was employed before detection by high resolution LC-APCI-MS/MS.

Generally, from the majority of the reports, enzyme hydrolysis, acid/base hydrolysis, solvent extraction, or solid phase extraction have been widely used as sample preparation methods and detection with either GC-MS or LC-MS have been the principal techniques.

Conclusions

The knowledge of the pathways of various agrochemical residues after consuming foods that are contaminated with these molecules is crucial for a number of reasons, as it will guide the analyst as to which are the best sample specimens to collect. It will also be helpful for the analyst to estimate the time needed from poisoning to analysis and which metabolites to expect from the various possible specimens (urine, milk, blood, serum, etc.). In addition to this, the choice and knowledge of the analytical instrument to use for analysis is important, as it will result in the correct analysis of the residues, both qualitatively and quantitatively.

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5
Food Forensics Cases Related to Food Bioterrorism/Food Bio-Weapons and Food Poisoning Agents: Biological Food Poisoning Agents

> *From the context of this chapter, biological agents that may be used in food bioterrorism or food poisoning include living organisms or infectious materials that may have their origin from living organisms and which are capable of multiplying in the human body system. Such agents can be used for the purpose of causing disease or inflicting health problems on either humans, animals, crops, or any other material regarded as a food source to humans, directly or indirectly (Spencer, 1993).*

Biological agents with the potential for being abused in food poisoning instances include those agents that are already known, such as microbial pathogens. The broader composition of such agents will include those created or manipulated in laboratories through molecular biology technologies. The molecular biology manipulated agents are normally created with chemical/biological features that enable them to be propagated easily and/or properties that resist antibiotics and are normally extremely virulent. These agents together with others such as those responsible for "severe acute respiratory syndrome" (SARS) (Guan *et al*., 2004); bird/avian flu (Normale, 2004 ; Parry, 2004); mad cow disease, swine flu, *etc.* have the potential to be deliberately introduced into foods that can enter the distribution supply chains and thus affect many people, even at distant locations or in multiple sites/cities/countries.

The use of biological agents to deliberately contaminate foods, sources of foods, or water is an example of indirect methods of food bioterrorism, which aims to inflict severe health problems, to kill (those who will consume the contaminated food), or instill fear and panic in other people.

Major Groups of Biological Agents with Potential for Being Used in Food Poisoning/Bioterrorism

Biological agents may be grouped according to their origin such that there are:

Biological agents may be grouped according to their origin such that there are:

1. microorganism-derived food poisoning biological agents;
2. plant-derived food poisoning biological agents; and
3. animal-derived food poisoning biological agents.

Microbial-derived Food Poisoning Biological Agents

There have been a number of incidences in the past that involved deliberate use of microbially-derived agents to poison food. For example, in 1984, there was intentional use of *Salmonella typhimurium* to contaminate a restaurant's salad bar, which affected more than 700 people (Török *et al*., 1997). Again, in 1996, there was an incident whereby muffins and doughnuts were deliberately contaminated with *Shigella dysenteriae* type 2, which were consumed by staff at a medical centre and resulted in health problems for all who consumed them (Kolavic *et al*., 1997).

During World War II, *Bacillus anthracis, Yersinia pestis, Vibrio cholera, Shigella* spp., and *Salmonella* spp. were used by the Japanese army to deliberately contaminate food items in Chinese towns (Harris, 1992).

Generally, different groups and classes of microorganisms have been used in food poisoning, either themselves or their secondary metabolites. Some are bacteria, fungi, and viruses (influenza virus A, etc.).

Bacterial-derived Microbial Food Poisoning Agents

There are two main broad groups of bacteria, namely Gram-positive bacteria (e.g. *Clostridium botulinum*, *Clostridium perfringens*, *Staphylococcus aureus*, etc.) and Gram-negative bacteria (e.g. species of *Shigella*, *Escherichia coli*, certain species of *Pseudomonas*, *Salmonella*, *Haemophilus*, *Neisseria*, etc.), which are known to cause food poisoning.

Gram-positive Bacteria and Antigenic Proteinaceous Exotoxins in Food Forensics

Gram-positive bacteria are associated with the production of powerful antigenic exo-neurotoxins of a protein nature. They are known as antigenic exotoxins because they are produced by these Gram-positive bacteria then secreted and diffused out of the cell wall into the external environment. These exotoxins have been responsible for food poisoning, especially foods of animal/avian origin, but other foods also (e.g. salads, potatoes, etc.).

Several Gram-positive bacterial genera (including *Clostridium* spp., *e.g. C. botulinum*, which produces botulinum toxin, and *C. perfringens*, which produces perfringens toxin, *Bacillus* spp. (e.g. *Bacillus anthracis*), which produce anthrax toxins, and *Staphylococcus* spp., such as *S. aureus*, which produces eneterotoxin B) are known to have been used or at least to have the potential to be used in food bioterrorism acts.

The different biotoxins produced by bacteria that have been used or have the potential for use in food bioterrorism are discussed below.

Bacillus cereus and its Toxic Metabolites in Foods

According to Turnbull (1981), there were more than 200 reported cases of food poisoning due to *Bacillus cereus* reported worldwide between 1950 and 1976. However, together with such a statistic, there are some reports that state that there has been under-reporting of the food poisoning cases due to *B. cereus*, because the illness is relatively mild and short-lived (<24 hours) (Granum, 1997 ; Kramer and Gilbert, 1989). Although a number of these cases may have been accidental, there are possibilities that criminals may use these microorganisms and the toxins they produce to inflict damage to innocent communities.

There are a number of bacterial species under the genus *Bacillus*, which are known for their pathogenicity and potential for food poisoning. A species like *Bacillus anthracis* is a causative agent for anthrax, and *Bacillus thuringiensis* possesses insecticidal properties, apart from it being associated with foodborne diseases. Another species, *Bacillus cereus*, when present in foods, causes two types of food poisoning that actually cause emetic and diarrheal syndromes, in addition to various local and systemic infections. The two toxins include cereulide and the tripartite hemolysin BL and its homolog, the nonhemolytic enterotoxin, which are known to cause diarrheal syndrome, with disease symptoms parallel to those of *Clostridium perfringens* food poisoning. The emetic syndrome normally associated with an emetic toxin pre-formed in food and cooked rice has been linked with poisoning, as it is a common vehicle. The symptoms for emetic syndrome are similar to those of *Staphylococcus aureus* poisoning.

Bacillus cereus is a spore-forming, motile, Gram-positive bacteria known to have heat resistance as well as a non-fastidious nature, which are advantageous features for the survival of the species and growth in different types of foods such as meat and vegetable soups, cooked meat and poultry, raw and cooked vegetables, pasta, milk, and ice cream (Shinagawa, 1990).

Isolation and Enumeration of *B. Cereus*

In order to verify an incidence associated with *B. cereus* food poisoning, one of the evidences can be obtained by the isolation and enumeration of *B. cereus* and another one is to extract and identify the toxins related to *B. cereus*.

The isolation techniques that have been reported involve the use of several selective growths. For example, a growth media for *B. cereus* made up of polymyxin B (5–10/~g/mL), mannitol, egg yolk, and an indicator has been reported widely (Bouwer-Hertzberger and Mossel, 1982 ; Johnson, 1984 ; Jinbo *et al*., 1978 ; Kozasa *et al*., 1977). An approach for surface plating onto other formulations of selective medium, for example the one with recipes such as mannitol egg-yolk polymyxin (MYP) agar, has been used by Mossel *et al*. (1967) for the isolation and enumeration of *B. cereus* from foods as well as clinical specimens. After culture, the isolates can be identified using several techniques. Traditionally, in the past, the identification was conducted mainly by morphological studies based on Gram and spore staining and biochemical tests such as the VP reaction, liquefaction of gelatin, starch hydrolysis, indole production, glucose fermentation, and failure to ferment mannitol, arabinose, and xylose (Shinagawa, 1990). Other methods including biotyping, serotyping, and phage typing, have been reported as useful for identification of microorganisms.

B. cereus produces a vomiting-causing emetic toxin that is produced by growing cells in foods and at least three types of diarrhea-causing enterotoxins that are produced by *B. cereus* vegetative cells growing in the intestine.

The Chemistry of Cereulide Emetic Toxin: Biosynthetic Pathways and Mode of Action

The compound known as emetic toxin has a molecular weight of 1200 Da and is a ring-shaped chemical peptide molecule comprised of three repeats of four amino and/or oxy acids: (D-O-Leu-D-Ala-L-O-Val-L-Val)3. This toxic compound, also named cereulide, has a chemical structure closely related to that of potassium ionophore valinomycin (Agata *et al*., 1994).

A study by Hughes *et al*. (1988) observed that this cereulide emetic toxin can be detected by using Hep-2 cells where it forms vacuoles. This cereulide emetic toxin, however, is not antigentic, but it is known to be resistant to heat, pH, and proteolysis (Kramer and Gilbert, 1989).

The mechanism of action and biosynthesis pathways for the cereulide emetic toxin has not yet been established with certainty. However, a report by Agata *et al*. (1995) suggested a possible biosynthesis route through the stimulation of vagus afferent by the emetic toxin through the binding of the toxin to the 5-HT receptor. However, it is still unclear whether the toxin is produced as a product of gene modification processes or its production is enzymatically mediated, although its structure may suggest more likely that it is a product of enzyme catalyzed processes rather than it being a gene controlled process product.

The Chemistry of *B. cereus* Enterotoxins

Several reports have shown that there are at least two different three-component enterotoxins produced by *B. cereus*, one being a hemolysin toxin HBL, which is comprised of three proteins, mainly protein B, protein L1, and protein L2, all of them exhibiting dermonecrotic, vascular permeability, and enterotoxic activity. They are known to cause fluid accumulation (Beecher and Wong, 1994, 1997 ; Beecher *et al*., 1995 ; Lund and Granum, 1996, 1997).

The other (second) three-component enterotoxin is a nonhemolytic enterotoxin (NHE), which is different from the HBL protein toxin (Lund and Granum, 1996).

Of the *B. cereus* strains, there are some that possess the ability to produce both of the three-component enterotoxins, while some produce only one of the two (Lund and Granum, 1997).

Mechanism of Action of HBL Protein Enterotoxins Produced by *B. cereus*

Beecher and Macmillan (1991) have suggested that HBL interacts with red blood cells (erythrocytes) and that the B component of the HBL is responsible for the binding to the target cells, while components L1 and L2 play a lytic function.

Mechanism of Action of NHE Protein Enterotoxins Produced *by B. cereus*

The NHE is believed to interact with vero cells, whereby a 105 000 Da protein plays a role in binding the complex's component (Lund and Granum, 1997).

Clostridium botulinum and its Toxic Metabolites

(Botulinum Toxins) in Foods

Botulinum toxin (BOTX) is a neurotoxic proteinaceous chemical compound produced by Gram-positive anaerobic bacteria of species *Clostridium botulinum* and other related bacterial species. The botulinum toxins are normally produced at an optimum temperature of 30 °C under anaerobic conditions. They are known to be heat labile but easily destroyed under oxidation conditions. Botulinum toxin is a white crystalline compound in appearance and has a high solubility in water, although when exposed to air it becomes inactive. Botulinum toxins are protein in nature with a chemical formula of $C_{6760}H_{10447}N_{1743}O_{2010}S_{32}$ (Mwt = 150 kg/mol (150 000 g/mol).

There are seven variants of BOTX represented by the letters A, B, (C, C1, C), D, E, F, and G. All these seven variants are structurally similar but they are known to be different from each other in terms of their antigenicity and serologicity (i.e. potency in terms of their toxicities). The types of foods that are highly susceptible to botulinum toxin (foodborne botulism) are mainly those characterized by low-acid components such as vegetables, meats, or fish (Shapiro *et al*., 1998).

Variants A, B, E, and in some cases F, are the ones capable of exerting toxic effects in human; however, variants C and D have been reported to exert toxic effects in animals. BOTX-A is normally associated with vegetables; BOTX-B with meat; and BOTX-E with fish (Shapiro *et al*., 1997).

The variant forms of botulinum toxins possess characteristics in terms of the toxicity potencies, but all of them cause botulism, which is known to be potentially fatal, especially to infants (Kessler and Benecke, 1997 ; Sing, 2000). Research has shown that a fatal BOTX dose in a human ranges from between 0.1 and 1.0 nanograms per kilogram (when taken orally); about 1–2 nanograms per kilogram (when taken intravenously) (Arnon *et al*., 2001), and 10–13 nanograms per kilogram (upon inhalation), and that these biotoxins are 15 000 times more toxic than VX nerve gas and 10 000 times more toxic than sarin (Arnon *et al*., 2001 ; Franz *et al*., 1997). This extremely high toxicity of botulinum toxins is attributed to the fact that they act as neurospecific enzymes in the sense that they are capable of utilizing just one single active toxin molecule inside the synapse to destroy the protein responsible for a functional neuroexocytosis (Rossetto *et al*., 2000).

From these data, it is evident that botulinum neurotoxins are probably the most potent toxic molecules of all bacterial biotoxins and due to their ease of

preparation and production, they thus pose a major bioterrorism threat (Arnon *et al*., 2001).

From their chemical structure point of view, each botulinum toxin molecule contains two chains (a heavy chain and a light chain), which are chemically linked by a disulfide bond (Figures 5.1a and b).

p y i e i n n g e

5′ ccguauauugaaauuaauaauggcgaa 3′

Figure 5.1a An example of a chemical structure of Botulinum neurotoxins (BoNT):- Amino acid sequence (top) and oligonucleotide sequence (bottom) of a specific fragment of BoNT/A using HE2 primer. The fragment is found in the amino acid position 635-643 and nucleotide sequence 2037–2062 (Thompson *et al*., 1990).

NB 1: These are mainly peptide toxins composed of sequences of amino acids. Such structures are derived from the nucleotide sequences using appropriate primers. The sequence is dependent on the strain used and also the culture conditions and can be deduced by a number of methods including PCR, DNA probes, immunochemical, mass spectrometry, *etc.*

NB 2: For complete sequences of BoNTs from various strains using various approaches, refer to the literature (Aranda *et al*., 1997 ; Boyer *et al*., 2005 ; Thompson *et al*., 1990 ; Whelan *et al*., 1992).

NB 3: The interpretation of the symbols is found below.

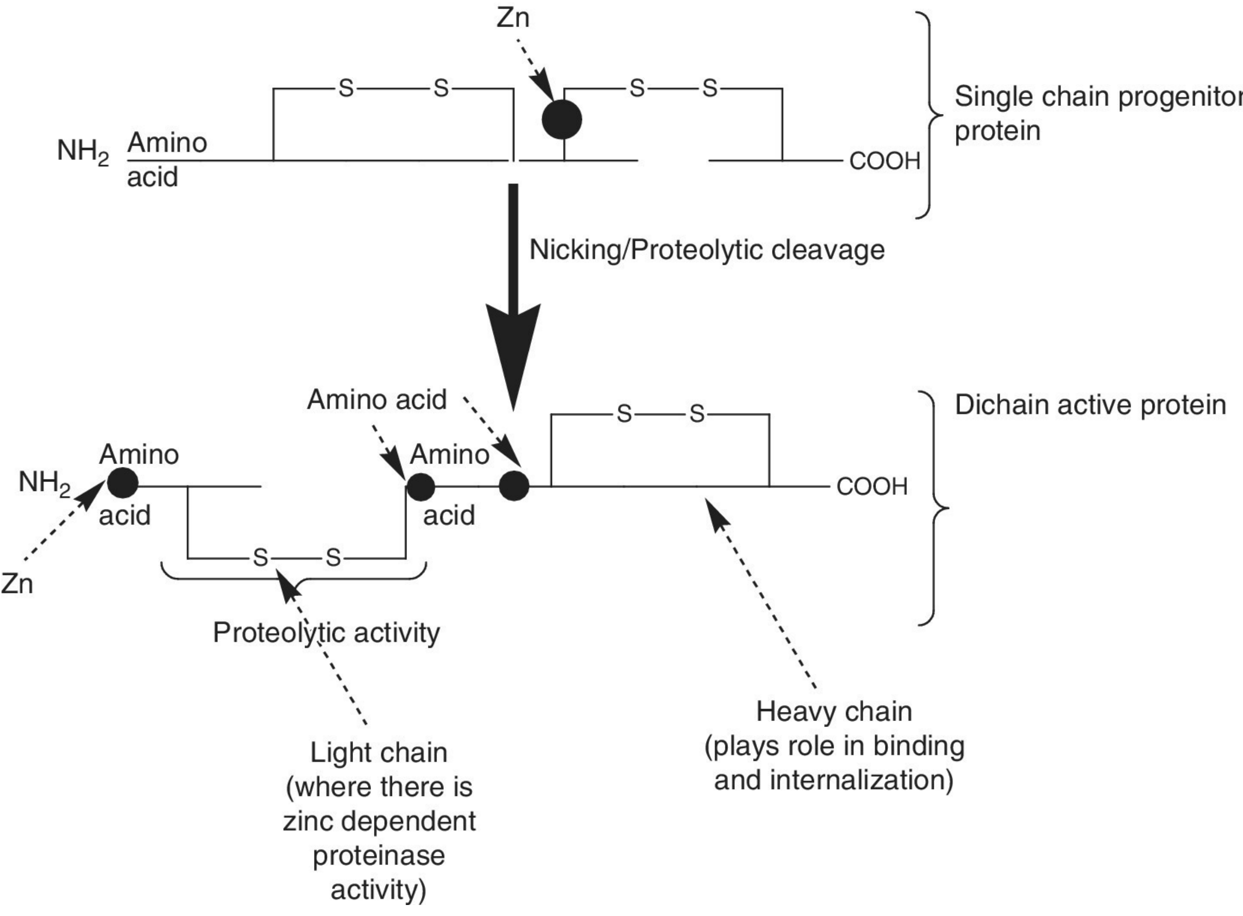

Figure 5.1b The chemical structure and the proposed cleavage of Botulinum neurotoxin to form an active dichain (Åberg *et al*., 2013; Boyer *et al*., 2005).

Of the two chains, the light one has an Mwt of about 50 000 Daltons, or amino acids numbering up to 448 and is located at the N-terminal end. It plays an important role as a zinc ions endopeptidase, and has proteolytic activity. The heavy chain of the botulinum toxin has an Mwt of about 100 000 Dalton, or amino acids numbering up to 1280. This heavy chain is the centre for cholinergic specificity and plays an important role in the binding of the toxin to presynaptic receptors. Moreover, the heavy chain facilitates the translocation process for the light-chain to move across the endosomal membrane. The light chain plays a role as a metalloprotease on SNARE proteins, a role that is dependent on Zn (II) ions to facilitate the cleaving of SNARE proteins, thus rendering their function in exocytosis redundant.

Mechanism of Action: Botulinum Toxin

The mode of action of botulinum toxin (proteolytic enzyme) involves presynaptically binding to high-affinity recognition sites on the cholinergic

nerve terminals, thereby decreasing the release of acetylcholine, a phenomenon that results in the blocking of neuromuscular activities. It should be noted that botulinum toxin is made up of two chains (the light chain and the heavy chain, which are linked to each other by a disulfide bond) and actually each of the two chains does contribute to the toxicity of the molecule. The heavy chain enables the toxin (which is protein in nature) to both bind and then penetrate and enter the neuron. The light chain behaves in the same way that enzyme protease acts and facilitates the cleavage of proteins that would have otherwise enabled neurotransmitters to escape from the cell, thus causing a characteristic flaccid paralysis (Dong *et al*., 2007 ; Magidan and Martinko, 2006).

In a more detailed way, the mode of action of botulinum toxin involves a 4-step mechanism:

1. binding the neuronal membrane: a step that is accomplished by the heavy chain of botulinum toxin that searches for the neuronal targets and binds to the gangliosides and membrane proteins of presynaptic neurons;
2. endocytosis: a stage where botulinum toxin becomes endocytosed into the cell membrane structure;
3. membrane translocation: a step that is accomplished by the heavy chain after it has undergone conformational changes required for it to translocate the light chain into the target of neuron's cytosol; and
4. proteolysis of SNARE proteins (Rossetto *et al*., 2011): this last step is accomplished by the light chain after it has been freed by the heavy chain (in a process involving the reduction of the disulfide bonds, which is catalyzed by NADPH-thioredoxin reductase-thioredoxin system) and arrives at the target active cleavage site on the SNARE proteins (Pirazzini *et al*., 2013 ; Rossetto *et al*., 2011).

With regard to botulism isotype variants, BOTX-As, also known as onabotulinum toxin A (Botox) and BOTX-Es, targets synaptic proteins whereby they cleave a presynaptic membrane protein known as synaptosome associated protein (SNAP-25), which is responsible for the fusion of neurotransmitter-containing vesicles (Hong and Lev, 2014 ; Kent, 1998). SNAP-25 is one of the members of the soluble *N*-ethyl-maleimide-sensitive factor attachment receptor (SNARE) protein family found in cell membranes and when it is cleaved with SNAP-25, its ability to form the SNARE complex necessary for the fusion of vesicles to the synaptic membrane becomes impossible.

BOTX-B (rimabotulinum toxin B (Myobloc)), D, F, and G target the vesicle

associated membrane protein (VAMP) (a SNARE protein found on cell vesicles), whereby they cleave VAMP, known as synaptobrevin, while BOTX-C targets syntaxin (another SNARE membrane protein found in the synaptic membrane), such that BOTX-C cleaves and degenerates syntaxin proteins, rendering them unable to play their normal functions.

Generally, all isotypes of botulinum neurotoxin are associated with functional damage to SNARE proteins, thus inflicting significant physiological problems to the victims, because when these SNARE proteins are damaged, the fusing of synaptic vesicles to the synaptic membrane is hindered and so the process of releasing their neurotransmitters into the synaptic cleft is also blocked. This will result in the failure of the propagation of the action potentials to stimulate muscle cells, hence resulting in paralysis and even death of the victims.

Figure 5.2a–c summarizes the mechanisms of all the variants of botulinum toxins.

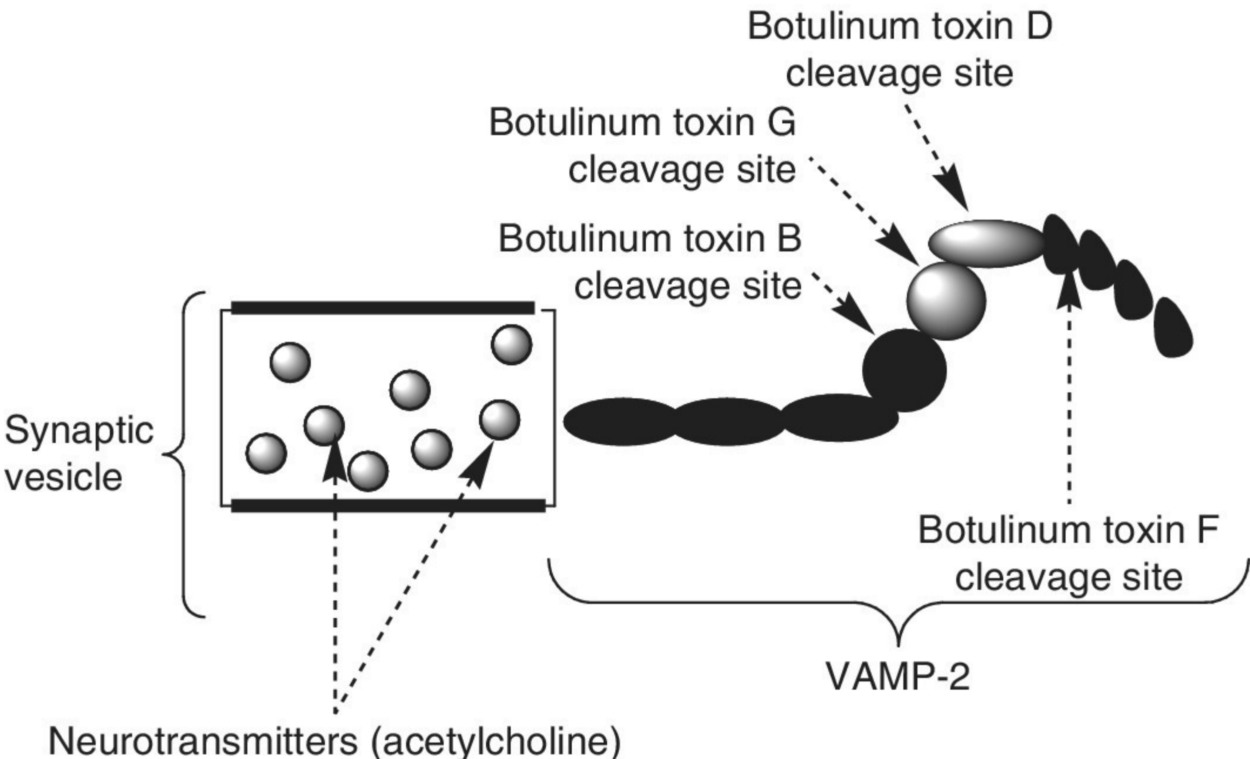

Figure 5.2a General mechanism of action of botulinum toxins in specific cleaving of VAMP-2 on the synaptic vesicle (Boyer *et al*., 2005).

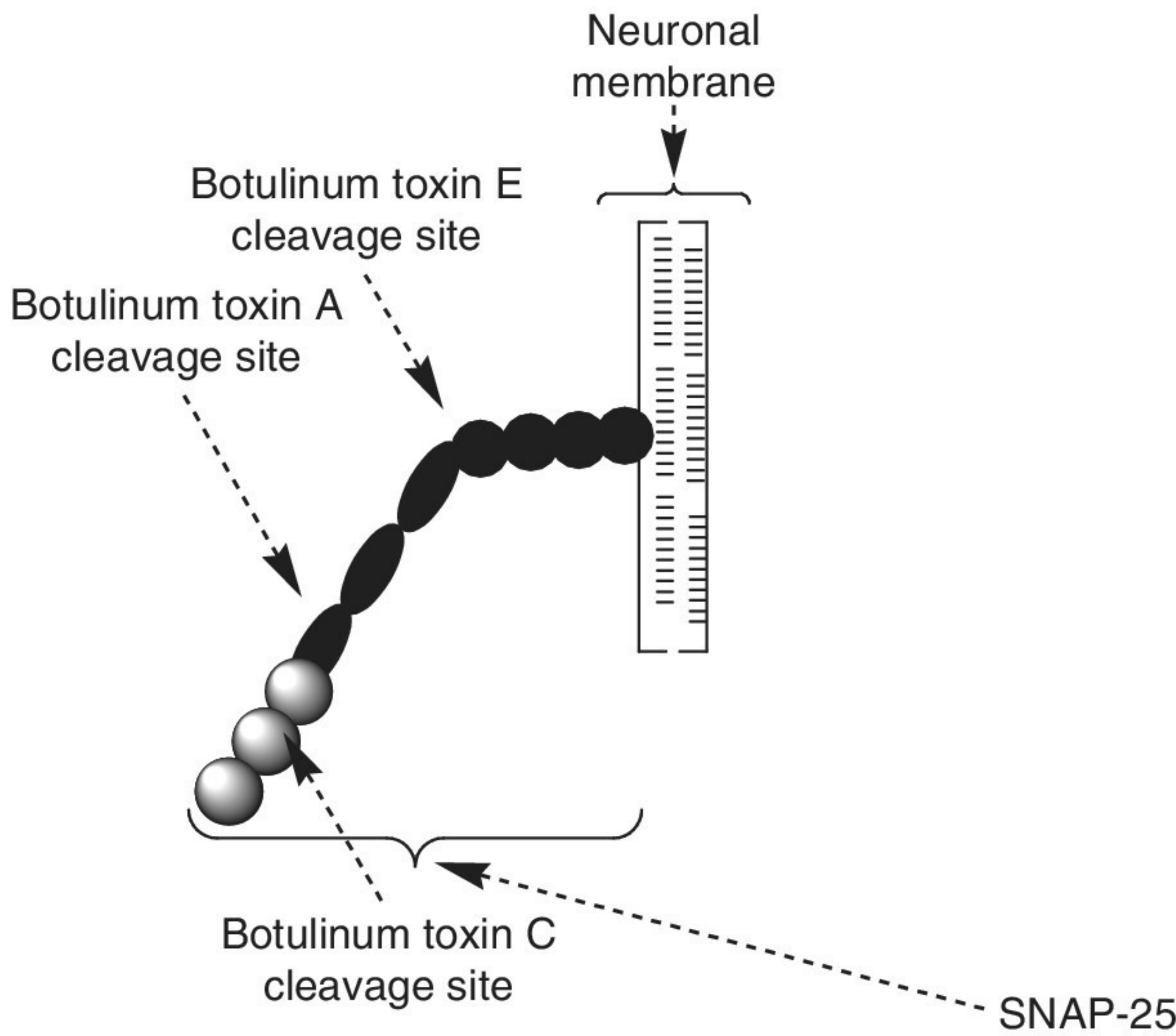

Figure 5.2b General mechanism of action of botulinum toxins in specific cleaving of SNAP-25 on the neuronal membrane (Boyer *et al.*, 2005).

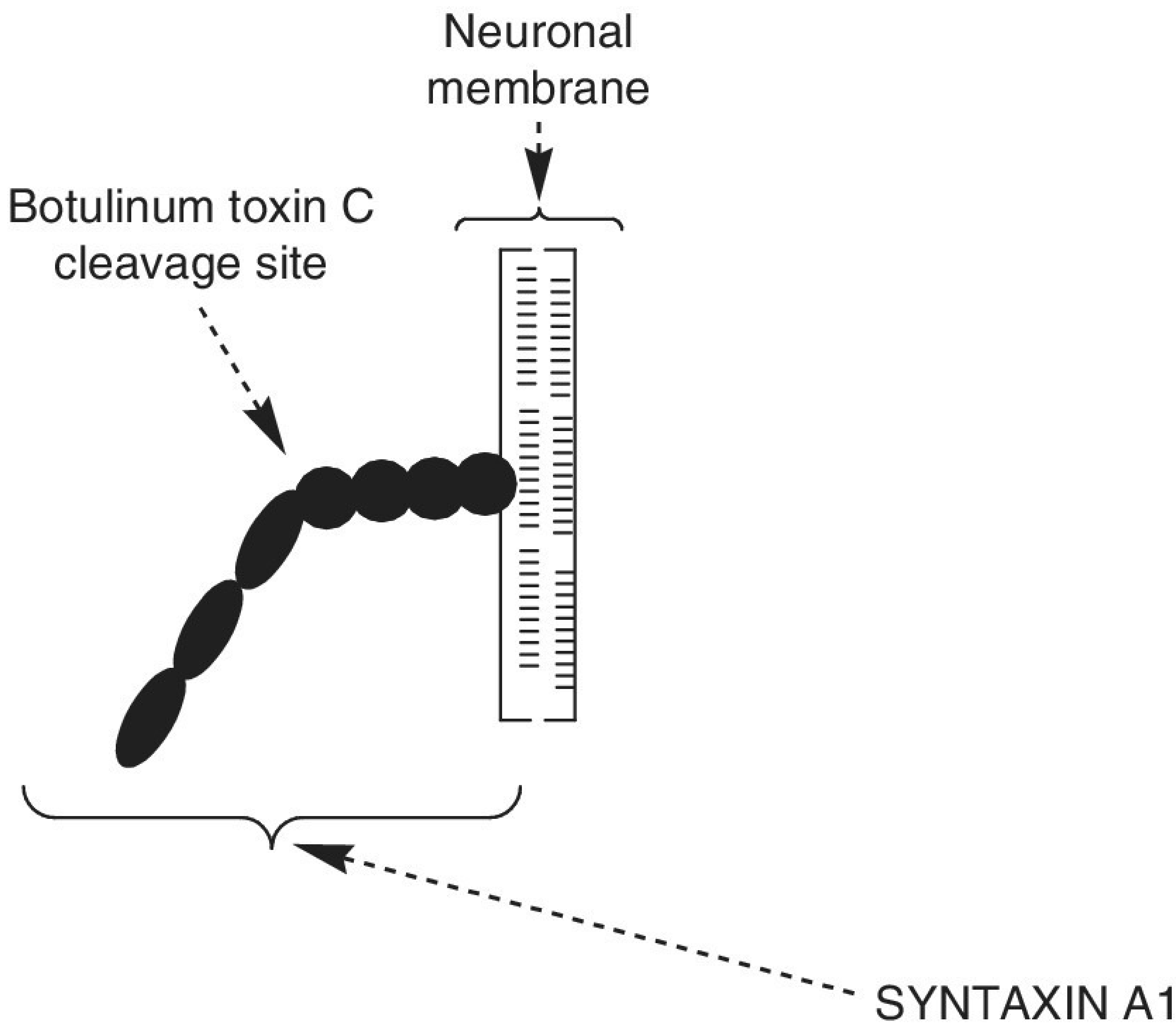

Figure 5.2c General mechanism of action of botulinum toxins in specific cleaving of SYNTAXIN A1 on the neuronal membrane (Boyer *et al*., 2005).

Clostridium perfringens and *C. perfringens* Toxins in Food Forensics

Clostridium perfringens is an anaerobic Gram-positive bacterial pathogen that possesses the ability to form tough, dormant, and heat-resistant endospores, allowing it to survive in incomplete cooking of foods; or offering protection during times of environmental stress such as unavailability of water, high temperatures, *etc*. It also possesses the ability to grow quickly in foods, enabling it to contaminate foods rapidly and widely. These species are also capable of producing intestinally active *C. perfringens* enterotoxin (CPE), a polypeptide

compound of 35 000 Da mol. Wt., which causes *C. perfringens* type A food poisoning (McClane, 1996). *C. perfringens* is commonly found in raw meat and poultry.

There are four major types of toxins that are produced by five main groups of *C. perfringens*, which are classified using the letters A, B, C, D, and E, based on the strain types as well as the type of toxin each produces. The four types of toxins are alpha toxin (α), beta toxin (β), epsilon toxin (ε), and iota toxin (i). There are also other minor toxins and enterotoxins that are produced by these strains, which do cause illnesses by affecting intestinal tissues (Gao *et al*., 2012; Sakurai *et al*., 2004). Moreover, *C. perfringens* can produce as many as 16 other toxins, which may exist in various combinations, for example perfringolysin O (PFO), enterotoxin (CPE), and beta2 toxin (CPB2) (Uzal *et al*., 2010). Almost all of the food poisoning cases caused by *C. perfringens* are actually mediated by either one or more of these toxins.

Type A of *C. perfringens* produces mainly one type of toxin, which is alpha toxin, while type B of *C. perfringens* produces three types of toxins, which are alpha, beta, and epsilon toxins, Type C of *C. perfringens* produces two types of toxins, mainly alpha and beta toxins. Type D of *C. perfringens* also produces two types of toxins, which are alpha and epsilon toxins. Type E of *C. perfringens* produces mainly alpha and iota toxins (Sakurai *et al*., 2004). The minor or secondary toxins that are produced by *C. perfringens* strains include enterotoxin and beta2 toxin (Sakurai *et al*., 2004).

Each of these toxinotype classes is known to cause a particular type of illness or disease. However, only toxinotypes A and C are the ones that cause disease and illness in humans, in addition to causing disease in animals. All toxinotypes A, B, C, and D cause disease to animals. Of all the toxin types of *C. perfringens*, type A (mediated primarily by a-toxin and secondarily by u-toxin and hydrolytic enzymes) is the one that is known to be ubiquitous, and very common among the *C. perfringens* toxinotypes.

There are several genotypes within each toxinotype associated with particular diseases. In toxinotype A, genotype plc is associated with gangrene in humans, while plc, cpe is associated with gastrointestinal diseases, and this disease is actually caused by an enterotoxin CPE, which is produced by *C. perfringens* during the sporulation phase; plc, cpb, cpe are associated with diarrhea. In toxinotype C, genotypes plc, cpb are associated with necrotic enteritis.

C. perfringens Alpha Toxins: Chemistry, Structure and

Function

Alpha toxin produced by all strains of *C. perfringens* belong to the clostridial glucosylating toxin family. Generally, like all glycosylating toxins, it is very large, with molecular masses ranging between 250 and 308 kDa in size and has structural similarities to a number of naturally occurring enzymes (Amimoto *et al*., 2007 ; Jank and Aktories, 2008 ; Morris *et al*., 2012).

From the crystallographic point of view, the structure of alpha toxin has been shown to consist of two domains, mainly the N domain, which is made up of nine tightly packed α-helices, and the second one is the C-domain, which consists of an eight-stranded antiparallel β-sandwich motif (Naylor *et al*., 1998 ; Sakurai *et al*., 2004). The N-domain has been shown to have a structural similarity to the one found in the alpha toxin produced by *B. cereus* (BC-PLC), with the exception that the alpha toxin produced by *C. perfringens* has an additional C-terminal domain consisting of 120 residues (Hough *et al*., 1989). Moreover, the structure of alpha toxins produced by *C. perfringens* has also been shown to contain three divalent cations that have zinc ions located in the active site, such that all the amino acid residues that take part in zinc coordination are highly important to effect the enzymatic activities (Guillouard *et al*., 1997 ; Nagahama *et al*., 2002).

Moreover, crystallographic studies have revealed that there is a structure-function relationship that exists between the amino acid moities and zinc ions of alpha toxin with its biological activity, such that it has been shown that one of the zinc ions is strongly bound to His-11 and Asp-130, while the other is strongly bound to His-148 and loosely bound to Glu-152, and a divalent cation is loosely bound to His-68, -126, -136, and Asp-130 (Nagahama *et al*., 1995, 1996). Another study by Nagahama *et al.* (1997) revealed that Asp-56 is essential for catalytic activity, a revelation that prompted the conclusion that the catalytic site of the toxin is found in the N-terminal domain.

The alpha toxin is most commonly found in animals, and is the toxin that is most commonly found in humans, more than any other types of toxins, causing toxicity to the victim by reducing blood supply to tissues, and thereby leading to illness and disease symptoms (Hunter *et al*., 1993 ; Sakurai *et al*., 2004).

C. perfringens Alpha Toxin: Mode of Action

Alpha toxins produced by *C. perfringens*, as well as beta and epsilon toxins, are known to either cause destruction of the cell membranes of the host or form

pores in the host's cell membranes (Sakurai *et al*., 2004) (Figures 5.3 and 5.4). On the other hand, the toxins produced by strains A-D (CPA, CPB, CPC and CPD), together with other hydrolytic enzymes are secreted, with the exception of CPE, which becomes released only after the *C. perfringens* cell has undergone lysis (Sakurai *et al*., 2004).

The ability of alpha toxins to damage the cell membrane comes from the fact that they possess two important enzymes, namely phospholipase C (PLC) and sphingomyelinase (SMase), which are associated with the required biological activities that result in hemolysis, lethality, dermonecrosis, and cytotoxic activities (Sakurai *et al*., 2004). The toxin phospholipase C (PLC) activity facilitates the hydrolysis of phosphatidylcholine to phosphorylcholine and 1,2-diacylglycerol (DG) (Barrett, 1986 ; Krug and Kent, 1984).

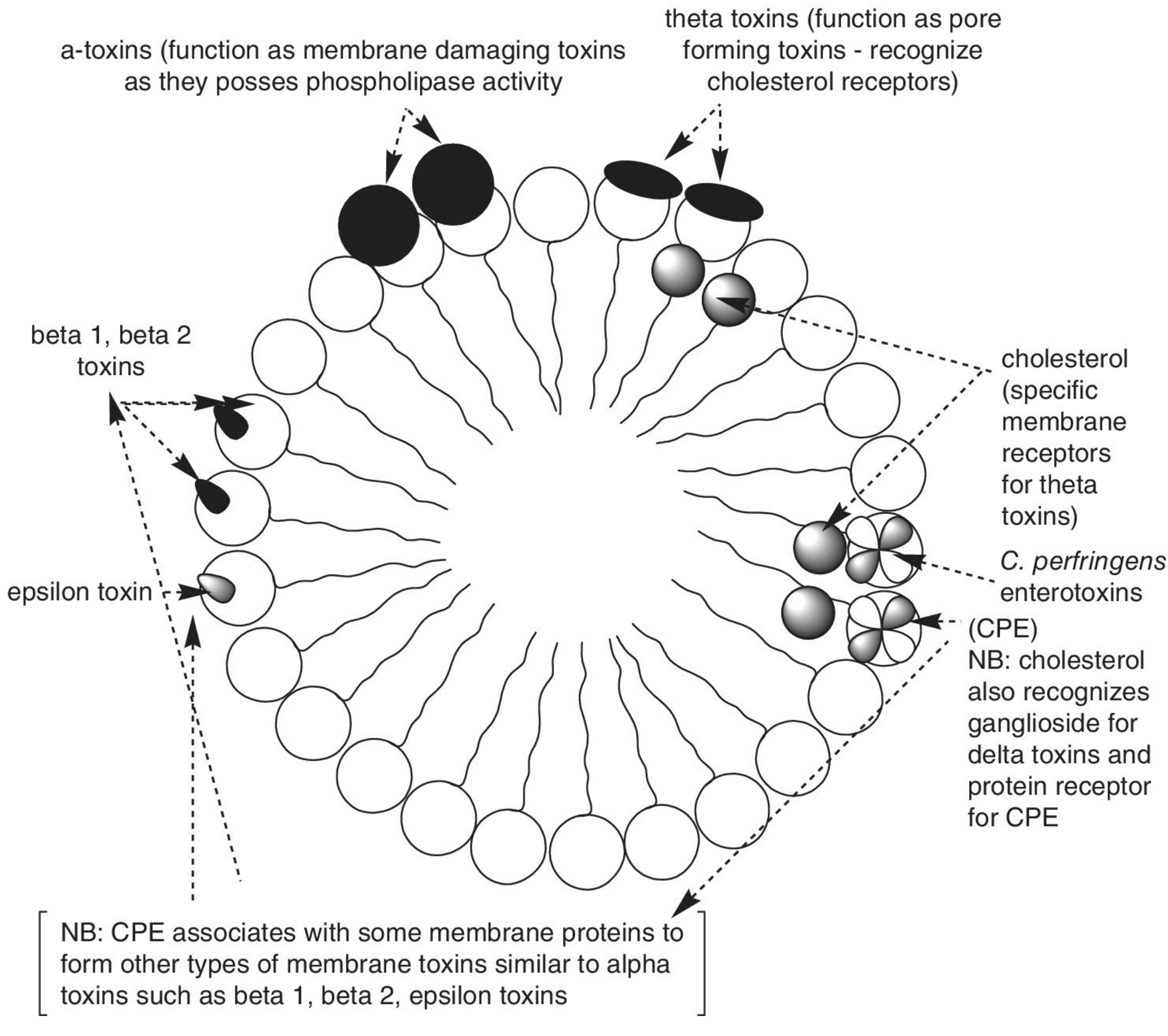

Figure 5.3 Targets and the proposed mode of action of toxins produced by *Clostridium perfringens*.

Active N-domain with active site cleft binds to the C-domain at the membrane surface

↓

The N-domain/C-domain complex inserts through the membrane

↓

The phosphatidylcholine is then cleaved into phosphorylcholine and diacylglycerol

↓

The membrane is then completely disrupted

Figure 5.4 Proposed mechanism of action of alpha toxin.

Mechanism of Action of Beta Toxins

Certain reports have suggested that *C. perfringens* beta toxins may have a certain degree of similarity to the primary structures of both alpha hemolytic and gamma-hemolytic toxins produced by certain *Staphyloccocus* spp., which are known to be pore-forming toxins (Shatursky *et al*., 2000 ; Steinthorsdottir *et al*., 2000). It is thus possible that *C. perfringens* produced beta toxins are likely to be membrane pore-forming toxins also. A report by Steinthorsdottir *et al*. (2000) showed that beta toxins produced by *C. perfringens* do form multimeric complexes of transmembrane pores, mainly in the human umbilical vein endothelial cells (HUVECS), something that can imply that beta toxins may be exhibiting the tendency to oligomerize, like many other pore-forming toxins (Steinthorsdottir *et al*., 2000). In another study by Shatursky *et al*. (2000), it was reported that beta toxins produced by *C. perfringens* are capable of inducing the secretion of arachidonic acid as well as the leakage of inositol from the HUVECS and that the beta toxin is also capable of forming potential-dependent and cation selective channels made up of phosphatidylcholine and cholesterol.

Pathogenically, beta toxins have been reported to act as a mechanism that involves the disruption of essential neurological pathways, consistent with their selectivity of the beta toxin channels, thus causing various diseases and illnesses (Shatursky *et al*., 2000).

Epsilon Toxin (ETV) and Food Forensics

The epsilon toxin (ETX) is an aerolysin-like, pore-forming toxin. Mainly produced by type B and type D strains of *C. perfringens*, it is rarely isolated in humans, but commonly in animals, such as sheep, goats, and cattle. The mode of action of epsilon toxin involves the formation of pores in the animal tissues, thus causing leakage of potassium ions as well as fluid, which results in the development of disease symptoms in animals, for example lamb dysentery, pulpy kidney diseases, and also colitis (Hunter *et al*., 1992 ; Oyston *et al*., 1998 ; Uzal, 2004).

The Chemistry of Epsilon (ETX) Toxin

ETX is normally secreted as a prototoxin of molecular weight 32 981 Da (Hunter

et al., 1992), which then undergoes enzymatic transformation catalyzed by proteases including trypsin, chymotrypsin, *etc.* into an active highly toxic molecule which has been reported to be almost 1000 times more toxic than the prototoxin (Bhown and Habeeb, 1977 ; Minami *et al*., 1997). The epsilon toxin (ETX) is known to be very potent, with a mouse lethal dose of 100 ng/kg (Sakurai, 1995).

The Genetics of ETX Toxin

Genetically, the ETX is encoded by the gene (*etx*), which is found exclusively in conjugative plasmids (Miyamoto *et al*., 2008 ; Sayeed *et al*., 2008). The *etx* gene in type B, as well as certain types of D isolates, has been reported to be flanked by IS*1151* in addition to a gene related to the transposase (*tnpa*) gene from Tn*3* transposons that are found in the upstream section of the *etx* gene.

The Chemistry and Mode of Action of Iota Toxin (ITX) and Food Forensics

The *C. perfringens* type E strains are those known to produce iota toxins. The ITX is a binary toxin in that it is made up of two unlinked proteins, the A protein and B protein (these A and B proteins are not covalently linked), and for that reason it is known as an AB toxin because of these two domains (Marvaud *et al*., 2002). The mode of action involved in ITX has suggested that domain A (with molecular weight of 45 000 Da) of the toxin is the active portion of the toxic molecule, while domain B (with 100 000 Da molecular weight), plays a role in enabling the toxin to bind to a receptor site on the membrane of the host cell (Marvaud *et al*., 2002). The main function of component A of ITX toxin is to break down the cytoskeleton through the mechanism that involves the inhibition of the actin polymerization reaction. On the other hand, the function of component B is mainly to bind to the surface of the cell receptors and also to catalyze the process responsible for the transport of the toxin into the cell.

The Genetics of ITX *C. perfringens* Toxin

The A and B protein components of ITX toxins have been reported to be genetically encoded by two sets of genes, mainly iap (~1160 nt) and iab (~2,630 nt), which are found on large, potentially conjugative plasmids of approximately 97 kb or close to 135 kb and these two sets of genes together form an operon with an additional intermediate portion of the gene that is composed of 243 non-

coding nucleotides (Perelle *et al*., 1993).

Clostridium perfringens Beta 2 Toxin (CPB2): Chemistry and Mode of Action

The *C. perfringens* toxin beta 2 toxin (CPB2) causes enteric diseases, mainly in food producing and also wild animals. Certain researchers have suggested that the CPB2 toxin acts in synergy with other various toxins produced by *C. perfringens* and thus causes disease symptoms related to necrotic and hemorrhagic enteritis (Perelle *et al*., 1993).

Clostridium perfringens Perfringolysin O (PFO)/Theta Toxin

Theta toxin or perfringolysin O (PFO) is a cytolytic toxin with a molecular weight of 54 000 Da, which contains 27 amino acid signal peptide, and when it has matured as a protein its chemical structure has been found to comprise of 472 amino acids (53 kDa) (Tweten, 1988). PFO or theta toxin executes its toxicity properties to a host cell by binding to a cholesterol-containing cell membrane. Like other *C. perfringens* toxins discussed throughout this chapter, PFO is known to form a network of pre-pores, which are oligomeric on the surface of the host’s cell membrane just before they penetrate the cell membranes of the host. The chemical structure of this toxin, which has been reported to have a strong affinity to cholesterol (Shepard *et al*., 1998), has revealed that it contains four domains, including a carbon-terminal that plays a role in the organization of water soluble monomers that then bind cholesterol, a molecule that causes the enrichment and therefore oligomerization of PFO at the surface of the cell membrane. This binding causes a conformational change in the third domain, which results in the mechanisms that exposes beta hairpin, which plays an important role in enabling the whole complex to penetrate the lipid-bilayer membrane of the host (Shepard *et al*., 1998, 2000). The properties of *C. perfringens* toxins are depicted in Table 5.1.

Table 5.1 Biological and chemical properties of toxins produced by *C. perfringens* (Sakurai, 1995 ; Sakurai and Duncan, 1978).

***C perfringens* toxin**	**ETX (34 kDa)**	**CPA (42.5 kDa)**	**CPB (35 kDa)**	**CPB2 (28 kDa)**	**IT kD**
Stability (time and heat/thermo)	Thermostable at room temperature for ~ 1 week; at	Thermostable	Thermostable up to 60 °C and up to two		Th

heat/thermo.)	for ~ 1 week, at –20 °C (months) or at –80 °C (years)		and up to two months if at –80 °C		
Stability under the action of trypsin	Activated/resistant	Susceptible	Sensitive	Sensitive	Ac
Biological activity	• Lethal • Dermonecrotic Edema • Mouth muscle contraction	• Lethal • Hemolytic • Necrotizing	Dermonecrotic • Edema • Enterotoxic • Cytotoxic		• •

The Genetics of Theta Toxins/PFO

The theta (PFO) toxin is encoded by the gene (PFOA gene), which is normally found on the chromosomal DNA that is located in close proximity to the origin of replication (Shimizu *et al*., 2002a, b).

Clostridium perfringens Enterotoxin (CPE): Chemistry and Mode of Action

Clostridium perfringens causes food poisoning in humans due to its ability to produce a 35 000 Da polypeptide known as *C. perfringens* enterotoxin (CPE), which is responsible for characteristic gastrointestinal symptoms. The ingestion of food containing *C. perfringens* causes food poisoning that results from sporulation of the *C. perfringens* bacteria inside the intestine, which then causes the accumulation of CPE to be released when the bacterial cells lyse to discharge the toxin to the intestinal lumen (McClane *et al*., 2006).

The sequence of CPE has shown that it has 319 amino acids and that this particular peptide contains an N-terminal domain, which is crucial for pore formation as well as for cytotoxicity. CPE also contains a C-terminal domain that plays an important role in mediating the binding process to the caludins receptor (McClane *et al*., 2006).

Generally, the CPE toxins form a number of small complexes, which then

oligomerize on the membrane surface to form CH-1 pre-pores, each containing at least six copies of CPE molecules, as well as claudin receptors and non-receptor claudins capable of interacting with the claudin receptors (Robertson *et al*., 2007). This process causes the influx of calcium, which then catalyzes the apoptotic and oncotic cell death pathways that bring about morphologic damage to the exposed biological cells. The consequence of morphologic damage is realized by the fact that it exposes the baso-lateral surface of the cell, creating the possibility for the CPE to interact with occludin and excess claudins to form another complex CH-2 in a process that has the potential to initiate the internalization of occludin and/or that of claudins, thus contributing to paracellular permeability changes and diarrhea (Figure 5.5).

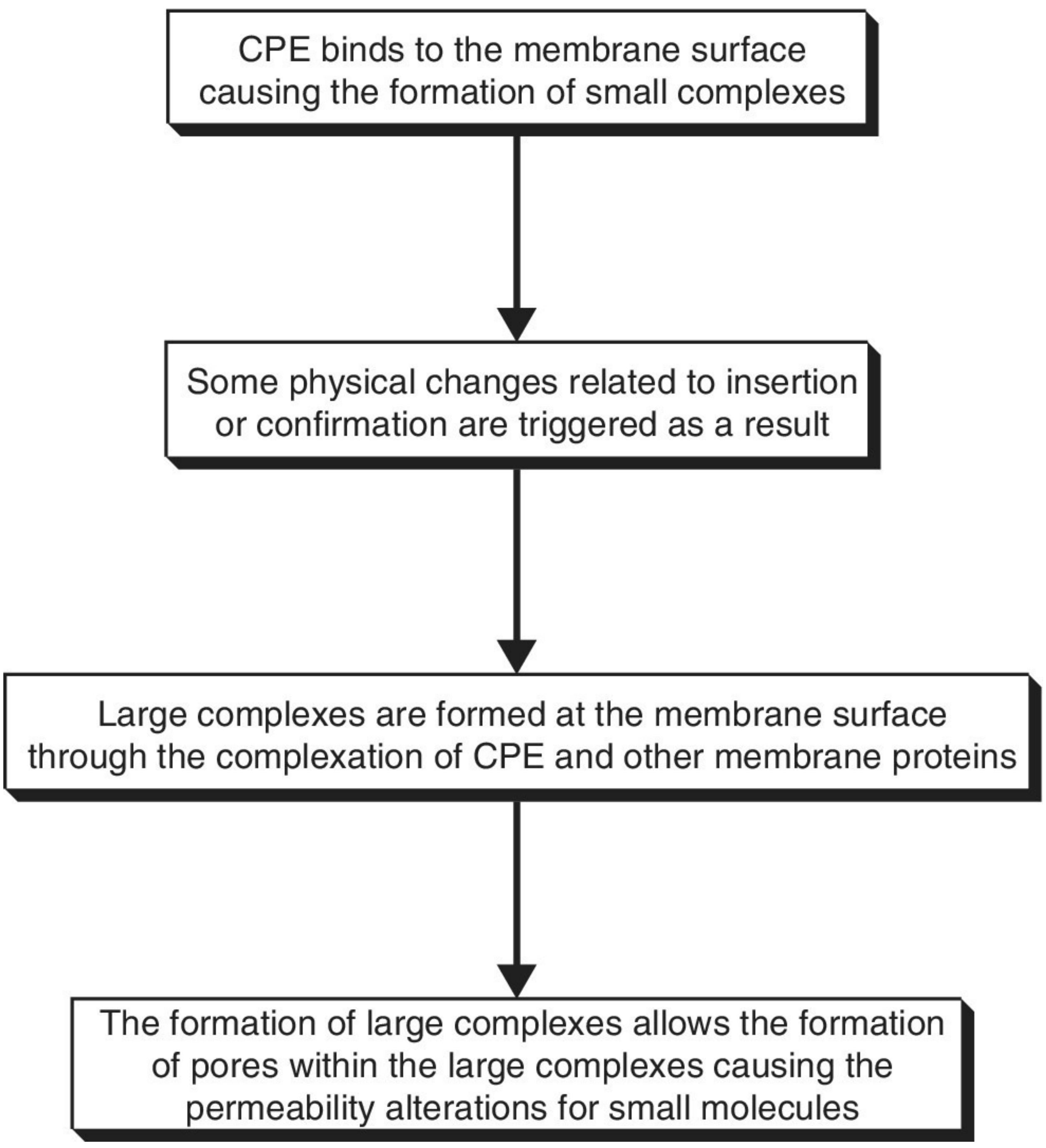

Figure 5.5 Suggested processes involved in CPE-induced cytotoxicity.

Humans become infected with these toxins by consuming food that contains these toxigenic microorganims (*C. perfringens*) (Labbe, 1989). Although the digestive system has mechanisms to eliminate microbial pathogens, such as the acid present in the stomach, in cases where there are too many *C. perfringens* contaminating the food that has been consumed, some of the cells may survive and get into the ileum (small intestine), where they can thrive, multiply, and

undergo sporulation, a stage in which CPE is genetically expressed and synthesized. The synthesized CPE becomes stored within the sporulating *C. perfringens* cells and they can only be released when the cells are ruptured or lysed at the culmination of the sporulation process, where the CPE enters the intestinal lumen to bind to the receptors found on the intestinal epithelial cells and causes food poisoning that can be exemplified through characteristic symptoms of diarrhea and abdominal cramps.

The Genetics of *C. perfringens* CPE Toxin

The gene that encodes for human poisoning CPE (the CPE gene) has been reported to be located on either the chromosome or on plasmids (Collie and McClane, 1998 ; Cornillot *et al*. 1995 ; McClane *et al*., 2006).

Staphylococcus Aureus Staphylococcal Food Poisoning (SFP) and Food Forensics

The Gram-and catalase-positive bacterial genus *Staphylococcus* is spherical, nonsporulating, and non-motile, which is a facultative aero-anaerobic species. Staphylococci have been classified based on their ability to produce coagulase such that there are two groups, one of which is coagulase-producing strains (coagulase-positive staphylococci (CPS)) and the other group is the non-coagulase-producing strains, (coagulase-negative staphylococci (CNS)). The majority of strains fall under the CNS group, which is comprised of strains that are considered to be beneficial in the food industry, because some members of CNS are responsible for fermentation of food products such as meat and milk (Becker *et al*., 2001 ; Rosec *et al*., 1997), and only a few have been reported to produce enterotoxins (Even *et al*. 2010 ; Vernozy-Rozand *et al*., 1996 ; Zell *et al*., 2008). On the other hand, the majority of strains that comprise the CPS group have proved to be responsible for many food poisoning incidents and thus in this chapter *S. aureus* will mainly refer to the strains that form the CPS group.

For this reason, among the possible microbial food terrorism agents that have the potential to be used is the one due to Staphylococcal food poisoning (SFP), which results in foodborne-related illness and disease following the consumption and/or ingestion of foods contaminated with staphylococcal enterotoxins (SEs), produced by enterotoxigenic strains of coagulase-positive staphylococci such as *S. aureus* (Jablonski and Bohach, 1997), *S. intermedius, S. cohnii, S. epidermidis, S. xylosus, S. haemolyticus, etc.* (Genigeorgis, 1989 ; Khambaty *et*

al., 1994).

Toxins produced by *S. aureus* are normally classified into groups, including staphylococcal enterotoxins (SEs) that are further subdivided into subgroups including SEA, SEB, SEC, SED, SEE; SEG …. SEI; SER …… SET). The SEs toxins are known to demonstrate emetic activity. Another group is that of staphylococcal-like proteins (SE*l*), which do not possess any emetic activity and these include mainly SE*l*L and SE*l*Q. There are those also that are yet to be tested and include SE*l*J, SE*l*K, SE*l*M …… SE*l*P, SE*l*U, SE*l*U2 and SE*l*V). Toxins that are grouped under SEs and SEls are those that have generally been classified (SEA to SEE), as well as the new classes SEG to SElU2.

The enterotoxins SEs are known to be potent gastrointestinal exotoxins and are generally produced either during the logarithmic growth phase or during the transition from the exponential to the stationary growth phase (Betley *et al*., 1992 ; Bergdoll, 1979 ; Czop and Bergdoll, 1974 ; Derzelle *et al*., 2009 ; Otero *et al*., 1990). The *S. aureus* cell wall and even SEs toxins have been reported to be resistant to heat treatment, drying, freezing, acidic and proteolytic enzymes (e.g. lysozyme), which make them survive the stringent digestive mechanisms in the alimentary canal and are also active at very low concentrations that are in between high nanogram to low microgram quantities (Bergdoll, 1983 ; Evenson *et al*., 1988 ; Larkin *et al*. 2009 ; Schantz *et al*., 1965). *S. aureus* species are, however, sensitive to lysostaphin, which is able to cleave their pentaglycin linkages. These species possess the ability to grow and thrive in different types of foods, even at high salt concentrations, and are thus susceptible for use by criminals in terrorism-related activities.

The Chemistry, Mode of Section, and Genetics of SE and SEls Toxins

The chemistry of SE and SEI toxins produced *S. aureus*, shows that they possess either superantigenic or emetic activity or both, and are encoded by accessory genetic elements that are located on several accessory genetic elements, mainly plasmids, prophages, *S. aureus* pathogenicity islands (SaPIs), genomic island vSa, and some are found next to the staphylococcal cassette chromosome (SCC) elements. Since these genetic elements tend to be mobile, and are different within various strains of *S. aureus*, it follows that they can also influence different profiles in terms of their ability to cause disease within *S. aureus* strains.

The mode of action of enterotoxins, as suggested by Sugiyama *et al*. (1965), involves the stimulation of the vagus nerve in the abdominal viscera, which then transmits the signal to the vomiting center in the brain, thus triggering an emetic reaction. This mechanism has been supported by Ho *et al*. (2007), who added that the receptors on vagal afferent neurons are very central to SEA-triggered emesis and that SE enterotoxins possess the capability to penetrate the gut lining and activate both local and systemic immune responses, which results in the release of inflammatory mediators such as histamine, leukotrienes, and neuroenteric peptide substance P, which are responsible for vomiting (Alber *et al*., 1989 ; Jett *et al*., 1990 ; Scheuber *et al*., 1987 ; Shanahan *et al*., 1985 ; Shupp *et al*., 2002). On the other hand, the diarrheal problems observed after ingestion of food contaminated with SEs may be due to the inhibition of water and electrolyte reabsorption in the ileum (small intestine) caused by these enterotoxins, which are believed to facilitate transcitosis, enabling the toxin to penetrate into the bloodstream, which brings about the possibility for the interaction with T-cells, resulting in superantigen activity (Hamad *et al*., 1997 ; Sheehan *et al*., 1970 ; Sullivan, 1969).

Their chemical structure is composed of globular single-chain proteins with molecular weights ranging from 22 000 to 29 000 Da. SEA (Mwt 27 100 Da) displays both superantigenic and emetic actions and is encoded by genes located in the prophage, while SEB (Mwt 28 336 Da), with both superantigenic and emetic actions, is encoded by genes located on chromosomes, plasmids, and *S. aureus* pathogenicity islands. Another superantigenic and emetic SE enterotoxin that is encoded by genes located in plasmids is SEC1-2-3 (Mwt ~27 500 Da). Other *S. aureus* enterotoxins, such as SED (Mwt 26 360 Da), SEE (Mwt 26 425 Da), SEG (Mwt 27 043 Da), and SHE (Mwt 25 210 Da), also possess both superantigenic and emetic activity and are encoded by genes located in plasmids, prophage/enterotoxin gene clusters (egc), chromosomes, and transposons, respectively.

Plasmids are genetic vehicles for the spread of resistance as well as virulence determinants through horizontal gene transfer and in *S. aureus* there are two kinds of plasmids that function to carry se/sel genes and both contain sej and ser associated with r sed (pIB485-like), as well as ses and set (pF5) (Omoe *et al*., 2003). *S. aureus* enterotoxins and enterotoxin-like toxins with genes encoded by plasmids include SEB, SEC1-2-3, SED, SEIJ, SER, SES, and SET.

Prophages are gene carriers and in *S. aureus* phages play a role in carrying se genes (sea, selk, selp, and selq). Pathogenicity islands refer to distinct genetic

elements located on the chromosomes of certain species of pathogenic bacteria species, which encode certain virulence factors. In *S. aureus*, they are highly conserved overall, and each one occupies a specific chromosomal site and always appears in the same orientation and they encode genes of SEB, SEK, SEIL, and SEIQ enterotoxins and enterotoxin-like toxins

On the other hand, enterotoxins such as SEI (Mwt 24 928 Da), have shown positive superantigenic action but weak emetic action. This enterotoxin is encoded by genes located in the enterotoxin gene cluster (egc) as well as on chromosomes. The enterotoxin SEIJ encoded by genes located in plasmids, with a molecular weight of 28 565 Da, has shown positive superantigenic action, but its emetic action is yet to be established. The same is true for SEK (Mwt 25 539 Da) and SEIL (Mwt 25 219 Da), which are encoded by genes located in pathogenicity islands, as well as for SEIM (Mwt 24 842 Da), SEIN (Mwt 26 067), and SEIO (Mwt 26 777 Da), which are encoded by genes located on egc/chromosomes. SEIP (Mwt 26 608 Da) encoded by genes found on prophages and SEIQ (Mwt 25 076 Da) encoded by genes found on pathogenicity islands, also show positive superantigenic action, but the emetic action has yet to be established. The same is true for SER (Mwt 27 049 Da), SES (Mwt 26 217 Da), and SET (Mwt 22 614 Da), which are encoded by genes located on plasmids as well as SEIU (Mwt 27 192 Da), SEIU2 (Mwt 26 672 Da), and SEIV (Mwt 24 997 Da), which are encoded by genes located on egc/chromosomes.

Gram-negative Bacteria and Lipopolysaccharide Endotoxins in Food Forensics

Gram-negative bacteria, unlike Gram-positive (which produce neuro-exotoxin proteins), produce intracellular lipopolysaccharide endotoxins that do not leave the cell but rather remain as part of the constituents of the cell membrane structure. Both exotoxins and endotoxins from Gram-positive and Gram-negative bacteria are known to cause food poisoning. Both live cells and dead cells (when they undergo decaying process) have the capability to produce endotoxins. Generally, the antigenic endotoxins have complex chemical structures and composition with proteins, lipids, and polysaccharides forming part of the complex structure. These endotoxins are heat stable and normally require no latent period to become active.

Vibrio Parahaemolyticus in Food Forensics

Several species in the bacteria genus of Vibrio are known to be food-associated pathogens (Dalsgaard, 1998). The *Vibrio parahaemolyticus* species is a halophilic Gram-negative, facultative anaerobic rod-shaped bacterium that inhabits mainly salty (marine and estuarine) environments. This species has been implicated in a number of food poisoning cases and is normally found in raw seafood, such as fish, crustaceans, and molluscan shellfish, where its presence is associated with foodborne illness in those who consume contaminated seafood (FAO/WHO, 2002). The ingestion infective dose has been reported to be between 105 and 107 count of viable cells (Daniels *et al*., 2000). Once a sufficient number of *V. parahaemolyticus* has been ingested, the victim may start experiencing symptoms that include self-limiting acute gastroenteritis as characterized by watery diarrhea and abdominal cramps, nausea, vomiting, headache, and fever and the incubation period from intoxication to symptoms can be between 12 and 24 hours and the sickness/illness can take up to 72 hours as patients do not suffer long-term sequelae illness (Heymann, 2004). The confirmation of *V. parahaemolyticus* normally necessitates the isolation of the bacteria from the victim's stool specimen, culture on appropriate media, normally thiosulphate citrate bile salt sucrose (TCBS) agar medium (Heymann, 2004), and then using molecular biology techniques to identify the genes that encode hemolysin (i.e. those that code for the major virulence factors of *V. parahaemolyticus* to upregulate the production of thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH)). The molecular methods appropriate for this task includes pulsed field gel electrophoresis (PFGE), a technique which involves the isolation of chromosomal DNA of a pathogen cut into large restriction fragments, then by using agarose gel electrophoresis to separate and identify the separated bands against a well-known genetic marker.

Salmonella Bacilli and Typhoid/Parathyphoid Toxins in Food Forensics

Salmonellae bacteria are Gram-negative, flagellated, facultative anaerobic bacilli microorganisms, characterized by having three major antigens, namely antigen H or flagellar antigen; antigen O or somatic antigen; and Vi antigen.

Salmonella species cause two basic forms of clinical disease:

1. gastroenteritis characterized by the abrupt onset of nausea, fever, vomiting, diarrhea, and abdominal cramps; and
2. enteric fever, a disease with greater systemic involvement with clinical

symptoms characterized by fever, mainly typhoid fever (generally caused by *S. typhi*).

Salmonella food-poisoning causes diarrheal disease as well as intestinal or systemic disease, when *Salmonella* infects the intestinal lumen. The poisoning in *Salmonella* food contamination is thought to be caused by endotoxins, which cause systemic infections (salmonellosis) or those secondary metabolites that are responsible for typhoid and paratyphoid diseases and dysentery. However, there is the effect exotoxin, which is an enterotoxin, produced by certain *Salmonella* species that may be responsible for the pathogenesis of salmonellosis (including several syndromes such as gastroenteritis, enteric fevers, septicemia, focal infections, and an asymptomatic carrier state). *Salmonella* species that normally cause salmonellosis include:

- *S. typhi*, *S. paratyphi A*, and *S. schottmuelleri* (cause enteric fever);
- *S. choleraesuis* (causes septicemia or focal infections); and
- *S. typhimurium* and *S. enteritidis* (cause gastroenteritis),

although, the practical observation is always that any *Salmonella* species/strain can cause any of the syndromes.

Mode of Action of Salmonella Endotoxins

Moreover, the cell membranes of Salmonellae contain a complex lipopolysaccharide (LPS) compound that can be released upon death or cell lysis, which functions as an endotoxin also playing the indicative role in terms of the virulence of the strain. The LPS endotoxin is made up of three components, the external *O*-polysaccharide coat, a middle portion (the R core nucleus), and an interior lipid A coat portion. The lipopolysaccharide structure is composed of repeating sugar units in the outer *O*-polysaccharide chains, which plays an important role related to O antigen specificity. The lipopolysaccharide moiety is also believed to be necessary in establishing the virulence of the *Salmonella* species or strains, a phenomenom that has been deduced from the fact that Salmonellae strains that do not contain a complete sequence of O-sugar repeats normally do not display virulence characteristics to the extent that those with this polymer show. The endotoxins produced by *Salmonella* species are known to cause fever, activate the serum complement, kinin, and clotting systems, depress myocardial function, and alter lymphocyte function.

There are several governing virulence factors that must be possessed by the *Salmonella* species to be able to cause salmonellosis and include the ability to

invade and infect cells and this takes place through the *Salmonella* cell binding to specific receptors on the epithelial cell surface, which then induces the enterocyte membrane to stimulate pinocytosis of the organisms.

When these pathogens have invaded the intestine, they induce an acute inflammatory response, which results in ulceration, elaborate cytotoxins that then inhibit protein synthesis, and also triggers inflammatory response. Moreover, invasion of *Salmonella* pathogens to the mucosa triggers the epithelial cells to synthesize and release pro-inflammatory cytokines, mainly IL-1, IL-6, IL-8, TNF-2, IFN-U, MCP-1, and GM-CSF, which invoke an acute inflammatory response and damage to the intestine. The intestinal inflammatory reaction as well as symptoms associated with inflammation cause fever, chills, abdominal pain, leukocytosis, and diarrhea (Figure 5.6). In many instances, it has been shown that the invasion of the intestinal mucosa is followed by activation of mucosal adenylate cyclase, causing an elevated level of cyclic AMP, which then induces secretion. The adenylate cyclase is stimulated in a series of mechanisms that involve production of prostaglandins or other components of the inflammatory reaction.

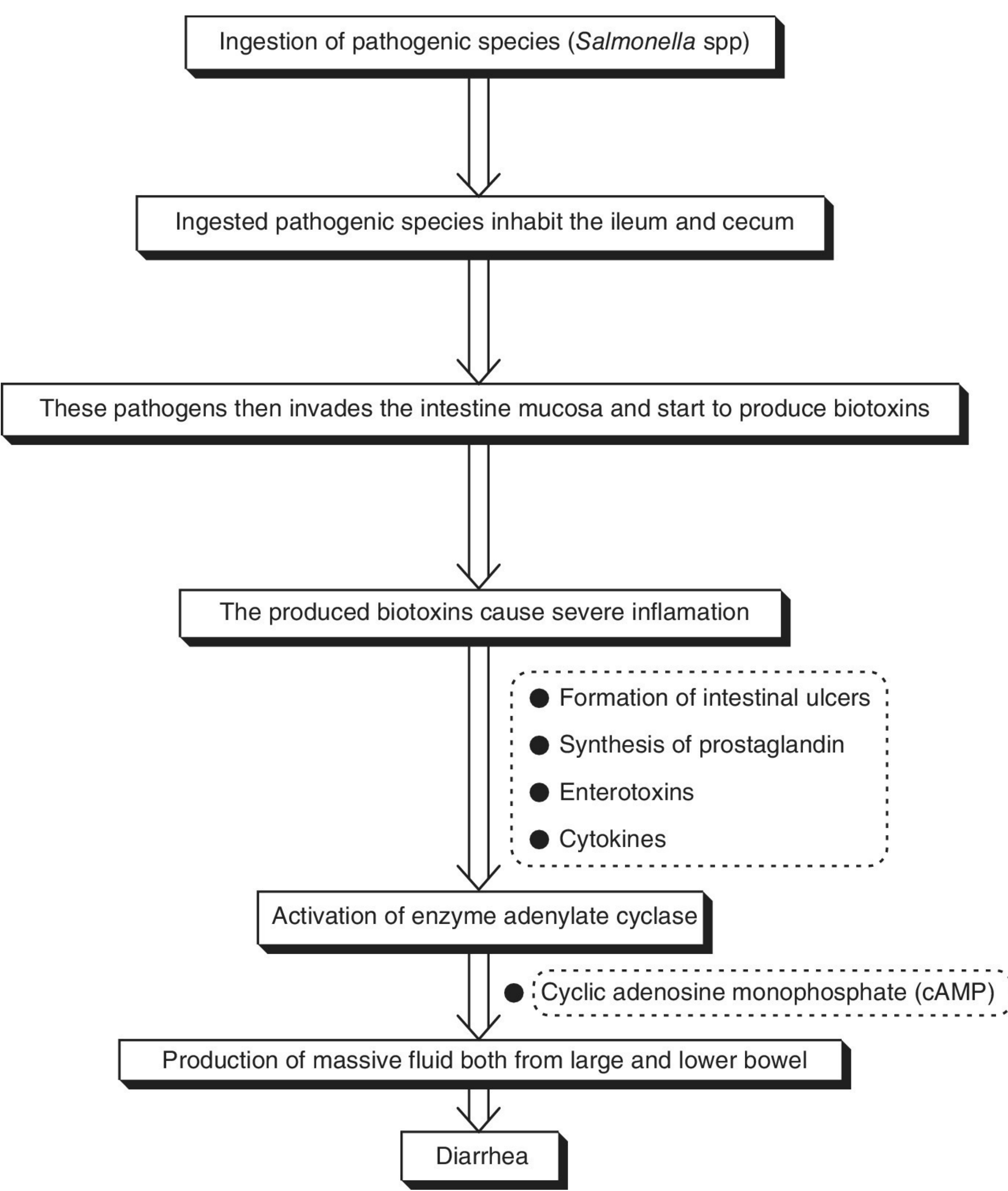

Figure 5.6 The proposed mechanisms of *Salmonella* gastroenteritis, enterocolitis, and diarrhea.

Other virulence factors necessary for salmonellosis include the presence of a complete lipopolysaccharide coat and the ability to replicate intracellularly. The mechanisms of *Salmonella* gastroenteritis, enterocolitis, and diarrhea are

summarized in Figure 5.6. The diarrhea occurs due to the secretion of fluid and electrolytes by the small and large intestines, due to tissue destruction and ulceration.

Campylobacter Species (*C. jejuni* and *C. coli*) and Food Forensics

Of the *Campylobacter* species, *Campylobacter jejuni* and *Campylobacter coli* are Gram-negative, spiral-shaped, motile, microaerophilic, thermophilic rods that grow best at 42 °C, which require low oxygen concentrations and complex growth media for their growth. These species are unable to ferment carbohydrates, although they are known to be catalyzed and oxidized positive, but urease negative (Blaser, 2000 ; Mandell *et al*., 1988). These *Campylobacter* species are among the pathogens that are a leading cause of foodborne enteritis, infectious fever, headache, abdominal cramps, vomiting, and bloody diarrhea diseases (Tauxe, 1997), with the main source of contaminated foods being poultry, dairy products, and water (Harris, 1986). Some reports have associated *Campylobacte*r infection with Guillain-Barré syndrome (GBS) as a post-infection complication (Koga, 2006). Campylobacters are known to have very slow cell multiplication and therefore cannot be isolated from fecal specimens, as is the case for other enteric bacteria and this requires the use of selective techniques of *Campylobacter* isolation (Blaser, 2000 ; Mandell *et al*., 2009). Certain strains of *C. jejuni* have been linked with the development of the neurological disorder Guillain-Barré syndrome. *C. jejuni* and *C. coli* are also known to be the main causative agents of acute human enterocolitis, as well as foodborne diarrhea diseases.

Campylobacter Species Toxins

There are two types of toxins produced by the *Campylobacter* species, which include cytotoxins and enterotoxins.

Campylobacter Species, Cytolethal Distending Toxin (CDT) in Food Forensics: Chemistry, Genetics, and Mode of Action

The *Campylobacter* species, like many other Gram-negative bacteria, produce

cytolethal distending toxin (CDT), which plays a central role as a virulence factor of the *Campylobacter* species (Asakura *et al*., 2008 ; Ceelen *et al*., 2006 ; Ge *et al*., 2008).

Cytolethal distending toxin activity causes certain cell types (i.e. HeLa cells and Caco-2 cells) to become slowly distended, which progresses into cell death. Although all *C. jejuni* and *C. coli* strains tested contain the cdt genes, research has found that there is a profound variation in CDT titres (Eyigor *et al*., 1999a, b ; Pickett *et al*., 1996). While most *C. jejuni* strains have a relatively high CDT activity, *C. coli* strains show mostly low activity (Pickett *et al*., 1996).

The cytolethal distending toxins (CDTs) are actually a class of bacterial protein toxins that present unique properties emanating from their ability to induce DNA double strand breaks (DSBs), which results in either an irreversible cell cycle arrest or death of the host/victim's cells. CDTs are encoded by three linked genes (*cdtA*, *cdtB* and *cdtC*), which have been identified among a variety of Gram-negative pathogenic bacteria. The CDT has been reported to be endocytosed via clathrin-coated pits and for it to exert cytotoxic effects, it requires an intact Golgi complex.

The chemical structure of CDT holotoxin shows that it is composed of three subunits that have been reported to be encoded by the cdtA, cdtB, and cdtC linked genes, and that these three linked genes (*cdtA*, *cdtB* and *cdtC*)/gene products are required to constitute the fully active holotoxin (Ge *et al*., 2008 ; Yamasaki *et al*., 2006 ; Zilbauer *et al*., 2008).

The cdtB component has functional homology with mammalian deoxyribonuclease I (DNase I). The Investigation of the roles of cdtA and cdtC genes has revealed that they combine with the bacterial outer membrane, a revelation that led to speculation that they are essential for cdtB delivery into the host cell by binding the CDT holotoxin to the host's cell membrane. Once the binding to the cdtB has been achieved, the cdtB active subunit, which possess DNaseI-like activity, induces the victim's DNA damage by breaking its double strand structure (Ceelen *et al*., 2006 ; Ge *et al*., 2008 ; Lara-Tejero and Galan, 2001).

It should be noted that the chemistry of the surface polysaccharide structures found in the outer membrane of most of the Gram-negative (*C. jejuni* and *C. coli* inclusive) is comprised mainly of lipo-oligosaccharide (LOS), which is composed of two regions, a lipid A molecule joined to a core oligosaccharide and lipopolysaccharide (LPS), which in addition to the components of LOS

contains an O-chain made up of repeating oligosaccharide. The LOS and LPS form important virulence factors that are involved in serum resistance, endotoxicity, and adhesion phenomena. The *C. jejuni* surface polysaccharide structures and flagella have been shown to be sialylated, which is thought to be responsible for the ganglioside mimicry leading to GBS (Nachamkin, 1998).

C. jejuni is also known (through its associated heat stable serotypes) to cause GBS (an autoimmune disorder of the peripheral nervous system that is responsible for an acute flaccid paralysis). These heat stable serotypes trigger the formation of antibodies that cause demyelination that results in GBS (Bersudsky *et al*., 2000 ; Endtz *et al*., 2000 ; Kuroki *et al*., 1991, 1993). The clinical symptoms associated with this disorder include progress weakness of the limbs and respiratory muscle, which lead to severe neurological deficits (van Vliet and Ketley, 2001).

Algae/Cyanobacterial Toxins in Drinking Water and Food Forensics

One of the greatest challenges in the aquatic system is the presence and occurrence of algae blooms due to species that belong to true algae, diatoms, flagellates, dinoflagellates, and cyanobacteria, which are ubiquitous in marine and freshwater bodies. Many reports have suggested that the majority of algal blooms are not associated with toxin production and that they are merely an aesthetic nuisance, but certain species of algae under certain stressing conditions do produce toxins that result in poisoning of aquatic species such as fish and shellfish, as well humans, livestock, and wildlife. Some algae species that inhabit marine environments and are pigmented bloom toxin-producing species, are also known as red tides, while those that inhabit freshwater systems are simply known as cyanobacteria blooms. Algal blooms occur under eutrophication conditions as well as when other physical environmental parameters such as temperature are optimal.

The known toxic species of algae produce different types and classes of toxins that exert different effects to aquatic organisms, livestock, and also to humans. For example, certain species of cyanobacteria and dinoflagellates produce toxins that can affect neurons but some affect the liver, whether in domestic animals or in humans. For example, certain species of cyanobacteria produce hepatotoxins and include *Microcystis* species, *Anabaena* species, *Nodularia* species (produce nodularin toxin), and *Aphanizomenon* species (produce saxitoxins,

cylindrospermopsins, and anatoxin-a). Other cyanobacteria species such as *Oscillatoria* species produce neurotoxins, mainly anatoxin-a, anatoxin-a(s), aplysiatoxins, microcystins (hepatotoxic), saxitoxins, lyngbyatoxin-a; *Cylindrospermum* species produce cylindrospermopsin and saxitoxin; and *Gloeotrichia* species produce microcystins (hepatotoxins). Some cyanobacteria species produce a mixture of hepatotoxins and neurotoxins, for example, *Anabaena* species produce saxitoxins (neurotoxins), anatoxins (neurotoxins), microcystins (hepatotoxins) and cylindrospermopsins (hepatotoxins). The route of entry for most of these toxins is mainly through drinking contaminated water. Diatoms such as *Pseudo-nitzschia* species produce domoic acid, a neurotoxin that causes amnestic shellfish poisoning. Domoic acid is known to accumulate in shellfish, sardines, and anchovies and this makes the consumption of contaminated food items (fish, etc.) one of the main routes of poisoning. Other dinoflagellates produce brevetoxins that are neurotoxic and consumption of seafood is the major route.

Fungi Derived Mycotoxins and Food Forensics

Mycotoxins are another group of natural toxins produced by fungi, especially those capable of growing on crops (grain, corn, nuts, etc.), either while still in the field or even after harvest. These mycotoxins, if they fall into the hands of the wrongdoer, can be used to inflict damage to the health of other people through poisoning. Mycotoxins can be present in food through natural contamination and affect people, but they can be used by criminals to deliberately and intentionally poison a number of foods such as cereals, nuts, fruit and dried fruit, coffee, cocoa, spices, oilseeds, and milk, and thus affect all consumers of the intoxicated foods. There are numerous known mycotoxins that possess a variety of different chemical structures, which display marked differing mechanisms of action. The different mechanisms are due to the chemistry and biology of the different organs/tissues the different mycotoxins target, as some affect the kidney, some target the liver, while some tamper with the immune system and there are those that are carcinogenic. Examples of mycotoxins (Figure 5.7) include aflatoxins, ochratoxin A, zearalenone, fumonisin, patulin, ergot alkaloids, trichothecenes, and deoxynivalenol/vomitoxin, *etc*.

Moniliformin/Semisquaric acid/
Hydroxycyclobutenedione/
Cyclobutenedione, hydroxy-/
3-Hydroxy-3-cyclobutenedione

Fusaproliferin/
[(2S)-2-[(1R,3E,5S,8E,12E,15S)-5,17-
dihydroxy-4,8,12,15-tetramethyl-16-
oxo-18-bicyclo[13.3.0]octadeca-
3,8,12,17-tetraphenyl]propyl] acetate

Beauvericin/
cyclo(D-a-Hydroxyisovaleryl-L-N-methyl-Phe)3

Patulin/Clavacin/Clavatin/Claviform/Expansin/Expansine/
4-hydroxy-4,6-dihydrofuro[3,2-c]pyran-2-one

Aflatoxin B1/(6aR,9aS)-2,3,6a,
9a-Tetrahydro-4-methoxy-1H,11H-cyclopenta[c]
furo[3′,2′:4,5]furo[2,3-h][1]benzopyran-1,11-dione

Aflatoxin B2/(6aR-cis)-2,3,6a,8,9,
9a-hexahydro-4-methoxycyclopenta[c]
furo[3′,2′;4,5]furo[2,3-h][1]benzopyran-1,11-dione

Aflatoxin G1/(7aR,cis)3,4,7a,
10a-tetrahydro-5-methoxy-1H,12H-furo[3′,2′:4,5]
furo[2,3-h]pyrano[3,4-c]chromene-1,12-dione

Aflatoxin G1/(7aR,cis)3,4,7a,
10a-tetrahydro-5-methoxy-1H,12H-furo[3′,2′:4,5]
furo[2,3-h]pyrano[3,4-c]chromene-1,12-dione

Aflatoxin G2/(7aR,10aS)-3,4,7a,9,10,
10a-hexahydro-5-methoxy-1H,12H-furo[3',2':4,5]
furo[2,3-h]pyrano[3,4-c][1]benzopyran-1,12-dione

Aflatoxin M1/(6aR-cis)-2,3,6a,
9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta[c]
furo[3',2':4,5]furo[2,3-h][1]benzopyran-1,11-dione

Aflatoxin M2/(6aR,9aR)-9a-Hydroxy-4-
methoxy-2,3,6a,8,9,9a-hexahydrocyclopenta[c]
furo[3',2':4,5]furo[2,3-h]chromen-1,11-dion

Ochratoxin A/(2S)-2-[[(3R)-5-chloro-8-
hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-
7-carbonyl]amino]-3-phenylpropanoic acid

Ochratoxin B/(2S)-2-[[(3R)-8-hydroxy-3-methyl-
1-oxo-3,4-dihydroisochromene-7-carbonyl]amino]-
3-phenylpropanoic acid

Ochratoxin C/ethyl (2S)-2-[[(3R)-5-chloro-
8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-
7-carbonyl]amino]-3-phenylpropanoate

Fumonisin B1/(2S,2'S)-2,2'-{[(5S,6R,7R,9R,11S,16R,18S,
19S)-19-amino-11,16,18-trihydroxy-5,9-dimethylicosane-
6,7-diyl]bis[oxy(2-oxoethane-2,1-diyl)]}dibutanedioic acid

Deoxynivalenol/(3|A,7|A)-3,7,
15-trihydroxytrichotheca-9,12-dien-8-one

Zearalenone/(S-(E))-3,4,5,6,8,10-Hexahydro-14,16-
dihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione

Figure 5.7 The chemical structures of some mycotoxin compounds (Nazari *et al*., 2015).

Fungi Producing Mycotoxins

The fungi species that are known to produce natural toxins are members of certain species of mushrooms such as the *Amanita* species. Moreover, certain molds (i.e. *Aspergillus*, *Fusarium*, and *Penicillium*) are known to produce non-protein heat-stable mycotoxins in crops such as cereals, grain, corn, and peanuts, especially in wet crop fields or if they are stored under moist conditions.

Of the mycotoxins, the consumption of foods contaminated with trichothecenes induces apoptosis and also causes emetic actions (Rocha *et al*., 2005). Other mycotoxins, such as deoxynivalenol, zearalenone, and fumonisin B1 have been reported to exert their toxicities into mitochondria, mainly in the human intestinal cell line Caco-2 (Kouadio *et al*., 2005). The exposure toxicity concentrations for deoxynivalenol, T-2 toxin, and zearalenone, as determined in three human epithelial cell lines, have been reported to be in the range of 100 ng/ml to 1 µg/ml, for the exposure duration ranging between 2 and 4 days (Calvert *et al*., 2005). The toxicity and mechanism of action of ochratoxin A involve the inhibition of mitochondrial respiration as well as tampering with cellular signaling and regulation (Ringot *et al*., 2006). A toxin known as moniliformin, produced by *Fusarium* molds, has been reported to cause kidney disease (it is cardiotoxic) and also affects smooth muscle cells (Kamyar *et al*., 2006).

There are also new and emerging *Fusarium*-produced mycotoxins, which include beauvericin (BEA), enniatins (ENNs) (A, A1, B, B1), fusaproliferin, and moniliformin, which have been found in corn, cereals, and grain, which exert their toxicity through forming cation-selective channels (Jestoi *et al*., 2004 ; Logrieco *et al*., 2002).

Plant Derived Phytotoxins and Food Forensics

Certain phytotoxins, such as ricin, are known to be allergens and are suspected of causing food poisoning related to the castor bean Ricin, found as part of castor oil seeds (species *Ricinus communis*; Family Euphorbiaceae), and are composed of two protein subunit-based phytotoxins with a molecular weight of 162 000 Da. Their toxicity and mechanism of action involve the inhibition of

cellular protein synthesis.

Analysis and Detection Methods for Protein Based Biotoxins in Food Poisoning Cases

A number of methods and techniques have been employed in the analysis of protein-based biotoxins such as botulinum.

Biological Food Poisoning Agents

It should be noted that the biological agents that contain all the properties and characteristics capable of being deployed as bio-weapons or used as bioterroristic materials can easily be found in nature or in human-modified forms and thus easy to access, transport, or be produced and stored. Materials that can be deployed as bio-weapons possess the following characteristics: they are either directly naturally toxic and lethal materials or can change to a toxic/lethal metabolite after ingestion, or they are easily transmissible and may possess an extensively longer incubation time. These agents are usually a product of biotechnology and may have dual use, making it difficult to control or ban but easy to smuggle (Einhorn and Flournoy, 2004).

Biological poisons refers to cellular secondary metabolites that are capable of exerting harm to humans/and or other eukaryotic cells (Russell and Orndorff, 1992). Biotoxic molecules may also be defined as cellular derived substances capable of inducing characteristic harm/lethality to the host eukaryotic cell (Poxton and Arbuthnott, 1990). The food we eat plays an important role in providing us with the energy we need to carry out life’s activities. However, the same food we consume happens to be a suitable energy source for many pathogenic or infectious microorganisms, which they use for their multiplication and growth. When microorganisms are contained in food, they secrete into the food matrix diverse metabolic by-products, which can lead to food spoilage and if this food is consumed it poisons the consumer. In some cases, the food substrate meant for human consumption may serve as a vector for a pathogenic microorganism, thus playing the role of a transmission agent.

Microbial Derived Food Toxins

Microbial food poisoning can be categorized as either toxicosis or infection. In the former category, the food will harbor toxic secondary metabolites released by microbes, while in the latter scenario, microbes present in the food are

consumed to cause health problems to consumers. Of the toxins produced by microorganisms, endotoxins (lipopolysaccharides/lipid A portions produced by the Gram-negative microbe's outer membrane) remain within the microbial cell, and those that are produced by the cell and then released (diffuse out of the cell) are known as exotoxins (extracellular toxic secondary metabolites are usually protein in nature). Another class of food-related toxins are known as enterotoxins, which mainly act on the gastrointestinal tract, resulting in symptoms typical of food poisoning.

Classification of Food Poisoning Agents

There are different ways of classifying food toxins, for example, they may be classified based on the type of cell in the host they target, such as neurotoxins (they affect the central nervous system – neurons); some are named after the microbe that causes the toxicity, *e.g.* cholera toxins (caused by *Vibrio cholerae*; and shiga toxins – caused by *Shigella dysenteriae*). Some food toxins are named after the type of activity they are involved with, for example lecithinase, a phospholipase enzyme produced by *Clostridium perfringens* and *Listeria monocytogenes* (acts on lecithin); and adenyl cyclase produced by *Bacillus anthracis* and *Bordetella pertussis*, acts on adenosine triphosphate (ATP) in a process that converts ATP to AMP (adenosine monophosphate)

As already pointed out, food poisoning is a general term that takes into consideration the aspects of infections as well as intoxication through the consumption of food. However, from a microbiology point of view, it may be desirable to distinguish between the two terms (food poisoning and food infection). Food poisoning, due to microbial attack, can only be considered if that particular microbe has been growing and multiplying in the food (using the food as its substrate) before the actual consumption. On the other hand, the case of food infection is when the food happens to be the carrier such that the microbes themselves do not depend on the food as a substrate (Sridhar, 2014). In other words, food intoxication is in many cases regarded as a result of the presence of secondary metabolites from microorganisms present in the food. For example, some species of *Amanita* mushroom are known to be poisonous when consumed and lead to a condition of mycetism, while consuming foodstuff contaminated with poisonous secondary metabolites from fungi is known to cause mycotoxicosis.

Conclusions

The presence of pathogenic microbes and their toxic secondary metabolites in foods is one the most notorious problems in food forensics. However, the analytical procedures to identify the actual microbial pathogen and/or respective secondary metabolite may require very experienced analysts and the application of multiple techniques to verify the correct identity of the pathogens or their respective metabolites.

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6
Food Forensics Cases Related to Food Bioterrorism Poisoning using Pathogenic Viruses

Viruses are infectious particles (agents) that lack the capability of self-replicating but can do so when they gain access to the inside of living cells of other organisms such as animal cells, plant cells and even microbial cells such as bacteria and archaea (Koonin *et al*., 2006).

There are different types of viruses which are grouped based on different criteria. One grouping is based on how the nucleic acid genetic material that is present inside the virus particles replicates. One of the virus types is the RNA viruses, in which the replication of their genetic materials takes place in the cytoplasm. The viruses in which the replication of genetic material takes place in the nucleus of the host cell belong to the type known as DNA viruses. There are several groups within the DNA viruses, with those with the capability to penetrate the host's cell membrane by diffusion controlled mechanisms through the receptors that may be present on the surface of the cell membrane of the host, and those that penetrate the cell membrane of the host through receptor-facilitated endocytosis mechanisms. Other types of viruses comprise those with single-stranded RNA, which are known as reverse transcribing viruses (Staginnus and Richert-Pöggeler, 2006).

Pathogenic Influenza Viruses and Food Forensics

Influenza viruses are a major concerns because of their potential to be used in food bioterrorism acts. In the 1990s to the 2000s, the world experienced outbreaks of different types of influenza viruses such as avian (e.g. highly pathogenic avian influenza A virus, H5N1, avian influenza A, H7N9 virus, H1N1) flu, swine flu, the virion that caused bovine spongiform encephalopathy (BSE), commonly known as mad cow disease, and when in humans the variant of this disease is known as Creutzfeldt–Jakob disease, *etc.* (WHO, 2008).

Avian Influenza Viruses and their Potential

Avian Influenza Viruses and their Potential Application in Food Bioterrorism

Avian influenza viruses are normally grouped into Influenza virus A, Influenza virus B and Influenza virus C. Influenza A viruses are further subdivided into several subtypes based on two proteins or antigens; hemagglutinin (HA) and neuraminidase (NA), which are found on the surface of the virus (Figure 6.1). For example, an influenza virus subtype H7N2 has an HA 7 protein and an NA 2 protein; the subtype H5N1 has an HA 5 protein and an NA 1 protein, *etc*. These are among the numerous influenza virus subtypes that have been reported to contain a mixture of HA and NA proteins.

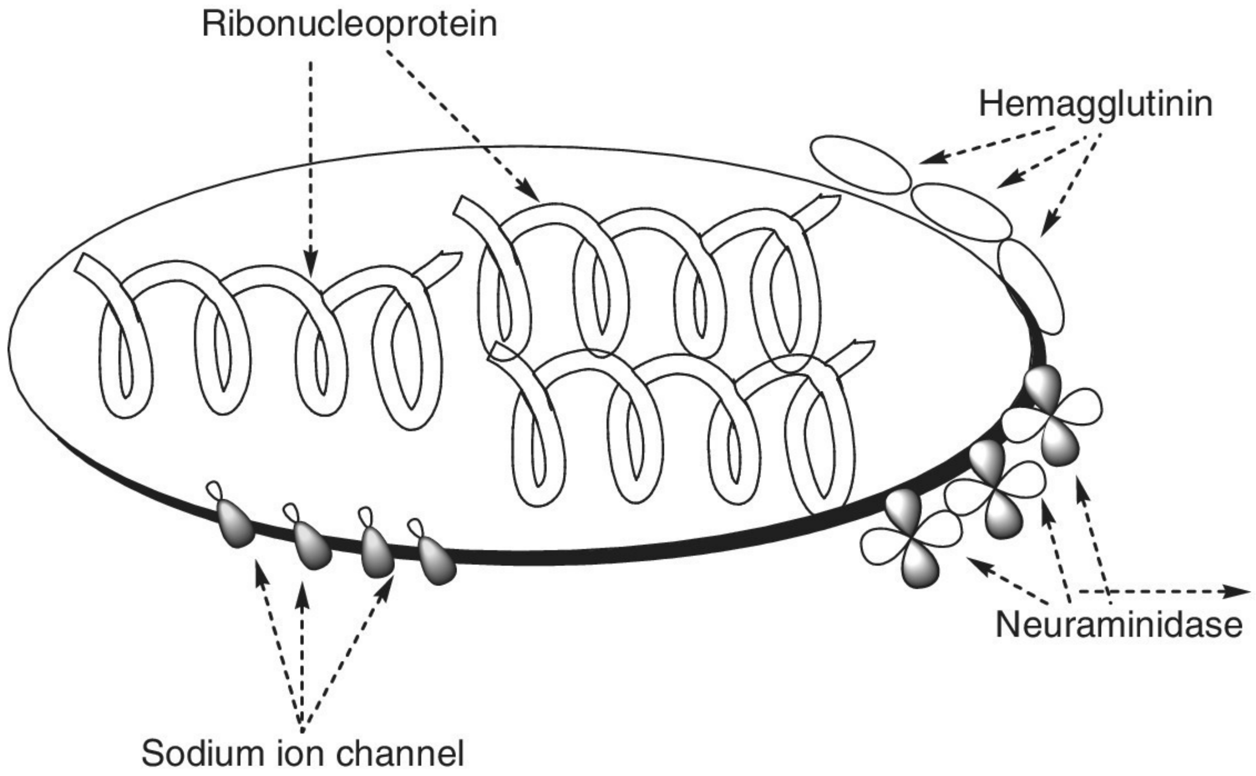

Figure 6.1 Influenza virus

(*Source:* http://www.cdc.gov/flu/images/virus/fluvirus-antigentic-characterization-large.jpg).

The Genetics of Influenza Virus Type A

The majority of influenza viruses are known to have the capability to infect birds and poultry, with the exception of two subtypes, which are H17N10 and H18N11 that infect bats and unlike subtypes H1N1 and H3N2, these subtypes (i.e. H17N10 and H18N11) have been reported to be incapable of growing in

human cells (Tong *et al*., 2012).

Based on the viral molecular attributes and the ability to cause disease and mortality in birds, avian influenza type A viruses are further subdivided into two groups, namely: (i) highly pathogenic avian influenza virus A (HPAI); and (ii) low pathogenic avian influenza virus A (LPAI) (Alexander, 2000). The HPAI types cause serious and severe diseases and high mortality in poultry that have been infected, while the infection due to LPAI does not result in any serious disease (Alexander, 2000).

Avian Influenza A Viruses that Infect Animals, Wild Birds, Poultry and Humans

Several subtypes of avian influenza A type H5 including H5N1, which was reported in Asian and Middle-eastern countries (and some other countries), have caused severe pneumonia with about 60% mortality worldwide; H5N2, H5N3, H5N4, H5N5, H5N6, H5N7, H5N8, and H5N9 are among H5 types that affect both poultry and humans.

Another avian influenza, the influenza A H7 does infect both birds, animals and humans. The H7 subtypes that cause diseases to poultry and humans include H7N1, H7N2, H7N3, H7N4, H7N5, H7N6, H7N7, H7N8 and H7N9. The majority of these H7 subtypes belong to LPAI group of influenza viruses.

Another influenza virus A known to infect animals, wild birds, poultry and humans is H9 and its subtypes are namely H9N1, H9N2, H9N3, H9N4, H9N5, H9N6, H9N7, H9N8 and H9N9. They generally cause mild upper respiratory tract illness.

It should be known that certain subtypes of influenza A virus show preference in terms of the host they infect, because some are very specific to certain species of animals but they are all capable of infecting birds without any exceptions. For example, H1N1 viruses have shown preference to pigs, while H7N7 and H3N8 virus have been more specific to horses.

The genome of influenza A viruses is comprised of up of eight separate single stranded RNA gene segments. The eight gene segments of influenza A virus encode 10 proteins: hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural (NS) proteins NS1 and NS2, the nucleocapsid, and the three polymerases, the PB1 (polymerase basic 1), PB2 and PA (polymerase acidic) proteins (Webster, 1992). In addition, certain influenza viruses contain

the PB1 gene, which has been reported to encode for the PB1-F2 protein (Chen *et al*., 2001).

Of these 10 genes in the genome of influenza virus A, segment 1 encodes for the polymerase proteins: basic polymerase 2 (PB2), segment 2 encodes for basic polymerase 1 (PB1), while segment 3 encodes for the acidic polymerase (PA). These proteins are encoded by segments 1, 2 and 3 and together they form the RNA-dependent RNA polymerase complex that drives the processes responsible for transcription and replication of the viral genome. In addition to this, segment 2 does encode for another protein, namely PB1-F2, which is known to induce cell death. Segment 4 encodes for the viral surface glycoprotein HA. The HA plays a crucial role in the process of binding to sialic acids (SAs), the viral receptors on host cells as well as the process of fusion between the viral and host cell membranes upon endocytosis. Segment 5 encodes for the nucleocapsid protein (NP), which is responsible for the binding to viral RNA. Moreover, the NA and polymerase proteins together form the ribonucleoprotein complexes (RNPs). Segment 6 encodes for the neuraminidase (NA), a sialidase, which is crucial for cleaving SAs from host cells and virus particles. Segment 7 encodes for the protein M1, which is actually a viral matrix structural protein as well as another protein M2, which is an ion-channel protein that is normally found incorporated in the viral membrane. Segment 8 encodes for the NS1 protein, a nonstructural protein, which is crucial as an antagonist protein of host innate immune responses and plays an important role as it interferes with host gene expression. The same segment 8 also encodes for the nucleic export protein (NEP), which facilitates processes that take part in the nuclear export of RNPs in the cytoplasm prior to the virus assembly.

One of the mechanisms that leads to the creation of even new influenza viruses is known as the antigenic shift, in which the segmented influenza virus genome mixes influenza A viruses from different species through the re-assortment of the genetic information process to replicate and thus create a new virus in cases where influenza A viruses from two different species infect the same individual or animal. This takes place mostly with influenza virus A subtypes, in which the majority of the population possess very limited or no immunity at all against it. This can happen, say in the case where a person is co-infected with an avian influenza A virus and at the same time with a human influenza A virus or an animal like the swine/pig which has co-infection due to: i) human influenza A virus; and ii) avian influenza A virus simultaneously, the re-assortment process will produce a new replicating virus that will be the result of a mixture of the existing genetic information such that the newly created virus will tend to

possess most of the genes from the human virus, but a hemagglutinin gene and/or neuraminidase gene and other genes from the avian virus. This will therefore result in a new virus with much enhanced capability and virulence to infect humans and spread easily from person to person and its virulence will be further enhanced due to the fact that it will now have different surface proteins (hemagglutinin and/or neuraminidase).

Mechanism of Action of Avian Influenza Viruses in Bird-to-Human Transmission, Pathogenesis and Host Restrictions of Interspecies Transmission

According to scientific research reports, the viral and host factors that control victims' barrier or restriction are determined by the viral HA and NA genes, several internal genes including the nucleoprotein, the PB2 genes and a combination of other diverse multiple viral genetic factors (Malik Peiris *et al*., 2007). Differences in the interspecies transmission mechanisms are depicted differently among different species. For example, the HA of human influenza viruses binds to cellular sialic acid that is directly linked to sugar molecule galactose via a α-2,6 bond (SA α2,6 Gal), but the avian viruses bind to the sialic acid linked to galactose by the α-2,3 bond (SA α2,6 Gal) (Rogers *et al*., 1983). It should be noted that the epithelial cells in the human trachea contain SAα2, 6Gal molecules on their surface, but they do not contain SA α-2,3 Gal molecules.

This observation in terms of the differences in the receptor specificity in different species plays an important role in the control of factors that govern the species restrictions that may prevent avian viruses from infecting humans easily and/or straightforwardly (Malik Peiris *et al*., 2007). Cases of interspecies transmission (e.g. birds to humans) may be aided by mutations that may occur and which could result in a change in affinity from SA a-2,3 and SA a-2,6 receptors (Yamada *et al*., 2006). In human respiratory tissues, the SAα2, 6Gal oligosaccharides are present largely on the epithelial cells of the nasal mucosa, paranasal sinuses, pharynx, trachea and bronchi, while upon viral infection to humans, the SAα2, 3Gal oligosaccharides are normally found located on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus, and also on cells lining the alveolar wall.

Receptor specificity is made possible due to the chemistry and biology of the amino acids that are present in the receptor binding pocket of HA protein

together with certain amino acids, mainly glutamine or leucine, which are found at position 226 and also amino acids glycine or serine, which are found at position 228, thus characterizing the specificity for either SAα2, 3Gal or SAα2, 6Gal, respectively. Moreover, in the case of H5N1 virus, there is an introduction of the human-type amino acid residues at positions 226 and 228 of the HA protein, a property that can be used to investigate and detect SAα2, 6Gal oligosaccharides in addition to SAα2, 3Gal oligosaccharides.

Generally it has been shown experimentally that restriction and pathogenicity of the influenza viruses has been demonstrated mainly with only a few proteins out of those 10 encoded by the 8 genes. These proteins include hemagglutinin (HA), PB2 (polymerase basic 2), NS1 (nonstructural 1) and neuraminidase (NA).

As explained above, the HA protein (antigen) plays a crucial role that enables the influenza virus to attach and also aid its fusion to the cell membrane of the host. Moreover, the HA protein is necessary for the establishment of the pathogenicity of avian influenza viruses. Normally the HA protein is synthesized as a single polypeptide (HA0), then the cellular enzymes (mainly proteases) cleave it into HA1 and HA2 molecules to expose the hydrophobic component of the amino terminus HA2, which actually plays the fusion role and it is therefore the HA2 that is known as the fusion peptide, as it mediates the fusion between the envelope of the virus and the endosomal membrane.

For the HPAI group of viruses, the HA proteins contain certain basic amino acids at the cleavage site, characterized by the presence of ubiquitous proteases, mainly furin and also proprotein convertase 6, which actually cause systemic viral infections. For the LPAI group of viruses, the HA protein is characterized mainly by the presence of single arginine and they are normally cleaved when they are in either respiratory or intestinal organs, implying that they only cause localized infection that in most cases is without any noticeable symptoms or very mild if any.

Another protein out of the 10 that are encoded by the 8 genes of the influenza virus A and which happen to demonstrate restriction and pathonogenecity, is the PB2 protein. This protein is actually a component of the viral RNA replication complex that plays an important role in the recognition and binding to type I cap structures of cellular mRNAs. Moreover, the PB2 segment is known to be involved in several restrictions of pathogenic avian influenza viruses in cell lines. The replication of a human virus containing an avian virus PB2 gene in mammalian cells is highly dependent on lysine, which is only present in human associated viruses. It is not dependent on glutamic acid, an amino acid mainly

found in avian species at position 627 of PB2.

NS1 is another protein that is encoded by the eight genes of the influenza virus A and which also demonstrates restriction and pathogenicity. It plays a crucial role as an interferon-antagonist, targeting the production of interferon-β and also the activation of interferon induced antiviral genes, thus playing an important role in enhancing the efficiency in virus replication in interferon-competent hosts. The NS1 protein of the H5N1 viruses demonstrates resistance to the antiviral effects of interferon and at the same time induces elevated levels of pro-inflammatory cytokines, including the tumor necrosis factor α (TNFα). For example, influenza viruses that contain the H5N1 NS1 gene and which also happen to be most pathogenic in pigs, were found to enhance the transcription of TNFα and that of interferon β, which are present in the primary monocyte-derived macrophages in humans. In the past, it was observed that the influenza virus interfered with the expression of interferon-regulated genes more than other viruses. In the case of the H5N1, it has also been established that the activation of cytokines and chemokines is regulated through cellular signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway. This implies that the viruses that belong to the HPAI group may be responsible for the activation of p38 MAPK and these observations suggest that NS1 of HPAI may be responsible for the cytokine imbalance (cytokine dysregulation) in cases of viral infection that results in a reactive hemo phagocytosis, which is a cytokine-driven disorder.

Neuraminidase (NA) protein is another protein encoded by the eight genes of the influenza virus A and demonstrates restriction and pathenogenicity. The activity of the enzyme sialidase of neuraminidase plays an important role of removing sialic acid from sialyloligosaccharides of HA, and the cell surface, thus simplifying the release of the virus and the removal of sialic acid from the mucin layer and enabling the virus to get to the surface of the host’s epithelial cells.

Neuraminidase protein of several influenza viruses has been linked to pathogenicity, as it has been found to be central to neurovirulence. Moreover, it has been observed that the absence of a carbohydrate chain at position 146 of neuraminidase (N2 numbering) and the presence of an amino acid lysine at the C-terminus, enables the neuraminidase protein to bind to and sequester plasminogen, which is a plasmin precursor and therefore assists in the cleavage of the HA protein causing virus pathogenicity.

Analytical Strategy for the Identification of

Influenza Virus A Infection Cases

Where there are suspected cases of influenza virus A infection, specimens that are normally collected from patients include respiratory specimens, blood and serum, as well as allantoic fluid. The extraction of viral RNA has been achieved mainly using specialized molecular kits, while the amplification of the gene is normally done using reverse transcription–PCR; the amplified gene is then sequenced and identification can be achieved. Purification techniques, which aim at separating monocytes and macrophages from blood samples, eliminating unwanted matrices, etc., are normally performed prior to identification using either molecular biology techniques (to identify the RNA genes), specific assays (e.g. microneutralization and horse red-blood-cell hemagglutinin inhibition assays, etc.) or specific analytical mass spectrometry (LC-MALDI-TOF-MS) for the analysis mainly of proteins associated with influenza virus A infection, as described above.

Conclusions

The possibility of using pathogenic viruses as bioweapons remains high. Bioterrorism may seek to eradicate lives *en masse* and one of the possible bioweapons to be considered by criminals may be pathogenic viruses. It may be tricky to multiply these agents for reasons that may be explained from a biological point of view (viruses can only replicate when they are in a living medium). However, with the spread of knowledge, it may be possible to encounter bioterrorism incidences that may try to make use of pathogenic viruses. This calls for preparation by having the infrastructure in place to prevent the possibility of anyone trying to use such agents as food bioweapons.

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7
Food Forensics Cases Related to Genetically Modified Organisms (GMO) Foods

Introduction

The world's human population currently sits at around 6 billion and the projections are that the statistic may even double in about half a century's time. Among many challenges that the Earth will face includes ensuring an adequate food supply for people. This challenge may further be strained by the further challenges that may stem from climate change, drought, floods and wars, which hinder food production through agriculture and thus cause severe food insecurity and acute food shortages. To ensure that the food supply is adequate throughout, other means of food production apart from agriculture and animal husbandry have been devised. One of these strategies is to use the knowledge of biotechnology and genetic engineering to manipulate genes, which will result in crops of better quality for the enhancement of production of food, thus genetically modified organisms (GMOs).

By definition therefore, genetically modified foods, also known as genetically modified organisms (GMOs), refer to crop plants created using biotechnology and molecular biology techniques specifically for direct consumption by either humans or animals. Moreover, processed foodstuffs in which ingredients of GMOs have been incorporated fall within the definition of Genetically Modified (GM) foods. The genetic modification of the plant is performed to ensure that the new crop plants have the desired enhanced traits, for example enhanced and improved resistance to herbicides and also quality and improved nutritional content. Genetic engineering, on the other hand, can be employed to create crop plants with the exact desired trait rapidly and more accurately. In the production of GMOs for example, genes that may be responsible for a certain desired trait (e.g. drought resistance, insecticidal properties) in crop plants can be isolated either from another different plant or even from bacteria such as B.t. (*Bacillus thuringiensis*) and inserted into the target crop plant and this newly created genetically-modified plant crop will gain drought tolerance. B.t. contains crystal protein genes that are known to enable crop plants to produce their own pesticides against insect pests.

The Process of GMO Production

The procedures involved in the production of GMO foods follow several steps (Figure 7.1), which may include:

1. Identification of the desired and appropriate characteristic traits from another suitable organism, which can either be an animal, a plant or a microbe, and location of the appropriate set of genes which code for the characteristic sought.
2. After the identification of the genes from an appropriate organism, molecular biology techniques can be employed to isolate and multiply this isolated set of genes. Generally, genes in organisms (e.g. microbes/bacteria) possess different types of switches (e.g. an "on switch" and other switches). These switches have specific functions related to that particular microorganism. This implies that when the genes have been isolated and inserted into the selected plant crop to be genetically modified, the original bacterial switches need to be replaced with different switches, in order to enable the gene to function in the genetically modified plant crop cells. The functioning of the switches is also specific; if they are inserted in plant cells located in the roots, then they will only function in that location and not in other cells in other parts of the same plant.
3. Therefore the addition of the desired and appropriate gene switches to the incoming selected gene of interest from a different donor organism is mandatory, in order to allow it to function in the cells at a chosen part of the recipient organism (e.g. in tissues of a plant crop).
4. When the new gene switches are in order, they can then be inserted into the recipient/host plant cells and the plant becomes a transformed plant/genetically modified organism, because it contains genes from the donor organism and also its normal genes. This can be performed using molecular biology techniques.
5. If all the above steps are successful, then the transformed cells can now be grown throughout the whole plant in the laboratory, where the growth of the cells can be properly done using selective nutrient-rich media and the whole process is monitored to ensure that the desired characteristics are expressed as anticipated.
6. The very last step will then involve breeding of the genetically modified plants with conventional plants of the same variety, in order to generate seed

en masse for propagation purposes.

The whole process to produce GMO takes a long time (many years) before certification and approval because it requires verification of all the risks that may be associated with the product healthy-wise, environmentally, *etc.* (Figure 7.1).

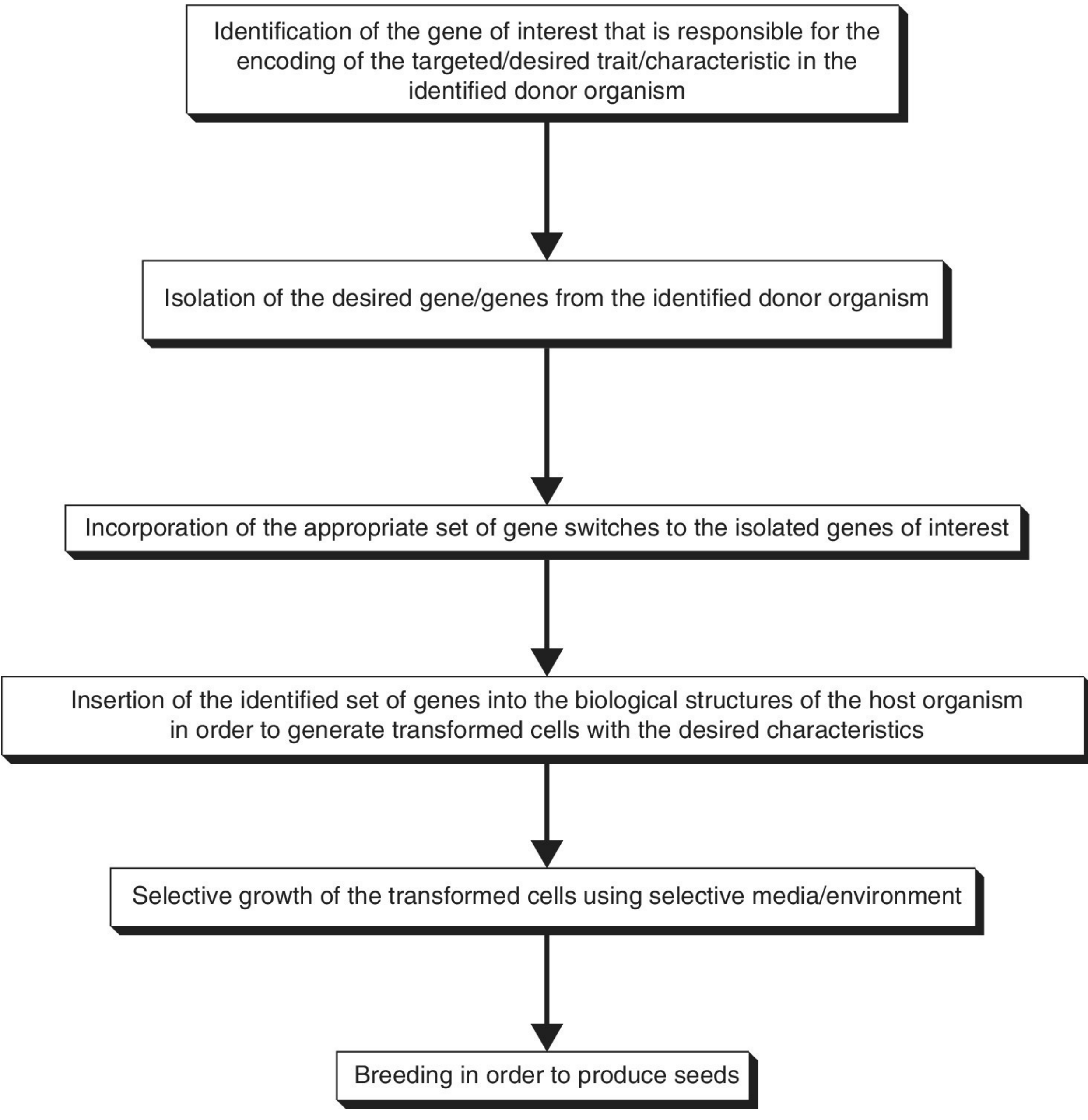

Figure 7.1 Summary of the GMO production steps

(*source:* Food Standards Australia New Zealand, 2005).

Currently there are opponents and proponents of GMO suitability in food and the feed supply chain. The opponents have serious concerns regarding the potential

long-term consequences to consumers that may threaten the very existence of humans and that before licencing GMOs for human consumption, a thorough scientific investigation of their long-terms effects must be conducted and there should be proper regulations regarding their production and use, while the proponents promote GMOs citing the promising advantages as follows:

GMOs have the capability to bring to a complete halt the loss of crop plants that arise from their lack of pest resistance from insect pests. There will also be no need to use environmentally hazardous and costly pesticides and insecticides, which are also potential health hazards if they contaminate food and water. The application of biotechnology and genetic engineering knowledge to grow and process GM foods, which may include the use of genes from *Bacillus thuringiensis* in the growing of corn, will no doubt discourage to a large extent the tendency to rely on chemical pesticides and insecticides, a move which will also be welcomed by environmentalists and nutritionists and will make the food production process a cost-effective process, which will provide farmers with more profit (Moellenbeck *et al*., 2001).

The proponents of GMOs also argue that GMO technology on crops results in the creation of herbicide-tolerant traits (Ohkawa *et al*., 1999) and this will discourage the practice of removing weeds by using physical means, as it is not cost-effective to remove weeds by rilling or other physical practices or the practice of spraying herbicides which is laborious, costly, time-consuming and has the potential to harm the food crops.

Proponents of GMOs also advocate that GMOs result in crops with traits that provide the genetically-engineered crop plants with disease resistance (Lynn *et al*., 2001; Scorza *et al*., 2001) and cold tolerance (cold resistance can be induced by an antifreeze gene extracted from, for example, cold water fish) (Kenward *et al*., 1999).

There are other enticing attributes that GMOs are believed to offer, which include the creation of drought and salinity tolerance traits in food crops/crop plants. This will enable crops to be planted not only on land but also in groundwater and in high salt content environments. Moreover, even with lack of rainfall, such plants can withstand long periods of drought (Tang, 2000 ; Zhang and Blumwald, 2001).

Biotechnology and genetic engineering on food crops have the potential to provide the solution to nutrition malnutrition by creating GMOs that contain vitamins or minerals that are missing in certain crops. One such example is the GMO strain of rice (known as golden rice) that was created with a gene to code

for an unusually high level of beta-carotene (vitamin A) (Beyer *et al*., 2002 ; Normile, 1999 ; Rockefeller Foundation, 1999). Moreover, apart from the potential solution to nutrition malnutrition, GMOs can be created containing edible vaccines (due to immune genes that may be inserted in food crops) in crop plants, plant vegetables, fruits, etc., thus serving as either pharmaceuticals, medicines or vaccines for certain target diseases (Daniell *et al*., 2001 ; Kong *et al*., 2001).

In 2001, a summary of scientific studies that had been conducted over the previous 15 years and which investigated the suitability, state of regulation and safety of GMOs, was presented (Kessler and Ioannis, 2001). In this summary report, which was conducted under the EU Research Directorate, it was concluded that there was no tangible evidence on the potential risks to human health or the environment (European Union (EU) Research Directorate, 2001). From this report, and many other reports from various EU countries, the proponents of GMOs argue that if something was being investigated for so long (15 years) and failed to find any problem, then the obvious conclusion is to accept that it is absent. However, opponents and skeptics who remain fearful of GMOs argue that the absence of evidence does not imply it as the evidence of absence (Paarlberg and Johnson, 2010).

Generally, there is still an intense debate in many countries with regard to the research and acceptance of GMOs. Figure 7.2 depicts countries in Africa that have research centers for GMOs and those that have ratified the biosafety protocol.

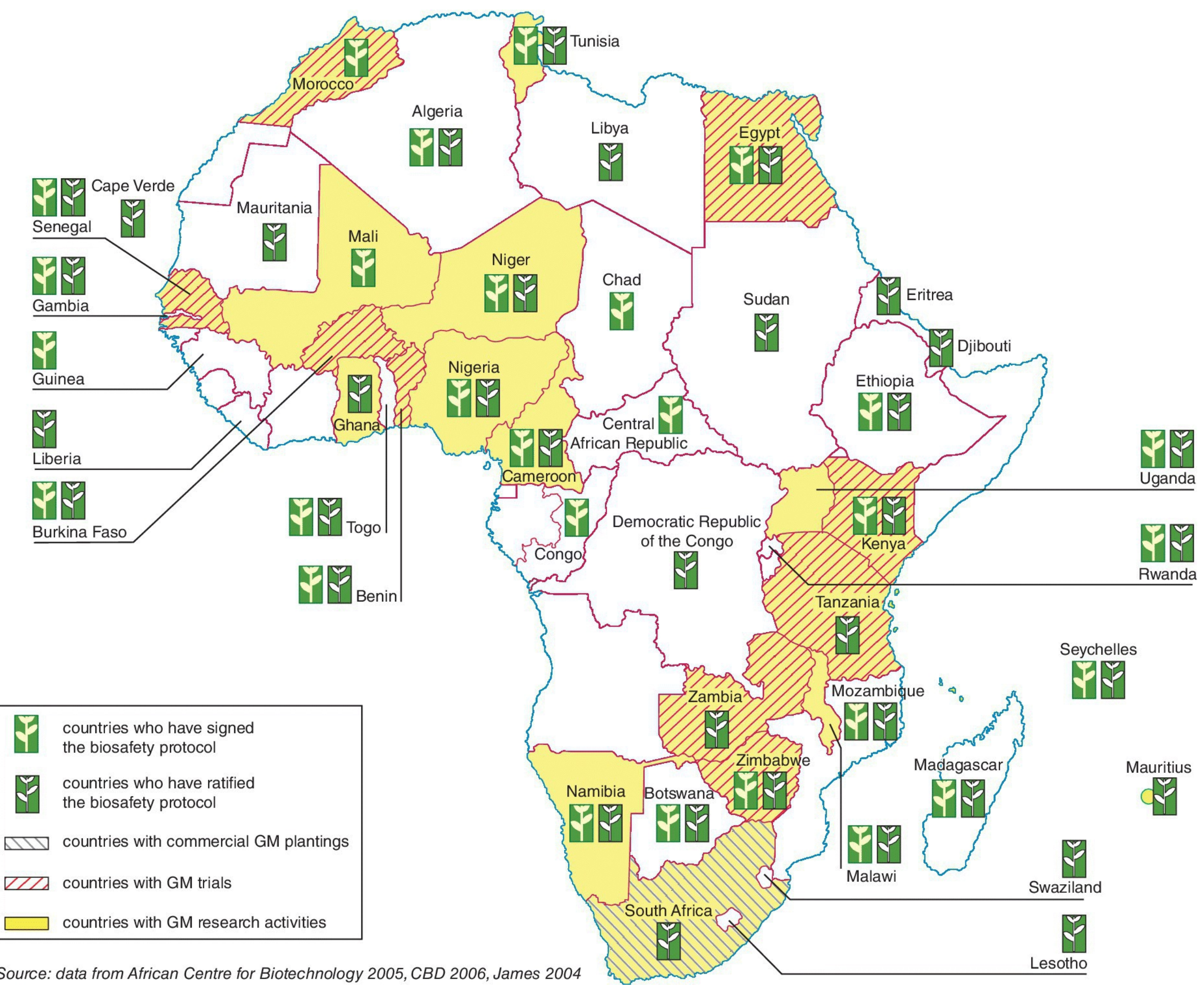

Figure 7.2 The status of GMOs in African countries.

Despite the fact that many arguments are still ongoing worldwide with regard to the acceptance of GMOs, some countries and regional bodies have regulatory mechanisms in place. These include the EU and the USA. The regulatory mechanisms in the EU prompt the distinction between regulation for genetically engineered (GMO) foods and regulation for GMO crops. In the USA, the regulation for both GMO foods, GMO crops and environmental safety concerns due to GMOs, is governed by the same regulation that was lawfully enforced for non-GMO foods and crops (Paarlberg and Johnson, 2010). The EU also requires that all GMO products must have identifying labels and that the approval for any product must comply with the requirement that the producing company produce solid evidence of the thoroughly tested associated risks such as toxicity, allergenicity and digestivity regarding the GMO product, which is contrary to the GMO regulatory requirements in the USA, which does not require labelling as long as the GMO foods have been approved.

The fact that there are regulations and that to some individuals GMO foods may be unethical, it may thus be tempting for unscrupulous business people and farmers who are seeking huge profits to bypass the law and sell uncertified or unapproved GMO products.

Sampling and Sample Preparation for GMO Analysis

Sampling of GMOs for analysis in food forensics cases has to ensure homogeneity of the sample specimen by collecting the appropriate sample size, which must be sufficient enough to allow adequate sensitivity and also be statistically representative and adhere to standard and acceptable sampling procedures (Gilbert, 1999). Depending on the type of raw sample material, sample preparation may take different steps, for example if it is seed, then the seed will be ground to obtain fine powders, the powder will then be homogenized and then the extraction step will follow for either protein or DNA, then analysis and detection.

Reference Materials in GMO Analysis and Results Validation

In order to generate results that are authentic and valid, it is imperative to use validated analytical procedures by employing appropriate reference materials for both positive and negative controls, which will also serve as the basis for assessing the performance of analytical methods and the reliability of the results from different laboratories. The ideal reference material needed for GMO analysis and validation has to be:

1. independent of the analytical methods; and
2. suitable for tracing either the raw material or the base ingredients and not tracing the finished food products.

In the process of GMO analysis, each GMO sample should have its specific reference material. Examples of GMO reference materials that can be used include grains, altered DNA, genomic DNA, plasmid DNA and expressed proteins. In the case of grains, not any grain can be suitable for use as reference material in GMO analysis, but rather grains for use as reference materials must meet certain criteria which include:

1. the potential/ability to realistically simulate/mimic the real-life test grain sample material in that they must possess the same or similar matrix effects and consistency to test grain samples;
2. it must prove to have stable and consistent quality as measured over a long enough period of time; and
3. it must show specific sample homogeneity, and content of the appropriate GMO and GMO stability (Yates, 1999). When protein-based methods are used for GMO analysis, it may suffice to use a single standard, but for the DNA-based methods, it is always recommended that a combination of a number of appropriate positive controls may be desirable in order to generate reliable and more authentic results.

Detection and Identification of GMOs in Food Forensics

The legislation that has been enacted by the EU countries, the USA, Canada, Australia, New Zealand and many other countries worldwide, to regulate the presence of GMOs in food crops, foodstuffs and ingredients, has prompted scientists to devise and develop reliable and sensitive methods for GMO detection. These analytical methods are grouped into protein-based methods such as those employing immunoassay as the principle of detection, molecular biology-PCR-based methods, DNA-based methods, which include those that employ protein immunoblots (commonly known as Western blots), enzyme-linked immunosorbent assay, lateral flow strips, Southern blots, *etc.*

Protein-based Methods for GMO Analysis: Immunoassay Techniques

Immunoassay techniques utilize antibodies in the principle of their detection and these techniques have proved to be ideal for both qualitative and quantitative analysis procedures meant for proteins, even in complex matrices, as long as the identity of target analyte in the sample matrix is known (Brett *et al*., 1999). Depending on the time given to produce results from the analysis, application (whether antibodies to whole protein or antibodies to specific peptide sequences) of amounts needed and the level of specificity of the detection, two types of antibodies can be employed for immunoassays: (i) monoclonal antibodies, which are known to be highly specific; and (ii) polyclonal antibodies, which are known

to be more sensitive. In cases where antibodies are attached to solid phases, the immunoassay analysis can either be carried out in a competitive assay mode, in the sense that the detector and analyte compete for the binding sites in the capture antibodies, or it can be carried out in a two-site mode (also known as double antibody sandwich assay), whereby the analyte is sandwiched between the capture antibody and the detector antibody (Cochet *et al*., 1998).

Protein Immunoblots (Western Blot Assay) Techniques

Protein immunoblots are techniques for the detection of specific amino-acid sequences in proteins (mainly insoluble proteins) (Brett *et al*., 1999). The technique employs gel electrophoresis to address the problems related to antibody cross-reactivity and also to separate proteins based on either their 3-D structure or by the denatured proteins based on the length of their polypeptides. After the separation process, the separated proteins are normally transferred to a nitrocellulosed PVDF membrane for staining with antibodies that are highly specific to the target protein that is being analyzed (Renart *et al*., 1979 ; Towbin *et al*., 1979).

The usefulness of this technique lies in the fact that it provides both qualitative and quantitative information about the sample and can also provide information as to whether the sample contains the target protein below or above the limits in the guidelines (Lipton, 2000). In this technique, the gel electrophoretic separation process is in many instances carried out under denaturing conditions, in order to counter problems that are caused by difficulties in solubilization, aggregation phenomena and also co-precipitation of the target protein being analyzed with other adventitious proteins (Sambrook and Russel, 2000). Generally, the procedures involved in the protein immunoblots begin with the solubilization of the sample with detergents and reducing agents and thus the gel electrophoresis is performed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). As indicated above, the separated peptides are then transferred to a solid support (either PVDF or nitrocellulose membrane) to enable the binding of immunoglobulin sites on the membrane which become blocked by dried non-fat milk. The specific sites on the solid support can then be investigated using either high-titer polyclonal antisera or a mixture of monoclonal antibodies raised against the denatured antigenic epitopes antibodies. The bound antibody can now be stained using either Ponceau, silver nitrate or Coomassie, or a secondary immunological

reagent, such as protein A coupled to horseradish-peroxidase (HRP or alkaline phosphatase (Yates, 1999)).

Immunoassay Methods for GMOs in Food Forensics

Immnunoassay methods in food forensics of GMOs can offer a reliable means for detecting new or modified proteins in food products that either contain GMO components or foods transformed into GMOs. Some of the requirements, when considering the use of the immunoassay, include:

1. the availability of genetically modified organisms (GMO);
2. the availability of proprietary proteins, which are required for test development; and
3. the availability of standard reference materials, which are necessary for method development as well as in the translation of the test results in terms of percentage GMO found in the test food sample.

Enzyme Linked Immunosorbent Assays (ELISA)/Enzyme Immune Assay (EIA)/Ligand Binding Assays

The enzyme linked immunosorbent assays (ELISA) technique is highly useful in the detection of the presence of an antibody or an antigen in biological wet/liquid samples. The enzyme immunoassay can be performed in different modes such as:

1. a microwell plate (or strip) mode;
2. a coated tube mode; or
3. magnetic particle coated mode (Fagan *et al*., 2001 ; Lipton *et al*., 2000).

In this technique, analytes in solutions are separated by being adsorbed onto an immobilized solid phase that possesses special binding properties and then multiple liquid reagents are added in a particular order, depending on the protocol being followed; the set is then incubated and washed. There will be some color development due to the formation of the enzymatic reaction product in the well, from which the quantity of the analyte is measured

spectrophotometrically (Gupta, 2000). It should be noted that in EIA/ELISA experiments, the analyte is also regarded as the ligand due to the fact that it binds/ligates specifically to a detection reagent, and for this reason some scientists classify ELISA in the same group with ligand binding assays.

Molecular Biology PCR-based Methods

In order to have successful design and method development procedures in the identification of GMO genetic components in foods using PCR-based molecular methods, it is imperative:

1. to have a thorough knowledge regarding the foreign genetic make-up within the GMO to be investigated/detected; and
2. to ensure the means, infrastructure, resources (including expertise) and the ability to extract adequate amounts of good quality DNA from the food sample under investigation.

Different PCR techniques do exist for the detection of GMOs in foods and they include quantitative PCR-based methods, which are based on the quantification of the DNA material subjected to PCR by photometric procedures and thereafter the measurement of the amount of transgene-specific DNA content can be performed by employing competitive PCR. The strength of this technique is that it tends to eliminate the possibility of generating false negatives that may arise due to inhibitory effects, because the approach incorporates a step which defines the sensitivity of PCR with the internal competitor. However, there are certain shortcomings associated with this method and they include the fact that the technique cannot eliminate the problems related to the quality of DNA after fragmentation and/or chemical modification processes and also for problems related to the extent of contained tissue-specific non-chromosomal DNA (tissue-specific). This necessitates the inclusion of standardization procedures for each single food matrix individually. Another limitation to this approach is that it is suitable for only pure matrices, in the sense that they should be derived from a single organism for identification using this approach, to generate reliable results related to the identity and quantity of the incorporated GMO.

This approach also faces challenges in cases where processed foods are to be analyzed for GMOs, because obtaining adequate and quality DNA is not easy due to the fact that the foods have undergone processes such as heat treatment, enzymatic action or pH changes, which may have caused fragmentation and other types of DNA modifications (Hupfer *et al.*, 1998). For all these reasons,

the procedures for the analysis of mixed samples from different organisms or for the processed products requires a special strategy that may include the determination of relative proportions of GMO versus certain food components in particular foods to be investigated. For example, if crop plants that have their DNA altered to enable them to withstand the herbicide glyphosate (generally known as Roundup Ready Soy (RRS)) are under investigation, then the proportion ratio to be considered will be that of RRS in the food sample portion within the sample matrix. To calculate the RRS ratio requires first to work out the RRS-specific DNA and also the general soy-specific DNA, which will act as a reference (Mayer *et al*., 1996). Roundup Ready Crops (RR Crops) are also known as glyphosate tolerant crops (Séralini *et al*., 2014).

Qualitative PCR Methods for GMO Analyses

Molecular biology PCR-based methods take advantage of the specificity of DNA polymerase to effect the process of selective amplification of specific segments within the gene that happen to occur at low levels (Ahmed, 1995). In the PCR process, normally two pairs of primers are used, such that there is a forward or sense primer or 5’ → 3’, and a reverse, antisense or 3’ → 5’. Primers play a vital role in the PCR process, as they hybridize on opposite strands of the sequence of interest, and as the polymerase chain reaction proceeds in a series of repetitive cycles, they will amplify the sequence between the primers into many copies. These amplified DNA fragments can be subjected to agarose gel electrophoresis to separate them based on their size. After gel electrophoresis separation, methods to confirm PCR results can then be applied and these methods include:

- the use of specific cleavage of the amplified product by restriction endonuclease digestion such as EcoRI, EcoRII, BamHI, TaqI, etc., which are known to have the capability to cleave DNA at or near certain specific recognition nucleotide sequences known as restriction sites (Lipp *et al*., 1999);
- hybridization with a DNA probe specific for the target sequence (Hill *et al*., 1999);
- direct sequencing of the PCR product (Sambrook and Russel, 2000); and
- nested PCR, a technique that uses two sets of primer pairs to bind specifically to the amplified target sequence (Van Hoef *et al*., 1998).

Quantitative End-point Pcr Methods for Gmo

Quantitative End-point Pcr Methods for Gmo Analyses

The quantitative end-point PCR method is another PCR-based technique that is quantitative in nature and is highly desirable since the regulations stipulate maximum threshold limits for GMOs in foods and thus the methods for GMO analyses must be capable of quantifying the GMO components, which actually form the basis for labelling (Hübner *et al*., 1999). Quantitative PCR employs an internal DNA standard that must be co-amplified together with the target DNA (McPherson and Møller, 2000).

Quantitative Competitive Pcr Methods in Gmo Analyses

In quantitative competitive PCR methods, the presence of PCR inhibitors can be recognized from the observation that the amplification of both internal DNA standard and target DNA will both be affected simultaneously (Studer *et al*., 1998). The procedures involved in QC-PCR consist of several steps:

- co-amplification of internal standard-DNA and target-DNA in the same vial;
- separation of the PCR products using agarose gel electrophoresis or any other appropriate technique of choice;
- staining the gel by ethidium bromide;
- analysis of the gel by densitometry; and
- estimation of the relative amounts of target and standard DNA by regression analysis (Ahmed, 2002).

Quantitative Real-time Pcr in Gmo Analyses

In essence, one would have expected the amplification of PCR products to proceed exponentially and indefinitely. However, practicably this is not the case, because in reality the amplification climaxes after several cycles, normally around 40 cycles due to certain reaction components becoming limiting (Ahmed, 1995). In conventional PCR, fragment products of the PCR reaction are measured at a single point in the reaction profile, resulting in a plot of the concentration at the measured point versus the initial amount of DNA present in each of the reactions (NB: this plot depicts the proportionality between DNA concentration (dynamic range) and PCR products over a limited range of DNA

concentration (dynamic range) and PCR products over a limited range of DNA concentrations, which presents limitations because it lacks precision in quantitation terms), the quantitative real-time PCR approach. However, the concentration of DNA in a real-time PCR reaction is taken to be proportional to the PCR cycle number during the exponential phase of PCR (Ahmed, 2000). In this technique, the estimation of PCR products can be worked out using a number of approaches including:

- the ds-DNA-binding dye SYBR Green I;
- hybridization probes or fluorescence resonance energy transfer (FRET) probes;
- hydrolysis probes (TaqMan∨ approach); and
- molecular beacons (Ahmed, 2000).

Quantitative real-time PCR is also attractive in that it can differentiate between specific and primer dimers or other nonspecific PCR products. This can be achieved through the use of probe hybridization techniques or by melt curve analysis of PCR products. The melt curve analysis of PCR is made possible due to the fact that the nonspecific products melt at a much lower temperature than do the longer specific PCR products (Ahmed, 2000).

Exhaustive Limiting Dilution Pcr Method in Gmo Analyses

The exhaustive limiting dilution PCR method is another PCR technique that relies on the optimization of the PCR procedures such that the amplification of an endogenous control DNA occurs from the terminal plateau phase of the PCR and also the technique is optimized such that one or more GMO components in the food sample give a positive result. Quantitation is normally done by employing multiple replicates at serial dilutions of the samples that are being analyzed with the aid of statistical techniques such as Poisson statistics (Sykes *et al.*, 1998).

Dna-based Methods for Gmo Analyses

DNA-based methods for the analysis of GM foods are enabled by the complementarity of two strands of ds-DNA that follow a specific hybridization pattern. As pointed out previously, the process of creating GMO involves the

engineering of the genetic material (the DNA) of the plant crop, such that this engineered crop will consist of several genetic entities that govern its functioning, and these entities include a promoter sequence, structural gene and a stop sequence for the gene. Of the DNA-based techniques, Southern blot has been widely used and also microarray-based methods, which provide the information on the gene (Hertzberg *et al*., 2001); these methods are normally used in conjunction with PCR-based methods (Ahmed, 1995 ; Sambrook and Russel, 2000).

Sample Preparation Methods for DNA-based Techniques in GMO Analyses

The sample preparation procedures for DNA-based techniques in the analyses of GMOs involve a number of steps, including homogenization of samples. Homogenization is a very important step, since only a small fraction of the sample of GMO origin is found to be distributed throughout the whole batch and moreover, there could be a low percentage of contamination that can be detectable. The contamination may arise due to a phenomenon such as addition cross-fertilization (e.g. maize-pollen) in the fields or cross-contamination of crops and dust that may occur during harvest, transportation, milling, food processing, *etc.*

Dna Isolation Methods in Gmo Analyses

DNA isolation for GMO analyses can be done using certain procedures, such as:

- the cetyltrimethyl ammonium bromide (CTAB), whereby food samples to be analyzed are incubated in the presence of the detergent CTAB (hexadecyltrimethyl-ammonium bromide), then extracted with chloroform and the DNA is precipitated with isopropanol (Tinker *et al*., 1993);
- the method that employs a DNA-binding silica resin to purify DNA directly from a solution obtained after enzymatic (proteinase K) and chemical (SDS) treatment of the food sample.

There are other DNA extraction methods based on the modification of these two.

Southern Blot for DNA Detection of GMO Samples

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Southern blot is very useful in the detection of specific DNA sequences in biological samples. The procedures in this technique transfer and fix the isolated gene fragments and electrophoresis separates gene fragments to a filter membrane, mainly a nitrocellulose membrane or a nylon membrane, then detect these fragments by probe using double-stranded (ds)-labelled nucleic acid probes specific to the GMO, before detection by hybridization radiographically, fluoremetrically or by chemoiluminescence.

Data Analysis and Interpretation

From the data and observations obtained, if validated qualitative approaches employed yield no positive results for GMOs, then these samples have to be subjected to analysis that will assist in revealing the presence of proteins, if any. If no proteins test positive, then the conclusion will be that the sample is not detectable for the presence of GMO components. However, if it happens that the qualitative PCR indicates a positive result, the food sample in question may be classified as a non-approved GMO product and this will necessitate validation procedures using Q-PCR, which will quantify the GMO product to establish whether it complies with the threshold limits set by the regulations and guidelines.

Other Non-molecular Biology-based Methods for Gmo Analyses

Nir Spectroscopy Technique for Gmo Analyses

Near-infrared (NIR) spectroscopy is an electromagnetic radiation technique based on the absorption of radiation at wavelengths in the range 780–2500 nm. When food samples are analyzed using NIR, they normally give rise to broad bands caused by overlapping absorptions related to overtones and vibrational transitions for chemical bonds found in foods that include C–H, O–H or N–H (Osborne, 2006).

The attractive features of this technique include the fact that it is fast, sample preparation is not mandatory, and it is cheap. The main limitations associated with NIR in GMO application include the fact that it lacks the capability to identify compounds, thus prompting the analysis of a large set of samples in order to generate meaningful spectra that can be used to predict the GMO

profile.

In addition, NIR may be sensitive to major organic functional groups such as the vibration overtones of C–H, O–H and N–H, but its accuracy is limited due to the fact that when this method is used for GMO analyses, it cannot detect a change in either DNA or a single protein, as it can only detect much larger unknown structural changes, for example those related to the parietal portion of the seed (e.g. lignin or cellulose) that are introduced by the presence of the new DNA.

Conclusions

Although the use of GMO is still controversial, with some viewing it as unethical and some with the opinion that it may be a solution for increasing food production worldwide, we need to have mechanisms in place to reverse the procedure, should it happen that GMO results in negative consequences and threatens the very existence of humans. There is no concrete proof of the negative consequences from the use of GMO at the moment. However, it may be plausible to continue with research on GMO and its possible consequences in the long run after repeated use.

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8
Food Forensics Cases Related to Poisoning of Food and Water using Radionuclides

On 26 April 1986, a nuclear power plant in the Soviet Union republic of Ukraine (Chernobyl) exploded, resulting in a nuclear disaster that affected humans and both plant-and animal-derived foods. The radiation released from the nuclear reactor had a relatively short half-life but included rapidly ingested types of radionuclides such as iodine-131. Others included radionuclide isotopes with longer half-lives such cesium-134 and cesium-137 (source: http://www.livescience.com/39961-chernobyl.html#sthash.NPIQczPe.dpuf; accessed 8 June 2016). According to Nesterenko *et al*. (2009), levels of I-131, Cs-134/137, Sr-90, and other radionuclides in food products such as milk, dairy products, vegetables, grains, meat and fish were found to be significantly elevated. The detectable measurements of radionuclides were being recorded in food products from many European countries and these were the radionuclide isotopes that could be traced to the Chernobyl disaster, even after several years had passed (Nesterenko *et al*., 2009). Another nuclear disaster took place in Japan that was caused by the Great East Japan Earthquake that took place on 11 March 2011, which damaged nuclear reactors leading to leaks of radionuclides such as Co-58, I-131, I-132, Cs-134, Cs-136 and Cs-137 into the environment.

In separate incidents, the suspected politically motivated death of individuals targeted by authorities for having different views or ideology using the polonium-210 radionuclide (a radionuclide with a short half-life, 138.38 days) has been reported to poison the food to be ingested by the target person (http://nuclearweaponarchive.org/News/PoloniumPoison.html; accessed 7 June 2016). This implies that for polonium-210 to be used in criminal or terrorist acts, it must be prepared shortly before the intended poisoning and in most cases it cannot be sourced from old discontinued radioisotope sources. However, other radionuclide isotopes with longer half-lives, such as cesium-137 and cobalt-60, may present substances of major concern if they fall into the hands of criminals. This shows that nuclear power plants and radionuclide isotopes, if accessed by criminals, can potentially lead to a serious disaster in food products.

Radionuclides of Concern in Food Poisoning

There are only a few radionuclide isotopes that may contribute significantly to human exposure through the consumption of contaminated foods. Mainly fission and activation processes are considered as the likely types of radionuclides that are capable of significantly contributing to human exposure in the event of food contamination, either directly or through nuclear accidents that may contaminate crops and water.

Food poisoning due to radioactive iodine (I-131) is of high concern due of its rapid migration into milk from cows that graze on contaminated grass or feed. This radionuclide has a tendency to accumulate in the thyroid gland and thus elevates the probability of the risk of thyroid cancer. Another radionuclide isotope of concern in food poisoning includes radioactive cesium (Cs-134 and Cs-137), which unlike radioactive iodine, has a long half-life. However, as with all radionuclide isotopes, exposure or consumption of food or water contaminated with cesium-137 has the potential to elevate the risk of cancer.

There are other radionuclide isotopes that may be of great concern, if they are used to poison foods, due to their long half-life. These include radionuclide strontium and radionuclide plutonium and unlike radionuclide iodine-131 or radionuclide casium, both radionuclide isotopes of strontium and plutonium are relatively immobile in the environment and are therefore of concern more locally where the contamination or poisoning first took place.

Food Products of Concern in Radionuclide Contamination

Vegetable and plant crops which are grown outdoors may be highly susceptible to contamination by the atmospheric release of radionuclides, from sabotaged nuclear reactors or other sources of radionuclides causing massive radioactive contamination and even over a wide area covering more than one country. Another food product that is of concern is milk in which there is rapid transfer of radioactive iodine and radioactive cesium from contaminated grazing grass/pasture or feed into milk. Radionuclides may be transferred through soil into crops or animals, or into rivers, lakes and the sea where fish and other seafood consumed by humans can adsorb the radionuclides. In many instances, it may be plausible to relate the measurements of radionuclides (e.g. radionuclide iodine-131) in pasture grass to that in milk, as the levels of pasture grass may provide a useful prediction of the type and concentration to be expected in milk and/or meat. Another group of foods of concern include those obtained from the wilder environment, which may include mushrooms, berries and game meat

wilder environment, which may include mushrooms, berries and game meat. Grain and rice may also be subjected to radionuclide contamination.

Types of Radionuclide Particles/Radiation

Radiation due to radionuclide particles may be classified into two main groups, such that the source of radiation may either be ionizing or non-ionizing. The radionuclide types which are ionizing are further subdivided as described below.

(i) Alpha Particles

Alpha particles are essentially helium nuclei consisting of two protons and two neutrons (Leikin *et al*., 2003a,b, 2007; McFee and Leikin, 2005). The alpha particles possess a significant mass and kinetic energy, enough to cause ionization of other atoms or molecules; however, due to the fact that they are massive and possess a +2 charge, they have relatively little penetrating power (compared to gamma rays or X-rays), such that they cannot penetrate the epidermis of the skin. For this reason, exposure or poisoning due to alpha particles or alpha radiation may hardly imply an internal contamination and internal irradiation hazard, except in cases that involve alpha particle inhalation, ingestion or injection by penetrating wounds where they can thus damage tissues (Christensen and Sugarman, 2007; Leikin, 2005). Of the alpha emitters of concern, polonium, americium and plutonium (radionuclide produced from uranium (^{239}U, ^{238}U)) may attract considerable attention as far as food forensics is concerned (Dyer, 2007). There are more than 20 isotopes of Po with ^{210}Po being the most stable form and unlike other radioactive elements, ^{210}Po has been reported to be relatively safe to transport, but it is very toxic and if its toxicity is compared to hydrogen cyanide for example, then ^{210}Po is several orders of magnitude more toxic than hydrogen cyanide. In terms of the radiotoxicity trend, $^{210}Po > ^{228}Ra > ^{210}Pb > ^{226}Ra > ^{234}U > ^{238}U > ^{224}Ra > ^{235}U$.

Contamination due to alpha particles/alpha radiation can be detected using a number of devices including Geiger-Muller detectors, of urine and blood samples from the victims.

(ii) Beta Particles

Examples of beta particles include cesium-137 (^{137}Cs) (McFee and Leikin, 2005). Beta particles, unlike the alpha particles, potentially do pose a significant hazard to internal organs and tissues and they can cause severe burns to the skin

and eyes on poisoning or contact occasions.

(iii) Gamma Particles/Rays

Gamma particles or gamma rays are high-energy emitting uncharged particles, which consist of electromagnetic radiation (Leikin *et al.*, 2007).

(iv) X-ray Particles

X-rays are like gamma particles, in the sense that they are also capable of emitting high-energy radiation.

Analytical Protocol for Radionuclides in Foods and Biological Sample Specimens

Just as with other analytical procedures, the analysis of radionuclides in food and biological matrices (e.g. blood or urine) involves steps such as sampling, sample preparation and detection measurements.

Sampling of Specimen for Radionuclide Analysis

Sampling for radionuclide analysis necessitates obtaining representative samples and the sampling regime is highly dependent on the purpose of investigation. For example, the analysts may be interested in establishing the source and type of poisoning, *etc.* Therefore samples can be collected from a localized area and/or from the single individual who may happen to be the victim or from a population if the act of terrorism has involved communities. Sampling may also involve collection from suspected routes where criminals may have traversed to the place where the radionuclide might have originated and the type of food that was contaminated.

Other precautions during sampling, such as the possibility for decomposition processes to occur and thus alter the evidence, are unlikely as the decomposition of food or biological matrices does not change the radioactivity. In order to maintain the integrity of samples during transport, freezing as well as the addition of formaldehyde and other additives are normally considered adequate as long as they are checked thoroughly that they do not contain significant amounts of the radionuclide being analyzed/suspected. After sampling, samples need to be subjected to treatment processes aimed at removing interfering and other unwanted molecules that may be present in the sample matrix and which may interfere with the signal of the analyte of interest during measurements.

may interfere with the signal of the analyte of interest during measurements. This step is known as the sample preparation step.

Sample Preparation Procedures for Radionuclide Analysis

Sample preparation in the process of radionuclide analysis is intended to simplify the matrix by reducing the bulkiness of the sample analyte material and also to improve the efficiency of the measurement, lower the detection and also improve accuracy and precision of the measurements. For example, foods and biological specimens such as urine, contain high water content, which is undesirable and thus must be removed before the sample is introduced to the analytical system for measurement. During sample preparation procedures, water can be removed by either drying at room temperature, elevated temperatures, or even by freeze drying. It should be noted that most of the radionuclides of interest in food forensic analysis are not volatile under drying temperature conditions, with the exception of radionuclides such as tritium and iodine. Moreover, for most radionuclides (except tritium and iodine), the water content can be further reduced by ashing, a process which is normally carried out at elevated temperatures, by employing cold ashing using activated oxygen, or by wet ashing using oxidizing acids. The limitation of dry ashing is that it may result in losses, especially if volatile radionuclides such as carbon-14, tritium, iodine, cesium, polonium and lead are the target analytes of interest and therefore extra care has to be taken to ensure that their integrity is retained.

The bulkiness of the sample can also be simplified by employing acids during the extraction procedures. The limitation of using acids in extraction is that they do not have the selectivity towards radionuclides and therefore all radionuclides (target and non target) will be extracted.

Another sample preparation procedure involves dissolution of the dried/ashed specimen in strong acids, especially if the specimen is to be subjected to chemical analysis. After sample preparation, the separation of the extracted radionuclides can begin.

Radiochemical Separations

The separation step plays a crucial role in converting the analytes of interest to a form suitable and compatible for detection and identification by the detecting counter device. The separation of radionuclides may be achieved through a number of processes such as elect redeposition and precipitation, to mention a few.

It should be noted that the real content of the radionuclide being analysed is normally decreasing and this may imply that chemical reactions that normally take place in other general chemistry setups such as precipitation do not have a chance under these circumstances. It is therefore plausible to incorporate small amounts of what is known as inert carrier material of the same element or incorporate a similar element to play a role as carrier (e.g. radium can be used in the place of barium), so that the inert form of the element will display the trend of the behavior of the element as it will proceed in the separation process, while carrying the radionuclide along with it. Another advantage of using the inert carrier is that it is vital in preventing undesirable co-precipitation or absorption of the analytes to the glassware.

Analytical Strategy for Measurement of Radionuclides in Foods and Biological Specimens

Biological specimens from victims may be urine or blood sample specimens. The selection of analytical measurement strategies and measurement protocols/methods depends entirely on the type of radiation/radionuclide particles, the form of the sample and/or matrix and the extent/quantity of radioactivity. The strategy for the complete analytical procedures and experimental design has to ensure that the sample is prepared to suit and meet the requirements for the intended analytical equipment to be used under its optimal conditions. For food forensic requirements, the measurements related to the total gamma, total beta, or even the total alpha activity on a sample of food are not desirable as far as radionuclide poisoning to humans is concerned. Normally, a proper and acceptable strategy and methods for radionuclide measurements and identification (e.g. for alpha and beta emitters) involve a radiochemical separation of the species (alpha and beta radionuclides) before the identification and it has to include (where necessary) the measurement of the magnitude of energy or half-life of the separated material for accurate and acceptable radionuclide identification.

Measurement and Identification of Alpha Emitters in Foods and Biological Specimens

In order to generate reliable measurements, alpha emitters are normally prepared as thin sample specimens to minimize the possibility of self-absorption

as thin sample specimens to minimize the possibility of self-absorption phenomena, which results in spectra signals with poor resolution. There are a number of techniques for the measurements of alpha radiation emitters (measurement of alpha activity) and they include:

1. thin-window counters;
2. descintillation counters equipped with zinc sulfide phosphor. These have the advantage of generating signals with minimal background and are associated with low detection limits;
3. liquid scintillation spectrometers. This technique, however, may be less sensitive as compared to other techniques used for the measurements of alpha emitters;
4. alpha spectrometers. These have various modes, such as:
    a. Frisch grid ionization chamber, which is normally used for sample specimens with a large surface area. This approach (Frisch ionization chamber) has limitations in that it is incapable of resolving closely separated energies;
    b. silicon diode solid state detector. The shortcoming of the silicon diode is that its small size has the potential to limit its applications to count samples with dimensions of less than 1 cm in diameter.

Measurement and Identification of Beta Emitters in Foods and Biological Specimens

The techniques used for the measurement and counting of beta particles in foods or biological specimens include:

1. Geiger counter;
2. thin-window proportional counter. This is widely used in more measurements of beta emitters than the Geiger counter due to the advantages it possesses, which include high efficiency and low background;
3. scintillation equipped with either:
    a. solid scintillators, which can be used for chemical precipitates; or
    b. liquid scintillators.

Liquid scintillators are attractive because they offer high efficiency, even for radionuclides with low energy emitting characteristics such as carbon-14 and

tritium.

Another technique that may be considered is the use of the beta spectrometer; however, this approach may not be ideal because each specific beta emitter species has a characteristic range of energies from a minimum of zero to a specific maximum that is applicable that particular beta emitting species.

Measurement and Identification of Gamma/X-ray Emitters in Foods and Biological Specimens

Gamma radiation is highly energetic and very penetrating and for that reason the detection system to measure gamma/X-ray emitting species must be built to handle a considerable mass capable of absorbing enough rays/radiation to generate a corresponding signal response. For this reason, solid detectors are the ones mostly used in gamma/X-ray spectrometric measurements, as they are capable of complete absorption of gamma/X-ray radiations. Sodium iodide is an example of such detectors; however, despite all the advantages which include high efficiency, sodium iodide detectors are characterized by poor energy resolution and this is their main shortcoming. To address the limitations of sodium iodide detectors, germanium diode detectors are normally employed to provide better resolution where the signal involves highly complex spectra.

Conclusion

Nearly all cases that have involved the use of radionuclides as food poisoning agents have been fatal resulting in terrible loss of life. It is thus expedient that efforts are made to come up with concrete ways of reversing the fatal effects of radionuclides. Moreover, due to their decaying nature, there is a need to have more reliable analytical methods to uncover the evidence precisely, even after the total decay of the radionuclide involved in the food poisoning.

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9
Food Forensics Cases Related to Nano and Novel/Intelligent Foods, Feeds and Agroproducts

Although nanotechnology has been applied in a diverse number of disciplines to introduce different properties and characteristics to various materials, the strategies to apply nanotechnology to the food industry differ markedly from those in other areas, due to the fact that food processing involves a diverse variety of raw materials, demands high biosafety requirements, and follows a well-regulated technological process. Food production and processing is currently employing nanotechnology in various areas, including the development of new functional materials, microscale and nanoscale food processing, food product/ingredient development processes, and also in the design of methods and instrumentation to produce improved food safety and biosecurity. There are, however, serious health concerns with regard to the incorporation of nanomaterials in foods. Certain countries and multinational/global organizations responsible for health have imposed strict regulations on the production and application of nanosized materials in foods. Transgressing these regulations and guidelines has serious legal implications to the manufacturers and distributors of food items, which violates the threshold or failure to meet labeling requirements.

Introduction

There has been an increase in the tendency for the food industry to commercialize nanofoods. This has to date had unknown effects on consumers, even though it may mean much economically. Several countries have come up with regulations for nanofoods and from these regulations, transgressors who are after profit may face legal actions for their behavior, which may include improper or false labeling. By definition, nanofoods/feed refers to any food substance created by the employment of nano technological techniques in any part of the food chain – cultivation, production, processing, or packaging – not just in the food itself. In other words, food is regarded as “nanofood” only when nanoparticles, or nanotechnology techniques or other tools, are used during cultivation, production, processing, or packaging of that food. Food does not

cultivation, production, processing, or packaging of that food. Food does not become nanofood in the sense that it is an atomically modified food or it has been produced using nano devices or nano machines.

The advent of nanotechnology has greatly influenced and revolutionized not only the industrial sector, but also agriculture and feed/food production sectors, where the share of nano-based products on the market has been on the rise (Euractiv.com, 2010; Project on Emerging Nanotechnologies, 2010).

Nanotechnology has shown the potential for a positive impact on food production, processing, and in agricultural/animal husbandry practices that are directed at the production of food/feed. Currently, numerous scientific and technical publications have reported on the potential of the application of nanotechnology to boost food security, disease treatment delivery methods, and new materials for crops and food crop pathogen detection (Ozimek *et al*., 2010). For example, nano sensors have been devised for application in the detection of food pathogens, as well as other food contaminants and thereby increasing security of food manufacturing, food processing, and shipping of food products. Nanomaterials in foods have also played an important role in preserving the integrity of foods as well as materials in the encapsulation and functional food ingredient delivery systems that carry, protect, and deliver functional food ingredients to their specific site of action (Ozimek *et al*., 2010).

Nanotechnology Derived Food Ingredients and the Role of Nanotechnology in the Pioneering of New Functional Food Ingredients

Foods in their natural state are composed of organic molecules such as protein, carbohydrate, fat, and lipids, with varying size and molecular weights, but they range from large complex polymers to simple molecules with sizes within the nano range. Nanotechnology has the potential to pioneer new functional food ingredients that may contain nanomaterials suitable for specific purposes, such as the encapsulation of nutrients that may play a vital function in increasing nutrient bioavailability, while some may play a vital role to enhance taste, texture, and consistency of foodstuffs, or mask an undesirable taste or odor.

Several nano-based agro/feed/food products, such as nano-encapsulated nutrients, antimicrobial nanoparticles, and active and intelligent food packaging, are already being produced and have already been commercialized. Other nano-based food/feed/agro products are currently under research and development,

and thus have the potential for commercialization in the future. However, producers/manufacturers of these nanofoods are obliged to prove the safe use of their products and to the environment before obtaining a license to distribute them to customers/consumers. For this reason, a number of national authorities in some countries have enforced regulatory mechanisms specifically for the application of nanotechnologies in foods/feed and agroproducts. Due to the regulatory enforcement, manufacturers and distributors of nano-based foods are obliged to include in the labeling of their products all information about the nanomaterials that are included (Cairns, 2006; Euractiv.com, 2010; FAO/WHO, 2009; International Center for Technology Assessment (CTA), 2008; Sandoval, 2009).

Manufacture of Nanomaterials for Application in the Food Industry to Create Nanofoods

There are two types of approach that are normally employed in the nanomaterial manufacturing processes:

1. Top-down approach: in which larger matter particles with dimensions in the nanometers range are broken down using either physical, biological or chemical processes. Examples of top-down approach in the production of nanofoods include physical/mechanical/dry milling of wheat bran to produce bioactive food nano ingredients (Zhu, 2010). Another top-down example is the application of the size reduction approach to improve the antioxidant properties of green tea (Shibata, 2002). Other techniques that fall under top-down approaches include homogenization, which involves pressure to reduce the size of fat globules in dairy products and the application of lasers together with vaporization and cooling (Brody *et al*., 2008).
2. Bottom-up approach: which encompass several techniques for nanomaterial production including crystallization, layer-by-layer deposition, solvent extraction/evaporation, self-assembly, microbial synthesis, and biomass reactions (Brody *et al*., 2008).

Classes of Nanomaterials and Nanostructures Used in Foods/Feed/Agroproducts

A number of functional nanostructures are known to be used as building blocks in the process to create novel structures and introduce new functionalities in

foods that are associated with the enhancement/improvement of certain categories of food properties. These functional nanostructures include food grade nanolaminate films, food grade nanoemulsions/microemulsions, nano-liposomes/liposomes, nanoemulsions, nanoparticles, nanofibers, monolayers, *etc.*

Food Grade Novel Edible Coatings and Edible Nanolaminate Films

Food grade novel laminate films and coatings are nanotechnologically created materials that consist of two or more layers of material with nanometer dimensions and are either physically or chemically bonded to each other. Their functional properties are governed by the characteristics of the 32 film-forming or coat materials used during their preparation. However, physico-chemical attributes such as composition, thickness, structure, and properties of the multilayered laminate or coats created around them can be controlled (Ozimek *et al*., 2010). For example, it is possible to change the type of adsorbing substances in the dipping solutions, vary the total number of the required/needed dipping steps, change the order that the object is introduced into the various dipping solutions, change the solution and environmental/experimental conditions such as the pH, ionic strength, dielectric constant, temperature, *etc.* and in all these possibilities, the properties of the resultant laminate or coat will be different from those in another set (Ozimek *et al*., 2010). Different types of mechanistic driving forces for adsorption of a substance to a surface can be involved, for example electrostatic, hydrogen bonding, hydrophobic interactive, thermodynamically incompatible, *etc.* But they are all highly dependent on the chemistry of the surface as well as that of the adsorbing substance.

Edible coatings and edible films find application in different types of foods, such as fruits, vegetables, meats, chocolate, candies, bakery products, French fries, etc., where they play an important role as moisture, lipid, and gas barriers (Cagri *et al*., 2004; Morillon *et al*., 2002). They are also useful in other functions such as to improve the textural properties of foods or as carriers of functional agents such as colors, flavors, antioxidants, nutrients, and antimicrobials (Ozimek *et al*., 2010).

Food-grade Nano/Microemulsions as Functional Nanostructures for Foods

Microemulsions refer to a spontaneously formed three-component homogeneous system that is a clear and thermodynamically stable kind of dispersion, composed of different ratios of oil, surfactant, co-surfactant, and water with dimensions not exceeding 100 nm (Feng *et al*., 2009; Radomska and Dobrucki, 2000).

The small droplet size imparts to nanoemulsions unique rheological and textural properties, which make them transparent and pleasant to the touch and thus improve the quality of food (Sonneville-Aubrun *et al*., 2004). In addition, nanoemulsions in food products (e.g. low fat nanostructured mayonnaise, spreads, and ice creams) play an important role in facilitating the use of less fat without a compromise in creaminess (Chaudhry *et al*., 2008). Also nanoemulsions provide added stability to foods, because as the size of the droplets in an emulsion is reduced, the less likely the emulsion will be to break down and separate, implying that nanoemulsification may reduce the need for the inclusion of certain stabilizers in food products (Cushen *et al*., 2012).

Other attractive features of microemulsions that make them suitable for incorporation as functional nanostructures in foods, and even other fields of science and technology, include their high solubilization capability of organic and inorganic components in foods, their thermodynamic stability, spontaneous formation, can easily be scaled up, large interfacial area, nanosize droplets, isotropy, and low viscosity (Solans and Kunieda, 1997; Stubenrauch, 2009).

Despite all these attractive features, the application of nano/microemulsions in foods is still limited due to the fact that large amounts/volumes of surfactants are required to form emulsions and it may not be economically viable and also compromises environmental safety (Zhong *et al*., 2009). Moreover, there are not many edible surfactants that can have direct application in foodstuffs (Flanagan *et al*., 2006).

In the food industry, microemulsion products find application mainly for food solubilization, in that they increase the water solubility of nutrients and vitamins, also for improving reaction efficiencies such as inter-esterification, hydrogenation, and for fortification of foods.

Nanotechnology for the Enhancement of Food Color

With the current trend in the application of nanotechnology in improving

rheological properties of foods, it may be possible to extend the application to improve food color, an area that currently has not seen much nanotechnology. For example, it may be possible to use oil-soluble pigment compound b-carotene to impart pigment color into aqueous-based foods by simply using nanoemulsion technology (Astete *et al*., 2009). There is also a high possibility of forming nanosized structures using alginic acid and calcium ions and this nanomaterial has the potential to open doors for application of fat-soluble colorants where it is possible to change the concentration of the b-carotene present in the nanomatrix and thus change food color (Cushen *et al*., 2012).

Nano-liposomes/Liposomes Functional Nanostructures in Foods

Liposomes are another class of useful nanostructure capable of adding functionality in food. These functional nanostructures have biological and chemical properties which can be explained as spherical bilayer membrane structures with aqueous cores. Nano-liposomes and liposomes can be useful in applications that require the aspects to contain and deliver hydrophilic, or water-soluble, food ingredients. One attractive feature that is characteristic to nano-liposomes/liposomes is that their internal pH is adjustable, and therefore they can contain and maintain stability of food ingredients. The disadvantages associated with lipopsomes include the fact that they are very fragile and leakage problems may occur. Otherwise, nano-liposome technology has found application in food technology, which involves areas such as encapsulation and controlled release of food materials, in areas where there is a need for enhancement of food/nutrient bioavailability, food stability, and improved shelf-life of sensitive food ingredients. These nanostructures have been used in the food industry to deliver flavors and nutrients and in the immobilization of antimicrobial ingredients in order to prevent microbial spoilage of food products.

Nanoscale Nutraceuticals

Nutraceuticals (e.g. bioactive proteins, etc.) play important functions in food, including the fact that they are nutrients themselves and also contain functional groups that improve the health of consumers, because they are bioactives in functional food systems (Chau *et al*., 2007). The small size of the nanoparticle bioactives is vital, due to the fact that this attribute tends to improve other properties of functional foods, such as improving the availability of the foods,

improving the delivery properties of functional foods, as well as the solubility of the bioactives and hence their biological activity, since the bioactivity is a function of the bioactive's ability to be transferred across intestinal membranes into the blood (Chen *et al*., 2006; Shegokar and Muller, 2010). The application of nanotechnologies is also vital in improving the stability of micronutrients such as nutraceuticals, omega-3 fatty acids, and certain beneficial probiotic bacteria species (lycopene, vitamin D_2, and beta-carotene) during processing, storage, and distribution (Chen *et al*., 2006; Neethirajan and Jayas, 2011).

Nanoparticles for Application as Food Additives, Food Supplements and in Water Purification

Nanoparticles, such as nano silver and nano zinc oxide have been applied as food additives or food supplements, where they enhance gastrointestinal uptake of metals. They are also useful in water purification works, where they play a role in the removal of contaminants or catalyzing the oxidation of certain contaminants (Ozimek, 2010). Moreover, filters with nano-pores have been highly useful in water purification works, where they play an important function in terms of pathogen removal from water.

Encapsulation in Delivery of Functional Food Ingredients

Encapsulation of functional food ingredients refers to the process that involves the isolation of the active food ingredient within the food product using food-grade materials. The processes to create capsules and then encapsulation of target ingredients takes into account the structuring of the active ingredient, mainly at either molecular or nanoscale levels, using food-grade ingredients capable of interacting with the active ingredient. Currently, there are numerous new functional food ingredients that are being integrated/encapsulated into various food matrix systems and the aim of encapsulation of these functional food ingredients is to improve the functionality of these ingredients in various food matrices. Moreover, encapsulation enhances the bioavailability as well as the ability to disperse these functional food ingredients far more than compared to their bioavailability and dispersal in their natural systems (Haruyama, 2003).

Generally, the vital components of functional foods, such as nutraceuticals, vitamins, probiotics, antimicrobials, essential oils, antioxidants, drugs, and

preservatives are rarely used in their pure natural form, but they normally form part of the delivery system. The ideal delivery system for functional food ingredients has to be compatible with the food product's attributes such as taste, texture, appearance, and shelf life of the final food product and the characteristics of the delivery system govern the efficacy of the functional food's ingredients in the food. A delivery system for functional foods, as is the case for the delivery systems of other substances such as drugs, etc., plays several important roles, including:

- to transport or act as a vehicle for the desired functional food ingredient to the target site of action;
- to protect the ingredient of the functional food in question from both chemical and biological degradation phenomena such as oxidation, temperature, spillage, microbial action, etc., during food processing, food storage, and utilization, in order to preserve and maintain the integrity of food and its functional ingredient in its natural active state; and
- to control the rate of release of the functional food ingredient under a specific set of environmental conditions, such as pH, ionic strength, or temperature.

The encapsulation of functional food ingredients in food is normally made possible by nanosized self-assembled structured liquids (NSSL) technology, which allows the addition of insoluble compounds into food, such as a healthier version of canola oil (Ozimek, 2010). There is a diverse variety of delivery systems that are currently in use to encapsulate functional food ingredients, depending on the type of functional food to be delivered, molecular form, as well as physical form of the functional food, and they include simple solutions (nanodispersions and nanocapsules), association colloids, nanoemulsions, nanostructured multiple emulsions, biopolymeric nanoparticle matrices, *etc*. Each of these possess unique and specific sets of attractive features, as well as shortcomings in terms of their suitability for encapsulation, protection, and delivery of functional ingredients, regulatory status, ease of use, biodegradability, and biocompatibility (Ozimek, 2010).

Association Colloids Based Delivery System for Functional Food Ingredients

By definition, colloids refers to a stable system of any substance that contains small particles that are uniformly dispersed throughout a particular matrix. In

cases where there are much smaller particles, which may range in size from 5 to 100 nm and which form a transparent solution (e.g. surfactant micelles, vesicles, bilayers, reverse micelles, and liquid crystals), this system is known as an association colloid (Bilska *et al*., 2009; Garti and Benichou, 2004; Garti *et al*., 2005; Golding and Sein, 2004). Association colloids have several advantages, including the fact that they form spontaneously, they are thermodynamically favorable, and are generally transparent solutions. However, they are associated with several drawbacks, including the fact that they tend to compromise food properties such as the flavor of the ingredients, because their formation requires the use of large amounts of surfactants, which may also lead to compromising the regulations and guidelines threshold limits. Another limitation is that they have a tendency to undergo spontaneous dissociation if diluted (i.e. their formation is concentration driven).

Nanoemulsions Based Delivery System for Functional Food Ingredients

Nanoemulsions can be defined as emulsions with droplet diameters of less than 100 to 500 nm and functional food ingredients are incorporated/encapsulated either within these nanoemulsion droplets (to facilitate the slowing down of chemical degradation processes by engineering the properties of the interfacial layer surrounding the ingredients of the functional foods), in the interfacial region, or in the continuous phase (Jasińska, 2010; McClements, 2004; McClements and Decker, 2000).

Nanostructured Multiple Emulsions Based Delivery System for Functional Food Ingredients

Examples of multiple emulsions include oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) emulsions, such that the ingredients of the functional foods can be encapsulated either within the inner water phase, the oil phase, or the outer water phase, and thereby create one homogeneous, continuous, and single multiple-functional system (Flanagan and Singh, 2006; Garti and Benichou, 2001, 2004).

Nanotechnology for Active and Intelligent Food Contact Materials

By definition, active packaging materials refer to those that are capable of releasing either nanoscale antimicrobial compounds, antioxidants, and/or flavors, which can also enhance the shelf life and sensory characteristics of foods (Cushen *et al*., 2012). An example of food contact materials-bound active nanomaterials include the nano-Ag embedded baby bottles (Alfadul and Elneshwy, 2010). The success in the area of active bound food contact materials is opening the possibility for the introduction of intelligent food contact materials in which nanosensors, which have the ability to identify specific microbial and/or chemical contaminants or environmental conditions, can be incorporated into food packaging matrices (Neethirajan and Jayas, 2011). These intelligent food contact materials are also capable of changing an environment in response to a stimulus such as pH, pressure, presence of gases, liquids, or products of microbial metabolism or spoilage accelerators, such as temperature or light intensity and thereby alert consumers or distributors to the contamination (Otles and Yalcin, 2008). There is also another possibility of combining food packaging materials with active substances, in order to obtain a composite that can be useful in controlling surface microbial contamination of foods and thereby extend the food's shelf life as well as improving its quality and safety (Vermeiren *et al*., 2002).

Food Grade Biopolymeric Nanoparticles in Food Packaging

Food-grade biopolymers such as proteins or polysaccharides can be used to produce nanometer-sized particles (Chang and Chen 2005; Gupta and Gupta, 2005; Ritzoulis *et al*., 2005). Several mechanisms, including aggregative (net attraction) or segregative (net repulsion) interactions, can be employed in the separation process of smaller bio-nanoparticles from a single biopolymer. The resultant bio-nanoparticles are then used in the encapsulation procedures for functional food ingredients and are then released in response to distinct environmental mechanisms.

Biopolymers in this context will refer to organic substances with molecular backbones that are composed of repeating units of either sugar moieties (saccharides), nucleic acids, or amino acids and in some cases various additional chemical side chains are attached to these functional groups and thus contribute to the shaping of the polymer's molecule. Biopolymers are thus a type of polymer produced or generated through natural means by living species.

Biopolymers can also be defined as types of polymers composed of monomeric units that are covalently bonded, forming chain-like molecules, and the prefix “bio-” implies that these types of polymers are biodegradable and the most likely degradation products (organic by-products) are mainly CO_2 and H_2O, which are safe when entering the environment (Liu *et al*., 2005; Muratore *et al*., 2005). Biopolymer nanoparticles which find application in foods are known to be highly bioactive solid particles with diameters of 100 nm or less and they may be used in foods to play various functions, for example as carriers of antimicrobial components. For example, nicin-containing biopolymeric nanoparticles display a more enhanced potent activity against *E. coli* O157:H7 than those particles that do not contain nicin.

Previously, before the advent of technology (nanotechnology), the food industry relied on natural biopolymers to cater for the needs of food packaging fabrics. The biopolymers that were mostly used included carbohydrate-derived biopolymers such as cellulose, chitosan, and agar; and protein-derived biopolymers such as gelatin, gluten, alginate, whey protein, and collagen. In the recent past, the food industry has witnessed the booming of synthetic biopolymers that possess more improved properties as compared to the natural ones. For example, synthetic biopolymers (i.e. polylactic acid (PLA); polycaprolactone (PCL); polyglycolic acid (PGA); polyvinyl alcohol (PVA); and polybutylene succinate (PBS)) have relatively better properties in terms of durability, flexibility, high gloss, clarity, and tensile strength (Rhim *et al*., 2013).

Classification of Biopolymers with Application in Food Packaging

There are generally three main groups of biopolymers that are normally used in the food industry, in a classification that is based on their origin, which include:

a. biopolymers that are derived from renewable sources;
b. biopolymers that are derived from petroleum sources, which include the synthetic biopolymers that are conventionally and chemically synthesized from petroleum products and a typical example can be polycaprolactone (PCL); and
c. bionanocomposites are the biopolymers that are derived from renewable sources, further grouped into three classes:
    1. natural biopolymers derived from biomass such as agro-based sources,

and examples include celluloses such as cellulose acetate, carboxylmethyl cellulose (CMC), and hydroxypropylmethylcellulose (HPMC) (Bruna *et al*., 2014; George *et al*., 2014; Mondal *et al*., 2013);

2. synthetic biopolymers derived from microbial production processes or fermentation, mainly chitosan (Pereda *et al*., 2014; Tripathi *et al*., 2011); agar (Kanmani and Rhim, 2014a,b); gluten (Rafieian *et al*., 2014); and alginate (Abdollahi *et al*., 2013). However, typical examples of synthetic biopolymers derived from microbial production processes can be polyhydroxy-alkanoates (PHA); and
3. synthetic biopolymers conventionally and chemically synthesized from biomass and a typical example can be polylactic acid (PLA).

Despite all the attractive features of using biopolymers in foods, they still present some shortcomings. especially with their use as food packaging materials if they are to be compared with the conventional non-biodegradable materials such as those derived from petroleum. The limitations include poor mechanics in the sense that they have low tensile strength and also are associated with barrier properties in the sense of high water permeability properties. Moreover, biopolymers are generally known to be brittle, have low heat distortion temperature, low resistance to extreme heat and humidity, low flexibility, and low resistance to prolonged process operations. To address these challenges, bionanocomposites have been introduced as new materials for food packaging, as they offer enhanced mechanical and barrier properties of biopolymers.

The bionanocomposite materials that are mostly used for food packaging applications are mainly starch and derivatives, which are considered to be safe because they are edible and this polymer is also known to be completely degradable and has the ability to stimulate biodegradability of other non-biodegradable materials that may be present in a starch blend or composite (Heydari *et al*., 2013; Nafchi *et al*., 2013; Pan *et al*., 2014; Sorrentino *et al*., 2007; Tang *et al*., 2008).

Nanophase/fillers – Bio-nanopolymers Blends for Use as Food Packaging

Nanofillers are also known as nanophase materials, and like other nanomaterials have reduced size that makes them possess large surface area to volume ratios,

and are therefore attractive for use as fillers in bionanocomposites that are intended for use as food packaging materials. The food packaging biopolymers depend to a large extent on the large surface area of the nanosized phases/fillers to provide them with a large interfacial or boundary area between the matrix or biopolymer and nanofiller. Generally, the bionanocomposites intended for use as food packaging are usually designed to have the ability to endure the mechanical and thermal stress during food processing, transportation, and storage. Thus, incorporation of nanophase/fillers in biopolymers is to provide the biopolymers with a large interface in order to enable the modification of molecular mobility, and the relaxation behavior besides mechanical, thermal, and barrier properties of the bionanocomposites to acquire such properties (Azeredo *et al.*, 2011: Rhim *et al.*, 2013a,b).

Classification of Nanofillers for Food Packaging

There are two general groups that classify nanofillers, based on the nature of their chemistry used as food packaging materials, and they include:

- organic based nanofillers; and
- inorganic based nanofillers.

The organic-based nanofillers are further subdivided into three main groups:

1. clay-based nanofillers: examples of these include montmorillonite (Pinto *et al.*, 2015), Cloisite-Na and Cloisite 10A (Lee *et al.*, 2014), Cloisite 30B (Shin *et al.*, 2014), and Cloisite 20A (Rhim *et al.*, 2011);
2. natural biopolymers: examples being chitosan (Martelli *et al.*, 2013) and cellulose (Shakeri and Radmanesh, 2014); and
3. natural antimicrobial agents: an example of this is nisin (Imran *et al.*, 2012).

The inorganic nanofillers are subdivided into two main groups:

a. metals such as silver, gold (Youssef *et al.*, 2014), and copper (Conte *et al.*, 2013); and

b. metal oxide nanofillers such as zinc oxide (Kanmani and Rhim, 2014), titanium oxide (TiO_2) (Zhu *et al.*, 2012), magnesium oxide (Sanuja *et al.*, 2014), and silver oxide (Ag_2O) (Tripathi *et al.*, 2011).

These metal nanoparticles are vital components of food packaging materials or food storage materials/devices, where they improve barrier properties of

packaging or storage materials, also serving as antimicrobial agents, or when they are created/engineered as active nanoparticles they function in foods by checking the migration out of packaging materials where they function as oxygen scavengers and prevent the growth of microbial pathogens.

Nanosensors in Foods

Nanoparticles that have been engineered and created as complex nanostructures are useful as nanosensors in food packaging, where they serve as detection systems in cases of food deterioration.

Nanoparticles/Nanofillers for use in Bioencapsulation Materials in Functional Food Delivery Systems

Nanoparticles or nanofillers that are engineered as nanosized nutrients, nanosized foods, or nano encapsulates, find application in food as part of the delivery systems for functional foods and therefore are highly useful as food additives or food supplements, where they play the important function of enhancing the uptake of functional foods and are also useful in the protection of targeted delivery of functional foods. The classification of nanofillers that find application in the food industry in food delivery systems based on their structural nature, groups nanofillers into a number of classes:

- nanoparticles (solid lipid nanoparticles);
- nanofibrils;
- nanorods; and
- nanotubes.

Solid Lipid Nanoparticles in Bioencapsulation Materials for Functional Food Delivery Systems

Solid lipid nanoparticles (SLNs) are crystallized emulsions made up of a high-melting point lipid and a bioactive lipophilic component. In foods, they are used as part of the composition of the delivery systems for functional foods as encapsulation components. Functional foods are normally composed of bioactive

ingredients such as carotenoids, amino acids, omega-3 fatty acids, vitamins, phytosterols, probiotics, etc., which play an important role in improvement of the health status of consumers. However, the incorporation of the ingredients of functional foods into the food matrices requires the facilitation of encapsulated matrices to serve as delivery systems that need to be designed and engineered specifically for each class of functional food ingredients. The encapsulation systems are attractive, as they are created with targeted properties such as high physical stability, ability to protect ingredients of functional foods against chemical processes such as chemical degradation, and moreover possess capability to ensure precise control over the release of encapsulated components during mastication and digestion to maximize adsorption. One form of encapsulation system, known to have the potential for use in functional foods, is solid lipid nanoparticles (SLN), which is composed of crystallized nanoemulsions with the dispersed phase composed of a solid carrier lipid–bioactive ingredient mixture.

Nanofibers and their Multi-applications in Foods

Although nanofibers and nanotubes (diameters range in size from 10 to 1000 nm) are not composed of food-grade substances, and thus have limited potential applications in foods, they still hold immense future potential application in the food industry. For example, it has been reported that under appropriate environmental conditions, certain globular milk proteins can self-assemble into structures that resemble that of nanotubes (Graveland-Bikker and de Kruif, 2006.; Graveland-Bikker *et al*., 2006). Moreover, nanofibers such as cellulosic nanofibers have found application in foods, especially in the immobilization of bioactive substances such as enzymes, vitamins, and antimicrobials; as delivery systems for nutraceutical and controlled release of other functional food ingredients (i.e. they are used as delivery systems of nutraceuticals and nutrients to protect them during processing and storage or in delivery systems for transferring the components to the target site in the body); and as biosensors.

Generation of Food-grade Polymeric Nanocomposites for Application in Food Contact Materials

Food-grade polymeric nanomaterials and nanocomposites can be created at the

nanoscale, but in most cases it is preferable to incorporate nanoscale materials in the structures of polymer matrices (Yang *et al*., 2007). Such nanoscaled materials can impart to food contact materials (FCMs) useful attributes such as enhanced flexibility, gas excellent barrier properties, and also temperature control and moisture stability due to the reinforcement contribution provided by nanoscaled nanomaterials and nanocomposites, which play an important role as nanoscale fillers (Alexandre and Dubois, 2000; Giannelis, 1996; Sinha Ray *et al*., 2002). Examples of such food-grade nanoscaled polymeric fillers include the clay montmorillonite, which is actually stacked silicate sheets known to have a high ratio of length to thickness (aspect ratio) and a plate-like morphology (Rhim and Ng, 2007); laponite (a synthetically prepared clay composed of sodium magnesium lithium silicate) is another example and has been reported to provide a higher aspect ratio as compared to montmorillonite (Chung *et al*., 2010).

Criteria for Establishment of Risk Assessment for Nanofoods

In order to establish risk assessment of nanofoods, it is required that the characterization of nanomaterials incorporated into foods be done appropriately and exhaustively, in order to establish accurate and precise data of the nanomaterial used. This is because the characterization of nanocomposites and nanomaterials used in foods is more complex, thus requiring a more detailed scope in terms of the analytical parameters to be investigated, the extent and range of properties required to generate detailed information, and in-depth data regarding the nanomaterials used in foods. Moreover, method development has to be done to establish standard analytical procedures and protocols that are capable of detecting the presence of nanomaterials in foods, food contact materials, food packaging, etc., at below the stipulated guidelines and threshold limits (Tiede *et al*., 2008). Moreover, certain crucial information is required, such as the possible accumulation of particulate nanomaterials in foods, and if the food that contains nanoscaled material is consumed, what could be the effects on the body of the victim/s after ingestion (exposure and absorption) (Handy and Shaw, 2007)?

In addition to knowledge about the possible accumulation of nanoscaled materials in foods, and their fate in the body, it is also imperative to establish the target organs/tissues where these nanoscaled materials accumulate, and their elimination profiles, *etc*. Currently, the risk assessment on food-grade

nanomaterials is yet to be refined, as it has to overcome all these obstacles, including the challenges in the reliable methods for characterization of the nanoparticles/nanomaterials, sensitive and selective methods for their detection, and also lack of reliable information and data related to the toxicology properties of food-grade nanomaterials (European Food Safety Authority (EFSA), 2009).

Potential Risks of Nanofood and Nanofood Regulations Concerning the Safety of Nanofoods

In as much as there are many potential benefits of nanofoods, nutritional-wise and economic-wise, there are also potential risks to human health that are associated with nanofoods (Pusztai and Bardocz, 2006; Siegrist, 2008). For example, some scientific reports have indicated that inhaled nanoparticles have the potential to accumulate in the lungs and cause chronic diseases (Chau *et al*., 2007; Poland *et al*., 2008). Generally there is still huge debate with regard to direct risks (due to direct ingestion of nano-containing foods/water) and the debate is mostly on their bioavailability. The bioavailability phenomenon in question is the one in which nanoparticles cross cellular barriers in the body, which are normally impossible to be crossed by normal foods in their natural state, then spread and accumulate in other parts of the body to cause unknown long-term health effects (Chun, 2009). If indeed bioavailability is enhanced with nanomaterials present in foods, then there are even more risks to human health, because this may lead to changes of the nutrient profile, greater absorption of nano-additives, and the potential introduction of foreign substances into the blood (Buzby, 2010; Chaudhry *et al*., 2008). Another potential risk may originate from nanosized materials, such as nanoparticles intended to function as antimicrobial agents in food packaging that may contaminate by leaching, which may cause such nanoparticles to migrate into the food (Chaudhry *et al*., 2008). There is also a potential for bioconcentration/bioaccumulate in the biological environment and thus the potential to uncontrollably contaminate the whole of the food chain (Buzby, 2010; Chaudhry *et al*., 2008).

In terms of regulations on the safety of nanofoods, there are no standardized procedures or universal regulations in place that govern the production, application, or utilization of nanofoods. For example, the Food and Drug Administration (FDA) in the USA regulates nanofood products but not the technology used to produce them, while the Institute of Food Science and Technology (IFST) in the UK recommends that nanomaterials in foods must be

Technology (IFST) in the UK recommends that nanomaterials in foods must be treated as new, potentially harmful materials until rigorous testing proves their safety.

Regulation Regimes for use of Nanoscaled Materials Foods

Generally, the regulation regimes for use of nanoscaled materials in foods fall within the scope of both the broad horizontal legislation as well as the specific vertical legislation. Different countries follow different sets of horizontal and vertical regulations. For example, in Europe, there is a horizontal regulation governed by the Directive 2001/95/EC known as the General Product Safety Directive (GPSD), which requires observance of the general safety of products that are being marketed and/or supplied to consumers (human safety rather than environmental safety). In addition to this, the EU came up with another piece legislation, known as the REACH legislation, in which a new labeling style for all marketed products has to include safety information and this legislation takes into account the ecotoxicity aspect of the product being sold. The EU has also imposed another regulation (Regulation (EC) No. 1272/2008) that supplements the REACH legislation and requires that consumers of potentially hazardous substances be notified of the possible risks by means of a new labeling system that includes the use of safety symbols as well as the inclusion of safety data sheets to consumers.

It should be noted that nanoscaled materials may not be directly mentioned in this legislation, but producers of edible products who incorporate nanomaterials into their products are to adhere to the legislation as well as the revision of the legislation, in order to include the use of nanomaterials in foods that have already been suggested (UBA, 2009).

As for vertical legislation, the EU has suggested to limit guideline values, labeling requirements, and risk assessment for products being marketed that include foods, and that where there is a change in the starting material used in the food production/processing or in the production method of an additive (e.g. a change of the particle size), this particular food product has to go through a new authorization process and safety evaluation. Moreover, the European Commission Directive 96/77/EC sets limiting standards to the quantity of certain impurities permitted within food additives, which must be adhered to. This directive controls the use of food additives, ensuring that manufacturers only use approved, quality grades of additives that have passed safety testing. With regard

to novel food (i.e. foods and food ingredients that have not been used for human consumption to a significant degree), Regulation (EC No. 258/97) requires them to undergo a safety assessment prior to being placed on the market. Another regulation on novel foods/nanofoods, Novel Food Regulation (EC No. 258/97), is even more specific in terms of the inclusion of nanoscaled materials in foods (EC No. 258/97, 1997).

The EU has another regulation that governs Active and Intelligent Materials, which requires that if legislation limits the quantity of a substance in a food, the total quantity should not exceed that limit, regardless of the source, *i.e.* originally included in the foodstuff or following release of that substance from the Food Contact Material (FCM). If it happens that a substance is released into the food in this way, it is required to be included in the ingredients list. For active food substances that are not designed to be released from the packaging and have no function in the food, there is a risk that these substances may migrate into the food.

Generally, in the rest of the world, legislation on the use of nanomaterials in foods tends to be cautious towards potential risks posed by the new applications (Chau *et al*., 2007).

Analysis and Characterization of Nanomaterials in Food Materials

As explained above, it is expedient to have a thorough knowledge regarding the potential of the risks of nanomaterials that are incorporated into foods and therefore it is imperative to have in place appropriate analytical methods capable of quantifying these nanomaterials within a food matrix, as well as analytical procedures that can provide data on the characteristics of the very same nanomaterial. The ideal methods ought to be unambiguous and must involve only limited manipulation of the samples, in order to avoid any possible introduction of artefacts.

Generally, due to the physico-chemical properties, nanomaterials in foods can be analyzed and characterized using different methods and techniques, which may include those based on microscopy, spectrometry (e.g. single particle inductively coupled plasma-optical emission spectroscopy/mass spectrometry); size separation with light scattering detection (e.g. field flow fractionation with dynamic light scattering (DLS)); multi-angle light scattering (MALS) detection; chromatography (e.g. hydrodynamic chromatography); surface characterization (e.g. small-angle X-ray scattering); as well as different variants and

(e.g. small-angle X-ray scattering), as well as different variants and combinations of the above-mentioned techniques. One important consideration during the analysis and characterization of nanomaterials in foods involves method validation and streamlining for detection, because of the possibility that different methods or techniques may yield different measurement data or information for a given sample.

Electron Microscopy Based Methods for the Characterization of Nanomaterials in Foods

An in-depth discussion about the application of electron microscopy-based methods in food forensics cases will be discussed in chapter 13. However, the application of electron microscope-based methods, especially those that are known for their great resolving power due to their ability to make use of an electron beam with wavelengths well below the nm range for the analysis and characterization of nanomaterials in foods, enables visualization and characterization of nanosized objects (Kachlicki, 2007). Of the electron microscopy-based techniques, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) seem to present the more likely potential application for the detection and characterization of nanomaterials in foods. The mode of operation of scanning electron microscopy involves the generation of the sample by scanning the sample surface with a low-energy beam of electrons, normally in the range that lies between 1 and 30 keV, and then detecting the electrons that are scattered from the sample (Denk and Horstmann, 2004). On the other hand, the mode of operation of transmission electron microscopy involves the transmission of a high-energy electron beam, normally in the range between 80 and 300 keV, across/through a very thin layer of the sample, and then detecting:

- the electron beam scattered from the sample; and also
- a beam of electrons transmitted due to change in either energy or direction.

Sampling for Electron Microscopy Analysis and Characterization of Nanomaterials in Food

Sampling is always the first step in any analytical procedure and is crucial in the sense that it determines accuracy, precision, and the detection limit of the method. The most important aspects in the sampling regime for the analysis

and/or characterization of nanomaterials in foods include obtaining a representative and homogenous sample with a uniform distribution in terms of its composition. Particular sampling protocols normally are dependent on the type of nanomaterials under investigation and also the matrix (Liu *et al*., 2009).

Sample Preparation for Electron Microscopy Analysis and Characterization of Nanomaterials in Food

Sample preparation is essential in electron microscopy analysis of nanomaterials in foods, because electron microscopes operate under vacuum conditions and therefore wet samples (samples containing any liquid/water) cannot be introduced into the scanning electron microscope without proper preparation to dry the sample and also to ensure that the sample is able to scatter the electron-beam. Several techniques are normally employed to dehydrate the sample and include chemical fixation, followed by either dehydration or drying and then subjecting the dehydrated sample to either conventional SEM or TEM. Another drying technique involves freezing followed by analysis using either cryo-transmission electron microscopy or cryo-scanning electron microscopy.

Dehydration of food sampled in the analysis of nanomaterials can also be achieved by using ethanol (Rahman *et al*., 2008). Despite the advantages that ethanol offers in terms of its dehydration power, it is not appropriate for use in food samples that contain high levels of saturated fats or even water-soluble carbohydrates. Other possible problems that may come from the extreme use of chemical dehydration include the potential introduction of artefacts or even possible loss of nanomaterials that may be associated with one of the phases that is being discarded. Moreover, there may be a potential triggering of reactions between these chemicals and the nanomaterials. It is therefore highly recommended that methods used for dehydration in the analysis of nanomaterials in foods be carefully examined and proved before using them. Alternatively, it may be advisable to employ sample-drying techniques to avoid all these potential problems (Lorenz *et al*., 2010; Novak *et al*., 2001). However, even drying methods in the analysis of nanomaterials in food samples have limitations in that they are mainly suitable if the food matrix is liquid and its density is low enough to allow the formation of a very thin film (Lorenz *et al*., 2010).

Other sample preparation techniques include the fixation of food samples with glutaldeyde, post-fixation using osmium oxide and then subjecting the samples

to a chemical dehydration process followed by critical-point drying (if samples are to be subjected for scanning electron microscopy analysis/characterization or resin embedding in the case where samples are supposed to be subjected for transmission electron microscopy). These methods are suitable for food samples such as fruits, meat, or vegetables, where there may be a need to investigate their intracellular structure. These sample preparation procedures are vital for retaining the integrity of the food samples by minimizing the possibility of changes that may occur in the sample matrices and thus enabling the uncompromised observation for the real distribution and possible interaction of the nanomaterials within the cellular/histological structure in the native state of the sample (Egelandsdal *et al*., 1999; Kaláb *et al*., 1996). For food samples such as those that are either fat containing or protein containing types of foods, the sample preparation for such foods in the analysis of nanomaterials they contain may involve post-fixing with osmium tetroxide, or using certain heavy-metal stains such as uranyl acetate, lead citrate, *etc*. In addition to these, mordant reagents such as tannic acid may also be used for staining and preservation enhancement of the food's ultrastructure (Musyanovych *et al*., 2008). Other staining techniques include negative staining (Maunsbach and Afzelius, 1999). Use of organic coatings on nanomaterials has also been reported for creatine and albumin coatings on gold NPs (AuNPs) (López-Viota *et al*., 2009).

The main limitation of staining procedures is that the technique is unable to make distinction for nanomaterials from densely-stained organic structures of the same size, for example between polystyrene NPs and vascular structures in cells (Mühlfeld *et al*., 2007). To avoid this problem, it is suggested that the analysis involves a sample that is known to contain particles with a blank sample, and the use of appropriate control samples in order to enable the distinction of samples.

Generally, drying and dehydration of food samples may result in a significant change in the structure of the food matrix. One of the methods that may be ideal, in the sense that it may not result in significant changes in terms of the structure of the food matrix, is physical fixation by freezing the sample. This is because during the sample freezing process, the ice crystals grow and only water molecules are incorporated into the ice crystals, and the specimen is thereby segregated into crystals of water and the ridges between containing enriched regions with the dissolved material (e.g. solutes and macromolecules). Formation of large ice crystals can even rupture and destroy whole cells in biological samples. As long as care is taken to prevent the possible formation of large ice crystals that may rupture the cell, the method may be the most

attractive to dehydrate the sample (Bruggeller and Mayer, 1980; Cavalier *et al*., 2009; Dubochet, 2009; Maunsbach and Afzelius, 1999).

Scanning Electron Microscopy (SEM) Analysis of Foods

Food samples that are meant for SEM analyses have to be mounted either on aluminum or carbon stubs by sticking with quick-setting glue, epoxy cement, wax, silver paint, or double-sided sticky tape. Where necessary, the tape may also contain carbon to avoid electrical charging of the sample. The resolution magnitude of scanning electron microscopy is normally good, but depends mainly on the type of sample that is being analyzed. Certain samples, for example organic samples (including the majority of foodstuffs), require a step whereby they are covered with a layer of electrical conductive substances such as metal, carbon, or gold in order to avoid charging effects during the process of imaging. When operating scanning electron microscopes, normally the SEM machines are equipped with several detectors that are capable of selecting specific energy ranges of the scattered signal, such that low-energy secondary electrons (SEs) can play a crucial role in providing information regarding the surface topography, while high energy backscattered electrons (BSEs) can be crucial for mapping contrast according to the differences in terms of the atomic number, Z, of elements to which the sample is composed, which makes it possible to establish a good contrast between heavy and light elements (e.g. AgNPs in cells) (Koh *et al*., 2008; Jóźwiak, 2007).

Among the most important observations in the analysis of nanomaterials in foods using SEM is the characteristic depth of field, as obtained from the SEM micrographs, because this data allows the analyst to deduce the effects of nanomaterials on the food structure and the same data can be used to locate nanomaterials within the food samples (Castaneda *et al*., 2008).

Transmission Electron Microscopy (TEM) Analysis of Foods

When using TEM in the analysis of foods, the resolution depends largely on the thickness of the prepared sample and also on the accelerating voltage for the electron beam, such that the higher the voltage, the better the theoretical resolution. For most biological samples as well as food samples that are

composed of structures that may be prone to electron damage, the optimal accelerating voltage of TEM is normally tuned to a magnitude of up to 100 kV (Kachlicki, 2007).

In summary, since the whole issue of nanofoods is still new and is growing rapidly, the scientific and technical question required is to shed light on whether there should be a green light to proceed with the application of nanotechnology in foods or not, or to implement universal regulatory mechanisms globally that will govern the definition of safety/toxicology and environmental impact of nanotechnology application in food production, processing, packaging and utilization, economics, *etc.* Eventually, the factor of consumer acceptance of nanotechnology application in foods will certainly make a strong contribution to the whole success in terms of the nanotechnology/nanoscience application in foods.

Field Flow Fractionation (FFF) Techniques in Nanofood and Food Macromolecule Analyses

Field-flow fractionation is a useful analytical separation technique for characterization of macromolecules such as proteins and protein complexes, and saccharides, as well as viruses, derivatized nano-and micron-sized beads, subcellular units, and whole cell separation (Karl-Gustav, 2013; Roda *et al.*, 2009). This technique is mainly based on the interaction of the analytes with a perpendicularly applied field and is normally performed using open-channel structures by a flow stream of a mobile phase of any composition such that fractionation, unlike chromatographic separation, takes place without any surface interaction of the analyte with packing or gel media, and without using degrading mobile phases. In simple terms, the FFF mechanism does not involve any interaction of the analyte being separated nor does it need a stationary phase, but rather the mechanism is dependent on the externally generated field, which is applied perpendicularly to the direction of the mobile phase flow. This makes the technique very attractive in food applications and other bio/macromolecules such as proteins, because they can be fractionated directly while preserving the integrity.

The theory and mechanism of FFF is based on several classical laws in physical chemistry, including Brownian motion, translational diffusion, laminar flow, frictional force, frictional coefficient, and drag-induced transport by flow, *i.e.* flow displacement, viscous forces (Caldwell, 2000; Karl-Gustav, 2013; Roda *et*

al., 2009; Schure *et al*., 2000). In other words, FFF separation of samples takes place within either a capillary or an empty channel, in which a laminar flow of mobile phase pushes sample components down the channel. As the field is applied perpendicularly to the parabolic flow to direct analytes into different laminar flows, based on the differences in their size, density, and surface properties, this results in different retention times. According to the principle and mechanisms of FFF, it is thus expected that the retention times will be shorter for lower molar mass/size analytes and longer for higher molar mass/size. However, in cases where the analyte diffusion becomes diminished or negligible, as sometimes happens with micron particles, then the elution order under these circumstances becomes reversed, implying that the larger particles are eluted first and the smaller particles are eluted last. The elution mode in FFF is normally known as steric or hyperlayer elution. The retention in FFF depends mainly on size, shape, density, rigidity, and surface features of the analytes.

There are several variants of FFF based on the field that is being applied, which include flow field-flow fractionation (Flow FFF or F4), also known as symmetrical F4 (SF4) (Ratanathanawongs Williams, 2000) or asymmetrical F4 (AF4) (Wahlund, 2000), in which a second flow stream is introduced as the hydrodynamic field to develop separation. Some variants of FFF involve cross-flow by employing cylindrical, porous channels, where either polymeric or ceramic hollow-fiber (HF) are used as fractionation channels to make a variant that is normally abbreviated as HF5 (Jönsson and Carlshaf, 1989), There is also centrifuge-based sedimentation FFF (SdFFF), in which the channel is positioned inside a centrifuge bowl (Moon, 2000). In other variants, gravity has been applied as the sedimentation field (gravitational FFF; GrFFF) (Giddings *et al*., 1979). Field flow fractionation variants utilizing fields such as thermal (thermal FFF; ThFFF), electrical (electrical FFF; ElFFF), and some utilizing split-flow thin cells (SPLITT) to provide continuous, preparative-scale fractionation of macromolecules and particles, have already been developed and applied in bioscience research (Giddings, 1985, 1992; Lu *et al*., 2004).

The commonly used mobile phase systems in FFF are aqueous in nature, although other solvents may be suitable and the composition of these mobile phases varies, depending on the nature of the analyte. However, in most cases, pure water is not recommended as a mobile phase for FFF, due to the fact that any electrostatic interaction that may occur during the fractionation procedures will be long range and may result in problems in the elution profile of sample components, as well as lack of reproducibility. For example, dilute buffer systems have been recommended as suitable mobile phases for neutral

nanofoods, while for ionic or charged nanofoods and their derivatives, higher ionic strengths in the mobile phase may be desirable, even though such a mobile phase system may influence the size and conformation of a sample. Buffer solutions may be the best option for proteinaceous, polysaccharide, or other macromolecule-based nanofoods.

FFF instruments have been coupled on-line to various detectors, such as the multi-angle light scattering (MALS) detector, dynamic light scattering (DLS) detector, refractive index (RI) detector, and spectrophotometric detector, and has been hyphenated to mass spectrometric-based techniques such as the ICP-MS. The MALS detectors detect the scattered intensity at different angles in relation to the incoming light. The light scattering data obtained from the detector is normally fitted using different models such as the Debye model (Debye, 1944), Zimm model (Zimm, 1948 a,b) and Berry model (Andersson *et al*., 2003; Berry, 1966).

When using FFF techniques, the aim is to obtain certain molecular properties that will be used to elucidate the important information about the sample. These molecular properties are mainly dependent on the type of detectors that have been used. Some of these properties include diffusion coefficient, hydrodynamic radii (size), and molar mass of the analyte. Generally, FFF techniques have the potential to offer the possibility to obtain vital information about molecular and conformational properties, as well as functional properties of nanoscaled foods or nanomaterials in foods and their effects in foods over a wide size distribution.

Hydrodynamic Chromatography for Sizing and Quantifying Nanofoods/Nanomaterials in Foods

Another technique that has the potential for application in the analysis and characterization of nanofoods/nanomaterials in foods is hydrodynamic chromatography (HDC), due to its ability to provide reliable size separation that is independent of the matrix. The mode of separation in HDC is based on different samplings of the flow velocity profile caused by differences in the effective diameter (Striegel and Brewer, 2012).

Size separation can be achieved using specific hydrodynamic columns such as PLPSDA. The detectors used with HDC are traditionally differential refractometers (Penlidis *et al*., 1983) or UV detectors (Striegel and Brewer, 2012; Williams *et al*., 2002). Other detection methods include particle-counting detection using laser scattering (Zarrin and Dovichi, 1985), MALS, DLS and

viscosimetry (Brewer and Striegel, 2009); and fluorescence and inductively coupled plasma mass spectrometry (ICP-MS) detectors (Philippe and Gabriele, 2014).

Analysis and Characterization of Nanofood Structure, Food Nanomaterials and Food Macromolecules Using Small-angle X-ray Scattering (SAXS)

Small-angle X-ray scattering is a technique for evaluating the X-ray scattering pattern of either nanoparticles or macromolecules at small angles, for the purpose of gaining information about their particle structure. With SAXS, the measurements are preferred to be done at small angles, because the larger the structures, the smaller the scattering angle. The parameters that can be looked at from SAXS measurements include size, shape, internal structures, crystallinity, orientation, porosity, *etc.* Therefore, SAXS may find application in the analysis and characterization of nanocrystals, emulsions, food macromolecules (proteins, enzymes, etc.), nanocomposites, and nanostructures surfaces, etc., where it can provide vital information about particle size, particle size distribution, and particle shape; size stability studies, structural studies of biomacromolecules, both in solution as well as in solid state, in their native state; can provide 3-D shape models, *etc.* (Burger *et al*., 2008; Cedola *et al*., 2006; Mollenhauer *et al*., 2003; Sasaki and Odajima, 1996a,b). In SAXS, the scattering of X-rays arises from differences in the electronic structure of the atoms (Lindner and Zemb, 1991).

Application of Neutron Scattering Techniques in the Analysis and Characterization of Nanofoods and Nanomaterials in Foods

Neutron scattering techniques encompass a range of techniques in which neutrons are used as probes in the analysis of structural and dynamic properties of materials, by measuring their change in direction and energy after interacting with a sample (Pynn, 1990). The scattering of neutrons is highly dependent on the nuclear structure of the atom (Lindner and Zemb, 1991). The atom of any element is composed of protons, electrons, and neutrons and the number of protons in an atomic nucleus defines the elemental type, while the number of

protons in an atomic nucleus defines the elemental type, while the number of neutrons defines the elemental isotope. Since neutrons are scattered by the atomic nucleus, this means that the scattering from different isotopes can differ significantly (Sears, 1992).

Neutron scattering techniques can be classified into two main groups, namely elastic neutron scattering and inelastic neutron scattering techniques. The elastic neutron scattering techniques involve a process in which the energy, or equivalently, wavelength of the neutron does not change as a result of the scattering event with nuclei in the target sample. Examples of elastic-based techniques include:

- small-angle neutron scattering (SANS);
- ultra-SANS;
- reflectometry;
- powder diffraction; and
- spin echo small-angle neutron scattering (SESANS).

The elastic scattering techniques are useful in providing information about the structure, ranging from the sub-Angstrom (<10_10 m) to supra-micron size range (>10_5 m), such as an ordered structure of a fiber, where neutron diffraction can be used for analysis; analysis of the structure of a casein micelle, where SANS can be used; the conformation of a protein at an interface, where neutron reflectometry technique can be used; and analysis of the arrangement of droplets in an emulsion, where ultra-SANS can be used. The spin echo small-angle neutron scattering (SESANS) technique employs the spin in its mode of operation.

The inelastic neutron scattering involves an energy change emanating from a scattering event, in which the neutron may either lose or gain energy by imparting energy to or from the sample through diffusion controlled processes. These inelastic scattering techniques are useful in generating information about dynamics across a broad temporal range with vibrational spectroscopy through to quasi-elastic neutron scattering and spin echo spectroscopy. Another attractive feature of inelastic techniques is that they are capable of providing simultaneous spatial information if angular dependent information is collected (Byron and Gilbert, 2000).

Neutron scattering techniques are important in the investigation of the properties and their effects on the final characteristics of the food for the purpose of preserving the integrity of foods as they are in the original setting.

Generally, unlike electron-based microscopic techniques, neutron scattering techniques provide bulk information, with the scattering representative of the whole food (Jacrot, 1976; Lu *et al*., 2007; Paciaroni *et al*., 2005; Porcar *et al*., 2004).

In the case of small-angle neutron scattering (SANS), it is possible to use this technique to probe structures over a size range from approximately 1 nm to several hundreds of nm present in food systems and as such it finds application in the elucidation of the quaternary structure of a protein, the conformation of a polysaccharide chain, and the lamellar structure in granular starches (Jacrot, 1976; May, 2002; Wignall, 1993).

Conclusions

The application of nanomaterials, novel/intelligent materials in foods, and food packaging has gained momentum and is now a reality. Regulations on their use vary from country to country. There is a need for all laboratories to be well equipped for the analysis and uncovering of any violations that involve the incorporation of these materials in packaging, or any steps involved in food processing.

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10
Food Forensics Cases Related to Application of Food Additives and Food Improvement Agents

> *Food additives are substances that are used in foods by being added, mixed or infiltrated into food, or by other methods in the process of food production, for the purpose of processing or preserving food, or for the purpose of performing certain specific roles for technological purposes in its manufacture, processing, preparation, treatment, packaging, transport or storage. Examples of the roles that may be played by food additives include preservation (to extend the shelf-life of foods by protecting them against microbes), coloring (to add or restore or sharpen color in a food), sweetening (to sharpen the taste and add flavor), antioxidation (to extend the shelf-life of foods by protecting them against oxidation (i.e. fat rancidity and color changes)), as flour treatment agents (to improve the baking properties), stabilizers (to stabilize food during its production, packaging or storage), flavorings and flavoring enhancers (to impart the desired aroma/odor or taste to food), etc. Therefore food additives ultimately become a component of the food. The term "food improvement agents" encompasses food additives, food enzymes and food flavorings.*

Introduction

According to Article 3 of the EU scope of legislation on food additives as controlled by the EU Regulation 1333/2008, the definition of food additives or food improvement agents is:

> Any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods (Food Additives Legislation, *Guidance Notes*).

There are certain items that are not part of this regulation and they include normal food/food ingredients, even if they are added to perform a controlled

function and therefore by this definition, they are not considered as food additives. Other items that are excluded from this regulation include processing aids, including filtration aids and release agents. Substances will fall under the definition of processing aid, if:

1. they are not consumed as a food by itself;
2. they are intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing; and
3. they have the potential to result in the unintentional but technically unavoidable presence in the final product of residues of the substance or its derivatives, provided they do not present any health risks and do not have any technological effect on the final product (Food Additives Legislation, *Guidance Notes*).

Food additives fall into four main categories and they include:

1. designated additives, which consist of additives apart from natural flavoring agents and substances that have generally been served for human consumption and that are used as additives;
2. existing food additives;
3. natural flavoring agents, *i.e.* substances obtained from animals or plants or mixtures thereof, which are used for flavoring food; and
4. ordinary food used as an food additive, *i.e.* substances that are generally provided as food and which are used as additives such as those used as coloring agents (pigments in red cabbage, etc.). Others are used as thickeners (gluten, etc.) or agents for quality improvement (gelatin, egg white, etc.).

In order to enforce the regulation and monitor strict adherence to the legislation, the European Union has established a number or coding system for all substances used as food additives (E numbers). The E numbers are codes for substances that are permitted to be used as food additives and these codes have to be found in the labeling information for all foods containing such additives.

For example, E number/codes that range from 100–199 are given to food coloring agents such that E100–E109 are for yellow food colors; E110–E119 (oranges); E120–E129 (reds); E130–E139 (blues and violets); E140–E149 (greens); E150–E159 (browns and blacks); and E160–E199 (gold and others).

Food additives that fall under the group of preservatives have E numbers

between 200 and 299, such that E200–E209 (sorbates); E21 E219 (benzoates); E220–E229 (sulfites); E230–E239 (phenols and formates/methanoates); E240–E259 (nitrates); E260–E269 (acetates/ethanoates); E270–E279 (lactates); E280–E289 (propionates/propanoates); and E290–E299 (others).

Antioxidants and acidity regulators have their E numbering between E300 and E399, such that E300–E305 (ascorbates/vitamin C); E306–E309 (tocopherols/vitamin E); E310–E319 (gallates and erythorbates); E320–E329 (lactates); E330–E339 (citrate and tartrates); E340–E349 (phosphates); E350–E359 (malates and adipates); E360–E369 (succinates and fumarates); and E370–E399 (others).

Thickeners, stabilizers and emulsifiers are classified with E numbers ranging between 400 and 499 as follows: E400–E409 (alginates); E410–E419 (natural gums); E420–E429 (other natural agents); E430–E439 (polyoxyethene compounds); E440–E449 (natural emulsifiers); E450–E459 (phosphates); E460–E469 (cellulose compounds); E470–E489 (fatty acids and related compounds); and E490–E499 (others).

Acid (pH) regulators and anti-caking agents have their E numbers ranging between 500 and 599 in the following pattern: E500–E509 (mineral acids and bases); E510–E519 (chlorides and sulfates); E520–E529 (sulfates and hydroxides); E530–E549 (alkali metal compounds); E550–E559 (silicates); E570–E579 (stearates and gluconates); and E580–E599 (others).

Flavor and flavor enhancer E numbering is between 600 and 699, such that E620–E629 (glutamates and guanylates); E630–E639 (inosinates); and E640–E649 (others).

Antibiotics have their E numbers ranging between E700 and E799, while other additives have their E numbers ranging between E900 and E999, such that E900–E909 (waxes); E910–E919 (synthetic glazes); E920–E929 (improving agents); E930–E949 (packaging gases); E950–E969 (sweeteners); and E990–E999 (foaming agents).

New additives that do not find a proper place within these classifications are classes between E1100 and E1599.

In this classification, some of the additives are permitted with specified maximum limits, while other have been banned and are not supposed to be used (zero maximum limit). For example, within food colors, there are a number that are known to be associated with hypereactivity reactions. For example, tatrazine (E102); alkanet, alkannin (E103), Quinoline Yellow (E104), Sunset Yellow

FCF. range Yellow S (E110); Cochineal/Carminic Acid (E120); and Cochineal/Carminic Acid (E122) have been banned as they have been implicated in causing hypereactivity in humans, especially children and those who suffer from asthma. Others include, for example, amaranth (E123), which has been labeled as a dangerous food color additive, while others including Ponceau 4R/Cochineal Red A (E124); Erythrosine BS (E127); Patent Blue V (E131); and Indigo Carmine/Idigotine (E132), are also associated with increasing hypereactivity and triggering of allergy in certain individuals. Green S/Acid Brilliant Green BS (E142) has been reported to be carcinogenic. Black PN/Brilliant Black BN (E151) and Carbon Black/Vegetable Carbon (Charcoal) (E153) fall in the same category (Magnuson *et al*., 2013).

In the case of preservatives, there are also many that cause hypereactivity and certain disorders; for example, sorbic acid (E200) and its salts, mainly sodium sorbate (E201), potassium sorbate (E202); calcium sorbate (E203); benzoic acid (E210) and its various salts (normally added in dairy products) mainly (E211–E219), *i.e.* Sodium Benzoate; Potassium Benzoate; Calcium Benzoate; Ethyl 4-hydroxybenzoate; Ethyl 4-hydroxybenzoate, Sodium Salt; Propyl 4-hydroxybenzoate; Propyl 4-hydroxybenzoate, Sodium Salt; Methyl 4-hydroxybenzoate and Methyl 4-hydroxybenzoate, and Sodium Salt, respectively. The sorbic and benzoic acids together with their salts are known to cause hyperactivity types of reactions in certain individuals.

The biphenyls and diphenyls (E230), also used as preservatives together with their derivatives, mainly 2-hydroxybiphenyl (E231); sodium biphenyl-2-yl oxide (E232); 2-(thiazol-4-yl) benzimidazole (E233) and hexamine (E239), are known to cause headaches as well as dermatological disorders. Other preservatives that are normally used in meat and meat products (nitrates and nitrites such as potassium nitrate (E249); sodium nitrite (E250); sodium nitrate (E251) and potassium nitrate (saltpetre) (E252)), as well as acetic acid (E260), and a few of its salt derivatives, mainly potassium acetate (E261) and potassium hydrogen diacetate (E262) and also carbon dioxide (E290), also cause problems in the intestines, skin disorders and headaches.

Antioxidants are the food additives that are in most cases safer, with the exception of Butylated Hydroxyanisole (BHA) (E320) and Butylated Hydroxytoluene (BHT) (E321), which are associated with hypereactivity reactions.

However, citric acid (E330) and its salt derivatives such as sodium citrates (E331); potassium citrates (E332) and calcium citrates (E333); tartaric acid (E334) and its salts, mainly sodium tartrate (E335); potassium tartrate (cream of

(E334) and its salts, mainly sodium tartrate (E335); potassium tartrate (cream of Tartar) (E336); potassium sodium tartrate (E337) and orthophosphoric acid (E338), cause constipation, intestinal problems and headaches.

The majority of emulsifiers and stabilizers are known to be safe, except for Tragacanth (E413), which is suspected to cause problems in individuals who suffer from allergies and intolerance reactions. Other food additives, such as Monosodium Glutamate (MSG) (E621), are known to cause skin disorders, headaches and intestinal problems.

Generally, some of the food additives have been declared safe, some have been declared dangerous and therefore banned/forbidden from their inclusion into foods, some are carcinogenic and therefore banned and others are suspicious. Labeling of all the ingredients in terms of their presence, grade and quantity is mandatory and monitored by guidelines and regulations.

Methods and Techniques for the Identification and Authenticity Verification of Food Additives

Different types of food additives have different analytical methods and approaches of analysis, depending on their chemistry, the aim of analysis, and the detection levels required, matrices, *etc.*

Analysis of Food Coloring Agents

Food colors as food additives are normally added to either make up for color loss due to exposure to light, air, moisture and variations in temperature; to enhance naturally occurring colors; or to add color to foods that would otherwise be colorless or colored differently. However, certain food coloring agents have been reported to trigger adverse reactions with respect to allergies, *etc.* Therefore food colors are regulated to ensure that they adhere to safety standards.

There are a diverse number of methods that have been devised to determine coloring agents used in foodstuffs, with the majority of them centering on the determination of numerous water-soluble foodstuffs. The methods include sample preparation methods based on solvent extraction, solid phase extraction, as well as separation and detection using chromatographic-based techniques such as paper chromatography, TLC and HPLC (Dennis *et al*., 1977, 1998; Chen *et al*., 1998; Ren *et al*., 1990; Wu and Zhang, 1992; Zhou and Li, 1990); and spectrophotometric-based methods (Berzas Nevado *et al*., 1998; Capitan *et al*.,

1996, 1988; Garcia Penalver *et al*., 1999; Lau *et al*., 1995; Ni and Gong, 1997; Sayar and Ozdemir, 1998; Valencia *et al*., 2000). Electrochemical-based methods include voltammetric methods (Berzas Nevado *et al*., 1997; Ni and Bai, 1997); and electrical migration driven methods such as capillary electrophoresis (Berzas Nevado *et al*., 1999; Kuo *et al*., 1998; Thompson and Trenerry, 1995; Wang *et al*., 1998), *etc.*

Analysis of Preservatives Used for Foodstuffs

Organic Acids

Organic acids used as food additives play several roles, including influencing taste, flavor, stability and the role of preservation because of their effects on bacteria, whereby non-dissociated or non-ionized organic acids possess the capability to penetrate the bacteria cell wall and thereby disrupt their normal physiologic functioning (Van Immerseel *et al*., 2006). However, the food industry and suppliers are obligated to meet the labeling requirements as enforced by the regulations.

There are numerous methods published for the determination of benzoic acid in foodstuffs. The majority of these methods are separation-based methods and they include gas chromatography (AOAC Official Method 983.16., 2000; Choong *et al*., 1995; De Luca *et al*., 1995; González *et al*., 1998, 1999; Lin *et al*., 1999; Ochiai *et al*., 1996); high pressure liquid chromatography (HPLC) (Andrade *et al*., 1999; AOAC Official Method 994.11, 2000; Castellari *et al*., 1997; Chen *et al*., 2001; Hannisdal, 1992; Heinanen and Barbas, 2001; Kantasubrata and Imamkhasani, 1991; Mihyar *et al*., 1999; Pylypiw and Grether, 2000; Silva *et al*., 2000; Willetts *et al*., 1996); Validation of Enforcement Methods Service (VEMS Method 0290); micellar electrokinetic chromatography (MEKC) (Boyce, 1999); the use of lanthanide-sensitized luminescence (Aguilar-Caballos *et al*., 1999); spectrophotometric (Hamano *et al*., 1997); high performance thin layer chromatography (HPTLC) (Khan *et al*., 1994); and potentiometric (Pezza *et al*., 2001).

Sulfites in Foods and Beverages

The sulfites used in foods and beverages as sulfiting agents encompass a group of compounds that includes sulfur dioxide and sulfite salts such as sodium sulfite, sodium bisulfite and sodium metabisulfite, where they are used as preservative agents to prevent the action of microbes that cause food spoilage as

well as browning reactions in food and beverage products (Wedzicha, 1984). In addition to the added sulfites, many other adduct species are generated because the sulfiting agents tend to undergo many different types of reactions in food/beverage matrices, which generate a variety of derivative species such as sulfite, bisulfite, metabisulfite, *etc.* (Adachi *et al*., 1979; Sullivan *et al*., 1990).

Despite their roles as preservatives in foods, sulfites have been implicated as the cause of allergic reactions as well as some asthmatic responses in certain individuals (Vally *et al*., 2000) and therefore regulations and legislation require that there must be proper labeling of any food or beverage that contains any sulfite above 10 mg/L or mg/kg (US Food and Drug Administration, 1986).

In order to enforce the regulatory compliance, a reliable analytical method with high selectivity and sensitivity needs to be devised in order to monitor low concentrations of sulfites in complex food/beverage matrices.

Methods for the determination of sulfites/sulfur dioxide in foodstuffs include both direct methods such as titrimetric, electrochemical techniques, mainly polarographic-based techniques, electrometric and colorimetric-based methods as well as indirect methods such as those which utilize distillation principles coupled to absorption of the sulfur dioxide in an oxidizing agent, mainly iodine or hydrogen peroxide and the quantitation is normally performed using techniques including volumetric, gravimetric, colorimetric or electrochemical techniques.

Of the many methods that have been devised for the analysis of sulfites in various food/beverage products, an optimized Monier-Williams Method (AOAC Method 990.28) has been used more frequently despite the fact that it is time-consuming, labor-intensive, and has been implicated to produce false positive responses under certain circumstances (AOAC Official Method 990.28, 2000; Kim, 1989). Another method, such as the AOAC Method 990.31, which involves the alkaline extraction of sulfites in foods and beverages followed by separation using ion-exclusion chromatography with direct current amperometric detection, has been reported to generate more accurate results (AOAC Official Method 990.31, 2000; Kim, 1990; Kim and Kim, 1986).

Other methods for the analysis of sulfites in foods and beverages are enzymatic-based methods where sulfite in liquid foods or extracts of solid foods is determined utilizing enzymatic principles which involve the reduction of NADH to nicotinamide adenine dinucleotide, and the decrease in NADH is related to the concentration of sulfite using spectrophotometric techniques (NMKL, 1993).

Another approach in the measurements of sulfites in food involves the application of differential pulse polarography whereby sulfur dioxide is measured from acidified samples, and then determined by differential pulse polarography. Ion exclusion chromatography has been used to measure sulfites in food by measuring sulfur dioxide released from food matrices by direct alkali extraction. Sulfites in foods have also been analyzed using capillary electrophoresis, where anionic forms of the sulfiting adducts are analyzed (Hirata *et al.*, 2000; Trenerry, 1996).

Nitrates and Nitrites in Foods

Nitrates and nitrites are normally added to foods such as cured sandwich meats, bacon, salami or sausages, in order to give them color and also extend their shelf life. However, nitrates and nitrites tend to form nitrosamines in the body, which has the potential to increase the risk of developing gastrointestinal cancer and, in infants, methemoglobinemia (Hord *et al*., 2009). The acceptable daily intake (ADI) for nitrites, expressed as sodium nitrite, is 0.1 mg/kg body weight. For this reason, there are regulations that govern the use of these additives in foods and therefore reliable methods have been devised and some are continually being added for the analysis of these species in food matrices

Methods of analysis for nitrates and nitrites in foods include spectroscopic techniques after enzymatic reduction (Haman *et al*., 1998; Korkmaz *et al*., 1993); ion-exchange chromatography (Bosch *et al*., 1995; Merino *et al*., 2000; Radisavljevic *et al*., 1996); differential pulse voltammetry (Mesaros *et al*., 1998); and capillary electrophoresis (Jimidar *et al*., 1995) among others.

Gallates in Foods

Gallates are artificial food additives that possess antioxidant properties and therefore help stop oxygen molecules from mixing with the oil in food, which would cause the food to go rancid. However, gallates such as propyl gallate, *etc.* have been implicated to have carcinogenic effects, stomach and skin irritability, as well as allergic reactions that impact breathing. They have also been linked to kidney and liver disorders. For these reasons, gallates as food additives are regulated and labeling requirements have been enforced.

There are a number of methods that have been reported for the determination of gallates (propyl, octyl and dodecyl) in foodstuffs and they include HPLC (Aparicio *et al*., 2000; Noguera-Orti *et al*., 2000; Yamada *et al*., 1993); micellar electrokinetic chromatography (Boyce, 1999; Hall *et al*., 1994);

spectrophotometric-based methods (Aguilar-Caballos *et al*., 1997, 2000); voltammetric electrochemical methods (Ni *et al*., 2000); and colorimetric methods (AOAC Official Method 952.09, 2000).

Polysorbate Food Stabilizers and Emulsifiers

Polysorbates are non-ionic emulsifying agents that are normally produced by copolymerizing sorbitan anhydride and ethylene oxide such that a fatty acid is esterified to one terminal hydroxyl group of the polyoxyethylene-oxide side chain. The type of the attached fatty acid used determines the numbers in the names of polysorbates. For example, if monolaurate is used then we have polyoxyethylene sorbitan monolaurate (polysorbate 20); polyoxyethylene sorbitan monooleate (polysorbate 80); polyoxyethylene sorbitan monopalmitate (polysorbate 40); polyoxyethylene sorbitan monostearate (polysorbate 60); and polyoxyethylene sorbitan tristearate (polysorbate 65).

Polysorbate 80 and other food additives that belong to emulsifiers (e.g. emulsifiers carboxymethylcellulose, often referred to as cellulose gum, and polysorbate 80 also known as Tween 80), which are used in processed foods, have been reported to promote inflammatory bowel disease and a cluster of obesity-related diseases known as metabolic syndrome.

Methods of analysis for the determination of polysorbates in foodstuffs include those which are based on gravimetry followed by colorimetry and gas chromatography (Daniels *et al*., 1982; Kato *et al*., 1989; Smullin *et al*., 1971; Tonogai *et al*., 1987) and high performance liquid chromatographic methods (Takeda *et al*., 2001).

Sweeteners in Foods

Sweeteners, such as sucrose acetate isobutyrate, are sometimes used in certain drinks to a certain maximum permitted as stipulated in the appropriate guidelines. Therefore labeling requirements have been enforced to ensure that the values for recommended acceptable daily intakes, toxicological data and limits are listed. Gas chromatographic based methods are normally among those used to analyze certain sweeteners in foods.

Conclusions

There are regulations in place for various additives. However, new ones are being introduced in the market. Adulteration due to food additives and food

improving agents has been gaining popularity among food vendors, manufacturers, food processing industries and business entities. This stems from the whole idea of making economic gains more than for other reasons. Food forensic laboratories ought to have method development schemes in place to continually detect adulteration due to disallowed additives, or new additives which are yet to receive certification.

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11
Application of Molecular Biology Techniques in Food Forensics

Food forensics is a diverse discipline that covers various techniques and methods throughout all major scientific spheres, such as molecular biology, microbiology, physics, biochemistry, chemistry, etc. Molecular biology techniques involve protein and DNA analysis for the identification, fingerprinting, and authenticity testing of food samples or species presented as evidence. Molecular biology methods rely on the presence of rare features of the species' genome or peptide sequence at a specific level of taxonomy that is highly dependent on the level of specificity needed as evidence that can be trusted beyond reasonable doubt for identification.

Molecular science methods are known to have a sensitivity high enough to correctly match the specimen found in the food to the corresponding species, even though the food material has been highly degraded during the multi-stage steps involved in processing or in cases where the sample contains very low quantities of the genetic material. These methods are both qualitative and quantitative, thus attractive for use in food forensics and food forensic-toxicology. This chapter will focus mainly on the usefulness of molecular techniques in the identification of forensic cases for the types of foods that originate from certain groups of animals and plants. Animal groups will include those from edible species of vertebrates, such as selected mammalian livestock (cows, sheep, swine, and goats), endangered mammalian wild animals (rhinoceroses and tigers), aves or birds (chicken, ostrich, and ducks), and actinopterygian (fishes, e.g. tuna, etc.), as well as edible aquatic turtles.

Introduction

Reports on food forensic cases are many and involve foods from a diverse range of species. However, some species or groups of animals have been more studied than others. According to Teletchea *et al.* (2005), the frequency of studies for various vertebrates follow the order, mammals > fishes > birds. In plant crops, only a few species have been reported on.

Molecular Biology Techniques in Food Forensics

Techniques in molecular biology may be grouped based on the information that is required and also the type of genetic processes that need to be investigated. Some of these techniques may involve dissecting the genome into manageable-sized segments for manipulation and sequencing of the DNA, while others require the separation of specific target macromolecules from the mixtures found in the cell. Also, in some cases, genetic analysis may involve the use of tools such as model organisms to substantiate the study and findings. All these methods can be used to qualify the composition and elements of different types of food products and provide the supporting evidence that may be used in forensic cases related to foods.

Molecular biological techniques for species identification and authentication takes advantage of the genetic diversity among organisms for the identification, classification, and authentication of species by estimating species diversity indices. A diversity index represents a measure of species diversity in a population. There are several genetic diversity parameters that may be used for species identification (Begon *et al*., 1996; Magurran, 1988, 2004; Rosenzweig, 1995; Roth *et al*., 1994) and they include the following:

Percentage or Proportion of Polymorphic Loci

This parameter provides a measure of the extent of genetic variation within the population under study. It can be computed as the ratio of the number of polymorphic loci to that of the total number of loci that have been investigated, both polymorphic and monomorphic. There are several measures that can give the magnitude of the proportion of polymorphic loci and they include a measure of heterozygosity as well as homozygosity, which are defined as the percentage of individuals observed in each locus. For example, if it can be assumed that N is the sample size in a chosen population, where Nhet is the total heterozygous individuals and Nhom is the total homozygous individuals, then the observed heterozygosity can be calculated as Nhet/N, while the Nhom/N ratio will be for the observed homozygosity. Another measure of the magnitude/extent of the percentage of polymorphic loci is the nucleotide diversity (denoted by the symbol π), which is actually a measure of genetic variation measured in terms of the variation in the DNA sequences. It represents the average number of nucleotide differences in every site in two DNA sequences being compared from

the population. A measure of nucleotide diversity has the same measure as that of the average measure of heterozygosity worked out over all nucleotide positions and is mathematically a nucleotide diversity that can be calculated, as shown in Equation 11.1:

$$\pi = \sum_{ij} x_i x_j \pi_{ij} = 2 * \sum_{i=2}^{n} \sum_{j=1}^{i-1} x_i x_j \pi_{ij} \quad (11.1)$$

where *xi* is the frequency of the *i*th sequence and d*ij* is the fraction of nucleotides at which sequences *i* and *j* differ.

Generally, it is possible to estimate the measure of nucleotide diversity by either examining the DNA sequences directly, or by making use of molecular marker data.

Shannon Diversity Index (H) and Simpson's Index (D)

The Shannon diversity index (H) enables the characterization of species diversity within a specific community. Shannon and Simpson's indices provide a measure and magnitude related to the abundance and measures of evenness of the species present in a population. Mathematically, the Shannon diversity index (H) is calculated as per Equation 11.2:

$$H' = -\sum p_i \ln p_i \quad (11.2)$$

where *i* denotes the proportion of species *i* and *p* represents the proportion of the total number of species (p_i).

The higher the value of the Shannon index, the more the richness and evenness within the population under study.

On the other hand, Simpson's index (D) has values between zero (0) and one (1), and provides a measure of dominance and is inversely proportional to diversity in the population such that if the magnitude of Simpson's index (D) increases, then diversity in terms of evenness decreases. Simpson's index also provides a measure of the probability that two individuals from an infinitely large population may belong to the same species, and is calculated as shown in Equation 11.3:

$$D = \sum p_i^2 \quad (11.3)$$

where again p_i is the proportion of individuals found in species i.

Jaccard's Index

Jaccard's index, which is important in cases where a comparison of biodiversity levels is required, has to be performed across some geographical sites and is calculated using Equation 11.4:

$$J = \frac{S_c}{S_a + S_b + S_c} \quad (11.4)$$

where S_a and S_b represent the numbers of species unique to samples a and b, respectively, while S_c represents the number of species common to the two samples.

Other parameters for measuring diversity include genetic similarity, F; mathematically, $F = 2m_{xy}/(m_x + m_y)$, where m_y represents the number of gene fragments shared by both species (X and Y), and m_x and m_y represent the gene fragments by X and Y respectively. F takes values between zero (0) and one (1). Another parameter, known as the genetic distance, is worked out mathematically as 1 – F.

Sampling

For forensic investigations related to the origin of wild animal species (tiger, white rhino, and elephant) and even domesticated animals, the types of sample specimen that are normally collected include hair/fur, muscle tissue, bloodstains, bone, ear, skin, urine, and feces. For food products of animal, fish, and bird origin, appropriate tissues or organs (e.g. blood, meat-and-bone-meal, meat-meal, skin, bones, tanned hides, fish gills, fins, cartilages, scales, whole organism for smaller species, *e.g.* sardines, etc.) are taken as sample specimens. These are then processed using various regimes of sample preparation methods utilizing solvent extraction principles of commercially available kits to eliminate unwanted components in the matrix before gene amplification with appropriate primers (e.g. 16S rRNA, cytochrome b, etc.). Table 11.1 summarizes solvent systems used for sample preparation methods and their applications as reported in the literature.

Table 11.1 Preparation of solutions and buffers for molecular analysis

(Nishiguchi *et al*., 2002).

Stock solution	**Preparation**
Ammonium acetate (**Mwt 77.08 g/mol; *****5 M*****)** ***Ammonium sulfate (*****Mwt 132.14 g/mol; *****1 M*****)**	Weigh accurately an appropriate mass of respective salt using an analytical balance and dissolve in small volume of nucleic acid free water in a volumetric flask of a desired volume, and after complete dissolution, make to volume.
5-bromo-4-chloro-3 indolyl-phosphate (BCIP; 50 ppb)	Dissolve BCIP in dimethyl formamide to make a stock concentration of 50 ppb BCIP. This stock solution should be kept in freezer at −20 °C.
Buffers: − **Carlos lysis buffer (100 mL)**	**Mix the following**:- Tris (0.1 M, pH 9.5) – by mixing Tris base (1210 mg) dissolved 700 mL of nucleic acid free double distilled water with hydrochloric acid (9.5 mL), 0.2 M ethylenediamine tetraacetic acid (EDTA, 760 mg) and 1.4 M sodium chloride salt (8180 mg). 2% Cetyl trimethyl ammonium bromide (CTAB), 1% polyethylene glycol (PEG-8000 or 6000 grade, 1000 mg) with stirring for 12 hr. Make up to volume (100 mL in a volumetric flask). NB: β-mercaptoethanol (2 µl/mL) shouod be added to the buffer prior to use. Mix 1 mM Tris (pH 8.0), 50 MEDTA and 5% (w/v) Chelex-100 resin.
− **Chelex-100 buffer**	Mix 0.1 M Tris-HCl (pH 8.0), 1.4 M sodium chloride, 0.02 M EDTA, 2% (w/v) CTAB, 0.1% (w/v) polyvinylpolypyrrolidine, 0.2% (v/v) β-mercaptoethanol (for buffer solution which is to be used immediately otherwise, do not add in cases where the buffer is not used immediately).
− **Cetyl trimethyl ammonium bromide (CTAB) extraction buffer**	Mix sodium chloride (0.1 M), Tris (pH 8.0, 0.1 M), and EDTA (0.025 M).

– **Digestion buffers (aliquots of 10 mL)**	
– **Dimethylsulfoxide (DMSO) buffer**	Use HPLC grade DMSO. Dispense in smaller volumes (e.g., 1 mL) and store them in a freezer (-20 °C). NB: DMSO buffer is used once.
– **Lysis buffer**	Mix EDTA (0.1 M), Tris-HCl (0.01 M, pH 7.5).
– **Liftons buffer**	Mix EDTA (0.1 M), Tris-HCl (0.025 M, pH 7.5) and sodium dodecyl sulfate (SDS) (1%).
Guanidium isothiocyanate (GITC) homogenization buffer	– **Prepare the following**: • 4 M of guanidinium isothiocyanate (Mwt. 118.16); 0.1 M Tris-HC1 (pH 7.5) and 1% β-mercaptoethanol by dissolving 50 g of guanidinium isothiocyanate in 10 mL of 0.1 M Tris-HC1 (pH 7.5) and nucleic acid free water in 100 mL volumetric flask. Heat at 65 °C to facilitate dissolution then filter the solution using Whatman No. 1 or by using a Nalgene Filtration Unit. • Alternatively, this solution may be prepared without any need for filtration by using 25 g of guanidinium isothiocyanate in 5 mL of Tris-HCl, and nucleic acid free water in a sterile 50 mL volumetric flask. NB: – *β-mercaptoethanol should be added to the final concentration of 1% (0.14 M) just before use.*
– **Maleic acid buffer (MAB)**	– Prepare MAB by mixing 0.1 M maleic acid and 0.15 M sodium chloride, pH 7.5.
[3-(N-morpholino)propanesulfonic	– Prepare MOPS by mixing MOPS (0.1 M, pH 7), sodium chloride (0.5 M) and Tween-

acid] – (MOPS) buffer	20 (0.1%).
Phosphate-buffered saline solution (10x PBS)	10xPBS is prepared by mixing sodium chloride (80 g), potassium chloride (2 g), of sodium phosphate (14.4 g) and potassium phosphate (2.4 g) and dissolve in 800 mL of nucleic acid free water, adjusting the pH to 7.4 with HC1 and make to volume (1 litre), autoclave and the solution is stored at room temperature.
Phosphate buffered saline, Triton X-100 (PBT)	PBT is a solution of 0.1% of Tween-20 in 1xPBS buffer.
10x PCR buffer	Mix:- molecular biology grade Tris base (8.116 g), 0.610 g of magnesium chloride (0.610 g) and ammonium sulfate (2.227 g) in HC1 (90 mL), stir to facilitate dissolution, make to volume (100 mL sterilized volumetric flask with H). Autoclave and dispense volumes of 1 mL eppendorf tubes.
PCR buffer w/non-ionic detergents	This is prepared by mixing potassium chloride (0.05 M), Tris-HCl (0.01 M, pH 8.3), magnesium chloride (0.0025 M), gelatin (0.1 ppb, i.e 0.1 mg/mL), NP-40 (0.45%) and Tween-20 (0.45%). *NB: This buffer cannot be filtered or autoclaved, it should therefore be prepared in aliquots of smaller volumes, eg.10 mL in sterile falcon tubes using sterile reagents.* *For example, prepare using smaller volumes and weights, such as potassium chloride (0.5 mL, 1 M), Tris-HC1(0.1 mL, 1 M, pH 8.3), magnesium chloride (25 µL, 1 M), gelatin (1 mg), NP-40 (450 µL), Tween-20 (450 µL) then make to volume (10 mL) using nucleic acid free water.*
PK buffer	PK buffer is prepared by mixing Tris-HC1 (1 M, 10 mL) and EDTA (0.5 M, 2 mL) and make to volume (200 mL) using nucleic acid free water.

RNA preparation binding buffer	Mix stock solutions of nucleic acid free sodium chloride (0.5 M), TrisCl (10 mM, pH 7.4), EDTA (1 mM, pH 8.0), autoclave, cool to 65 °C then add 0.1% SDS from a 10% stock solution.
RNA preparation elution buffer	Mix nucleic acid free stock solutions of Tris-HC1 (10 mM, pH 7.4 and EDTA (0.1 mM, pH 8.0), autoclave the solution and cool to 65 °C.
RNA preparation washing buffer	Mix nucleic acid free stock solutions of Tris-HC1 (10 mM, pH 7.4) and sodium chloride (100 mM) cool the mixture to 65 °C. Then add sodium lauryl sulfate sarcosinate (also called SDS) from a 10% stock solution and EDTA (1 mM, pH 8.0).
Sarkosyl buffer (5%, 500 mL)	Prepare by dissolving sarkosyl (25 g) in sodium chloride (15 mL, 5 M), Tris-HC1 (25 mL, 1 M, pH 8.0), EDTA (15 mL, 0.5 M) and 400 mL of H then make to volume (500 mL sterile volumetric flask) using nucleic acid free water. *NB: Do not refrigerate or autoclave this solution*.
SDS buffer (4%)	Dissolve sodium dodecyl sulfate (20 g) in sodium chloride (30 mL, 5 M), Tris-HCI (25 mL, 1 M, pH 8.0), EDTA (100 mL, 0.5 M) and nucleic acid free water (300 mL).
Sodium Chloride-Tris-EDTA buffer (STE)	STE is prepared by mixing sodium chloride (0.1 M), Tris-HCI (10 mM, pH 8.0) and EDTA (1 mM, pH 8.0).
50x Tris-Acetate Buffer (TAE)	50x TAE is prepared by dissolving Tris base (242 g) in glacial acetic acid (57.1 mL) and EDTA (100 mL, 0.5 M pH 8.0) in nucleic acid free water and make up volume (1 litre). *NB 1: The 50x TAE is a highly concentrated stock solution.* *NB 2: Dilute this solution to make lx TAE (0.04 M Tris-acetate, 0.001 M EDTA) and use it as your working solution*.

5x Tris-Borate/EDTA buffer (TBE)	Dissolve 54 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 M EDTA (pH 8.0) in H2O up to 1 liter. Stir until dissolved. The 5x TBE is the concentrated stock solution. Use 0.5x TBE (0.045 M Tris borate, 0.001 M EDTA) as electrophoresis buffer. *NOTE: A precipitate forms when concentrated solutions of TBE are stored for long periods of time. Discard any batches that develop a precipitate.* *NOTE: 10x TBE buffer is commercially available, and it constitutes a good solution for laboratories not using much TBE buffer, or for laboratories with high budgets.*
TBST solution	135 mM NaCl 2.7 mM KCl 25 mM Tris HC1 (pH 7.5) 0.1% Tween-20 2 mM levamisole (add on day of use).
TE Buffer Solution (pH 7.4)	10 mM Tris-HCl, pH 7.4 1 mM EDTA, pH 8.0
TE Buffer Solution (pH 7.60)	10 mM Tris-HC1, pH 7.6 1 mM EDTA, pH 8.0
TE Buffer Solution (pH 8.0)	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0
CTAB + NaCl solution (10 % : 700 mM)	Mix CTAB (appropriate weight, e.g 10 g for 100 mL stock volume) and 700 mM sodium chloride (appropriate volume), heat with stirring to dissolve, make it to volume (eg., 100 mL, 1000 mL etc).
H-treated diethyl pyrocarbonate (DEPC)	Dissolve 0.5 % DEPC in H, then homogenize by thoroughly stirring in the fume cabinet then allow the solution to sit for sometime then autoclave. NB: Composition for HM include calcium chloride (1 mM), sodium bicarbonate (1.5 mM), magnesium chloride (0.1 mM), magnesium

	sulfate (0.08 mM) and potassium nitrate (0.03 mM) all dissolved in Arrohead spring water.
Dodecyltrimehylammonium bromide (DTAB)	Mix 8% DTAB with sodium chloride (0.015 mM) and Tris (100 mMn pH 8.8) and EDTA (50 mM).
Ethylene diamine tetraacetic acid (EDTA)	Mix disodium ethyl anediaminetetraacetate (168.1 g) with H (800 mL) with thorough stirring, adjust to pH 8 using NaOH (or instead add ~ 20 g NaOH pellets) in order to enhance complete dissolution of EDTA, then autoclave the solution.
Ethidium bromide (10 ppb)	Mix ethidium bromide (0.2 g) and 20 mL of H (refer above), stir to dissolve the dye and then store at room temperature in the dark room wrapped in an aluminium foil.
Glycine (2 ppb)	Dissolve 2 mg in 1 mL PBT (phosphate buffered saline, Triton X-100) then store in a freezer at −20 °C. NB 1: PBT is prepared by dissolving 0.1% Tween-20 surfactant in PBS (phosphate buffered saline solution). NB: 2: PBS is prepared by dissolving sodium chloride (80 g), potassium chloride (2 g), sodium phosphate (14.4 g), and potassium phosphate (2.4 g) in a sufficient amount of nucleic acid free water (e.g., 800 mL) then ajust the pH of the solution to 7.4 using HC1. Make to volume using nucleic acid free water (in a 1 litre sterile volumetric flask). Autoclave the solution and store the solution at room temperature.
Guanidium thicyanate (5 M)	Mix guanidium thiocyanate (59 g) with nucleic acid free water (in a sterile 100 mL). Heat at 65 °C to dissolve the salt. Filter the solution using Whatman (No 1, or using Nalgene filtration unit).
Lithium chloride (4 M)	Prepare by dissolving an appropriate amount of lithium chloride (Mwt, 42.39 g/mol), e.g 169.56

	g in an appropriate volume of nucleic acid free water then make it to 1litre (steriled volumetric flask).
Levamisole (1 M)	Preapare this solution by dissolving levamisole (0.06 g) in 0.25 mL of nucleic acid free water. This solution should be prepared only when is needed to be used (should not be stored!).
Magnesium chloride (1 M)	Prepare by dissolving an appropriate weight of magnesium chloride hexahydrate (Mwt 203.31) in an appropriate volume of nucleic acid free water and make it to volume (eg 100 mL, volumetric flask, 1 L, etc). For example use 203.31 g of MagCl2 and 800 mL of water the make it to volume (1 L) with water. NB: Magnesium chloride is highly hygroscopic, do not store and also do not open the bottles for a long time.
4-nitroblue tetrazolium chloride (NBT)	Prepare NBT solution by dissolving 75 mg/mL dimethyl formamide (70%). Store in a freezer at −20 °C.
NTMT solution	Prepare NTMT by mixing sodium chloride (0.1 M); Tris-HCl (0.1 M, pH 9.5) magnesium chloride (0.05 M) and Tween-20 (0.1%). When using NTMT solution add 0.002 M levamisole at that time.
Paraformaldehyde (4 %)	This solution prepared by dissolving paraformaldehyde (10 g) in DEPC-treated H (200 mL), heated at 65 °C in a fume hood and then cooled on ice. Then using NaOH (5−10 µl) correct the pH to 7.5. Add 10x PBS (25 mL) and make volume up to 250 mL with DEPC treated nucleic acid free water then store the solution at −20 °C.
phenol:chlorophora:isoamyl alcohol (PCI)	The PCI solution is prepared by mixing phenol, chloroform, and isoamyl alcohol, at a ratio of 25:24:1. at a pH of 7.5−8.0. NB: For molecular analyses, a commercial PCI

	may be better.
Potassium acetate (5 M)	Prepare by dissolving potassium acetate (49.1 g, Mwt 98.15) in 90 mL of nucleic acid free water then adjust the pH to 7.5 using 2 M acetic acid. Make to volume (100 mL) and the solution can be stored in a freezer at −20 °C.
Proteinase K (20 mg/mL)	Prepare proteinase K by mixing Tris (pH 7.8), EDTA (0.005 M) and 0.5% sodium dodecyl sulfate (SDS), incubate the solution at 37−56 °C and then store Proteinase K at −20 °C. NB: Proteinase K should be used at a concentration of 50−60 µg/mL with a reaction buffer containing 0.01 M of the prepared solution (Tris + EDTA + SDS).
10% SDS (100 mL)	Prepare by dissolving sodium dodecyl sulfate (10 g) in nucleic acid free water and then make it to volume (in 100 mL sterile volumetric flasks).
20x SSC (pH 4.5)	Prepared by mixing sodium chloride (3 M) and sodium citrate (0.3 M).
Silica solution	Prepare by dissolving silica dioxide (4.8 g) in nucleic acid free water (40 mL) in a polypropylene tube of appropriate volume, then homogenize by agitating the tube with contents. Allow it to stand 24 hrs then dispense 35 mL accurately (using a pipette), add 5 mL to make a volume of 40 mL with nucleic acid free water (or distilled water), agitate and allow this solution to stand for 5 hrs. Pipette 36 mL and add to it 48 mL of HCl. Dispense smaller volumes of 1.5 mls and store in the dark room.
3 M sodium acetate (NaOAc) (pH 5.2 and 7.0)	Dissolve 40.82 g of sodium acetate trihydrate (CH3COONa•3H2O; M.W. 136.08) in 80 mL of H_2O. Adjust the pH to 5.2 with glacial acetic acid or adjust the pH to 7.0 with dilute acetic acid. Adjust the volume to 100 mL with H_2O.

	Sterilize by autoclaving.
2 M sodium acetate (pH 4.0)	Dissolve 27.22 g of sodium acetate trihydrate (CH3COONa•3H2O; M.W. 136.08) in 80 mL of H_2O. Adjust the pH to 4.0 with glacial acetic acid. Adjust the volume to 100 mL with H_2O. Sterilize by autoclaving.
• **5 M sodium chloride**	Dissolve 292.2 g of sodium chloride (NaC1; M.W. 58.44) in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Sterilize by autoclaving.
• **Solution I**	50 mM glucose 25 mM Tris-HC1 (pH 8.0) 10 mM EDTA (pH 8.0) Solution I can be prepared in batches of 100 mL. Autoclave for 15 minutes on liquid cycle. Store at 4 °C.
10 mM Tris-HC1 (pH 7.4, 7.6 and 8.0)	Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust pH to the desired value by adding concentrated HCl. pH 7.4 add 70 ml. pH 7.6 add 60 ml. pH 8.0 add 42 ml. Other pHs desired can be obtained by titrating the HCl. Adjust the final volume of the solution to 1000 ml with H_2O.

Preparation of the Genetic Materials

The preparation of genetic materials involves three main steps. The first step involves the preparations of buffers and solutions needed in the molecular analyses, while the second step deals with the extraction of the nucleic acids; the third step involve the purification of the genetic material to ensure that it is of high quality. Normally, when the nucleic acid material has been extracted and purified, it is re-suspended in either distilled water or TE buffer solution (1 µg nucleic acid/µL).

Preparation of Common Solutions for Molecular Analysis in Food Forensics

Sample preparation also involves the extraction and purification of the gene (DNA fragment) for molecular identification and fingerprinting purposes. The DNA extraction and molecular analysis requires the use of some solutions, which can be prepared in the laboratory. Some of the most common solutions are displayed in Table 11.1.

Molecular Methods for Genetic Materials Extraction

Molecular methods for genetic materials extraction can be carried out by using commercially available kits. These kits can be obtained from various vendors/companies that offer various commercial kits such as QIAamp® DNA Mini Kit #51304, #51306; DNeasy® Tissue Kit #69504, #69506, etc.; Nucleospin® (http://www.clonetech.com), Isoquick® (Microprobe Corp.); and Bio 101® (http://www.bio101.com). Commercial nucleic acid extraction kits contain reagents and solutions required for nucleic acid extractions. For example, commercially available kits may include enzymes such as proteinase K, extracting buffers such as non-phenol/chloroform buffers, and purification columns such as silica-gel membrane spin-column, as well as extraction protocols that have been simplified.

DNA Marker Fragments

There are several targeted DNA/genetic markers that are normally used for species identification, as well as other food forensic-related analytical applications. They include cytochrome (Cyt) b gene (mainly mitochondrial cyt b genes), 16S rRNA, 12S rRNA, 5S rRNA, D-LOOP, ATPase, COI, ND3/ND4, ATPase 8, NADH2, ND4, *etc.*

Sample Preparation Methods Suitable for Molecular Biology Techniques

These are summarized in Tables 11.2 and 11.3.

Table 11.2 Sample preparation methods for the molecular forensic analysis of freshwater and marine animal food products (Taggart *et al*., 1992).

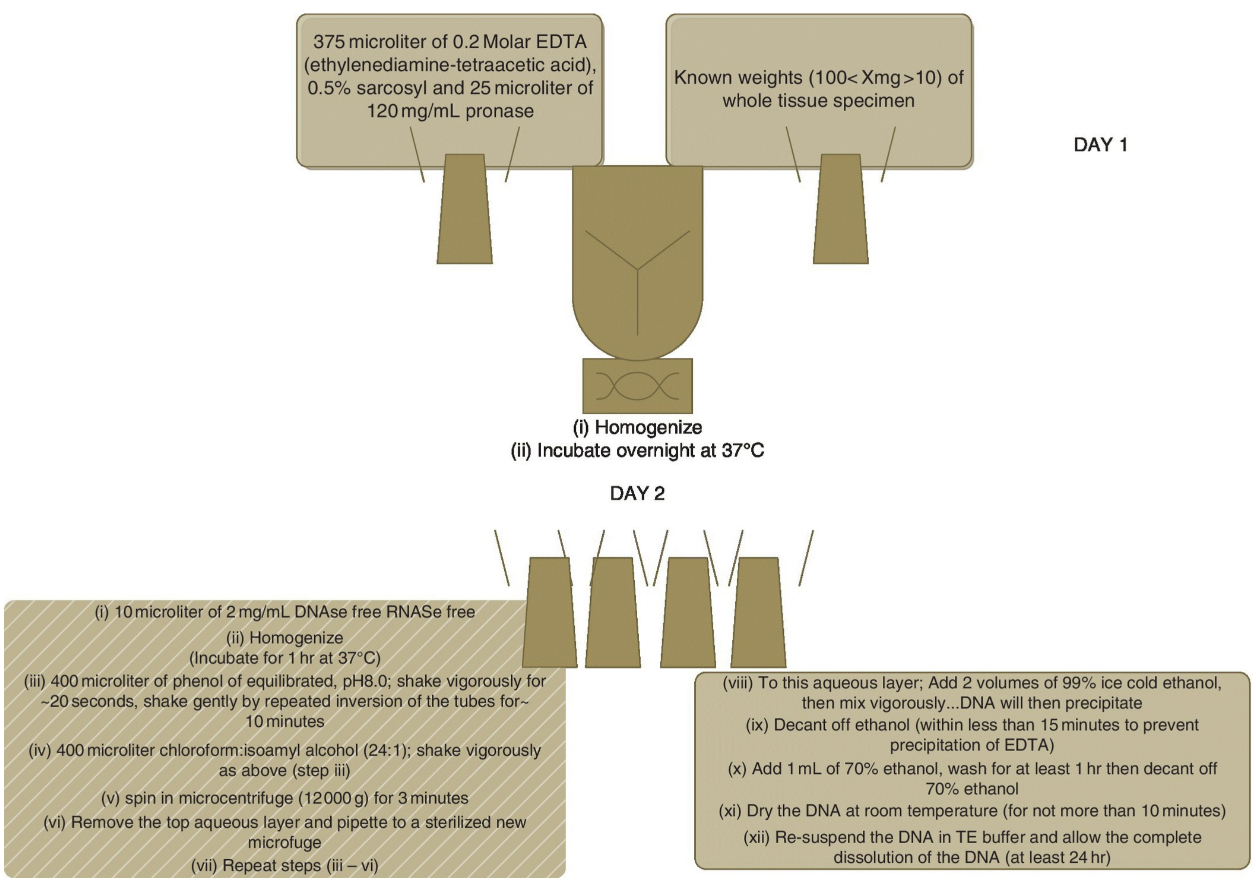
Solvent system	Targeted DNA marker fragment and species
Chloroform, methanol, water	− Cyt b gene (171 bp); − specimen from canned tuna fish (Terol *et al*., 2002)
Chloroform, phenol, isoamyl alcohol + Molecular biology grade Chelex resin	− Cyt b gene (158 bp) − specimen from canned sardines (Jérôme *et al*., 2003)
Phenol-Chloroform	• – Cyt b gene (875–876 bp) • – Sea turtle (muscle, blood, eggs, skin) (Moore *et al*., 2003)
Phenol-Chloroform	• – Cyt b gene (155–188 bp) • – Shark (soup, dried fin, cartilage pills) (Yan *et al*., 2005)

Table 11.3 Nucleic acid extraction methods and the target specimens for food forensic molecular analyses.

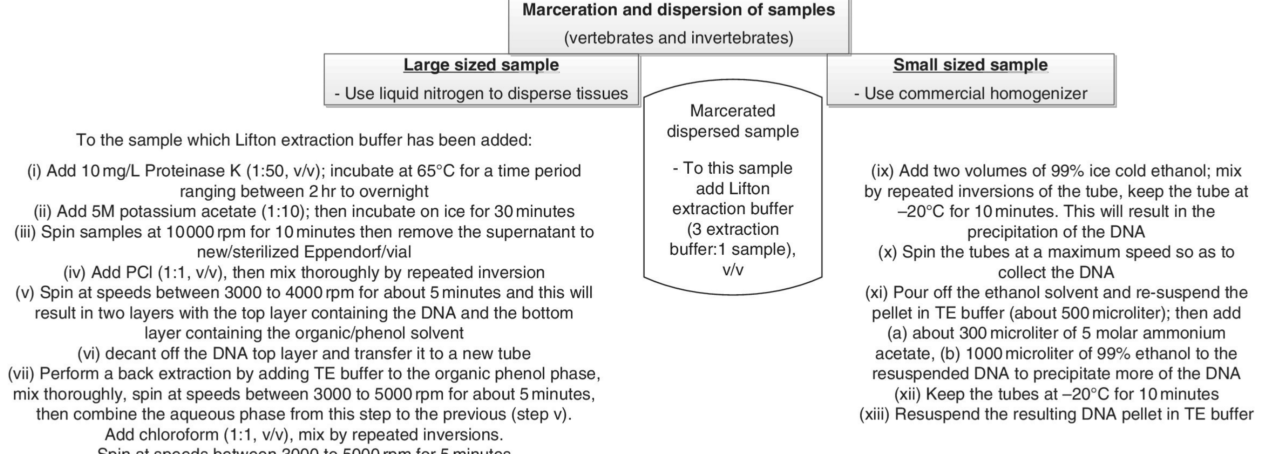
Forensic specimen, target species and fragment	Recommended Extraction protocol
Dried/salted/unfrozen strips of whales, with the target fragment being the D-loop	Miscellaneous
Embedded human tissues, which are formalin/paraffin	Chelex

Embedded human tissues, which are formalin/paraffin fixed, with the target fragment being microsatellites, amelogenin	Chelex
Sea turtle muscle, blood, eggs, and skin, with the Cyt b being the target fragment	Phenol-chloroform
Shark soup, dried fins, and cartilage gills, with Cyt b being the target fragment	Phenol-chloroform
Tuna canned products, with Cyt b being the target fragment	Chloroform, methanol, water
Sardines canned products, with Cyt b being the targeted fragment	Chelex, phenol, chloroform and isoamyl alcohol
Beef, mutton, pork, chicken meat/bone meal, with tRNA, ATPase being the targeted fragments	Guanidium thiocyanate

Schemes 11.1 to 11.8 also summarize sample preparation methods.



Scheme 11.1 An example of the phenol-based protocol for DNA extraction.



Scheme 11.2 Protocol for crude total cellular miniprep.

≥100 microliter/100 mg of tissue specimen

- Grinding buffer (200 microliter)
- Tissue douncing (using a dounce tissue grinder)

- Mini lysis solution (200 microliter)
- Incubate at 65°C for 30 minutes

- 5M potassium acetate; mix thoroughly by repeated inversions
- Incubate on ice for 30 minutes

- Spin at maximum speed
- Transfer supernatant to new/ autoclaved tubes

- Add ice-cold 95–99% (1 mL)
- Mix thoroughly by repeated inversions
- Allow tubes to stand at room temperature for 30 minutes

- Spin at maximum speed to precipitate the DNA
- Remove the supernatant part which contains the DNA

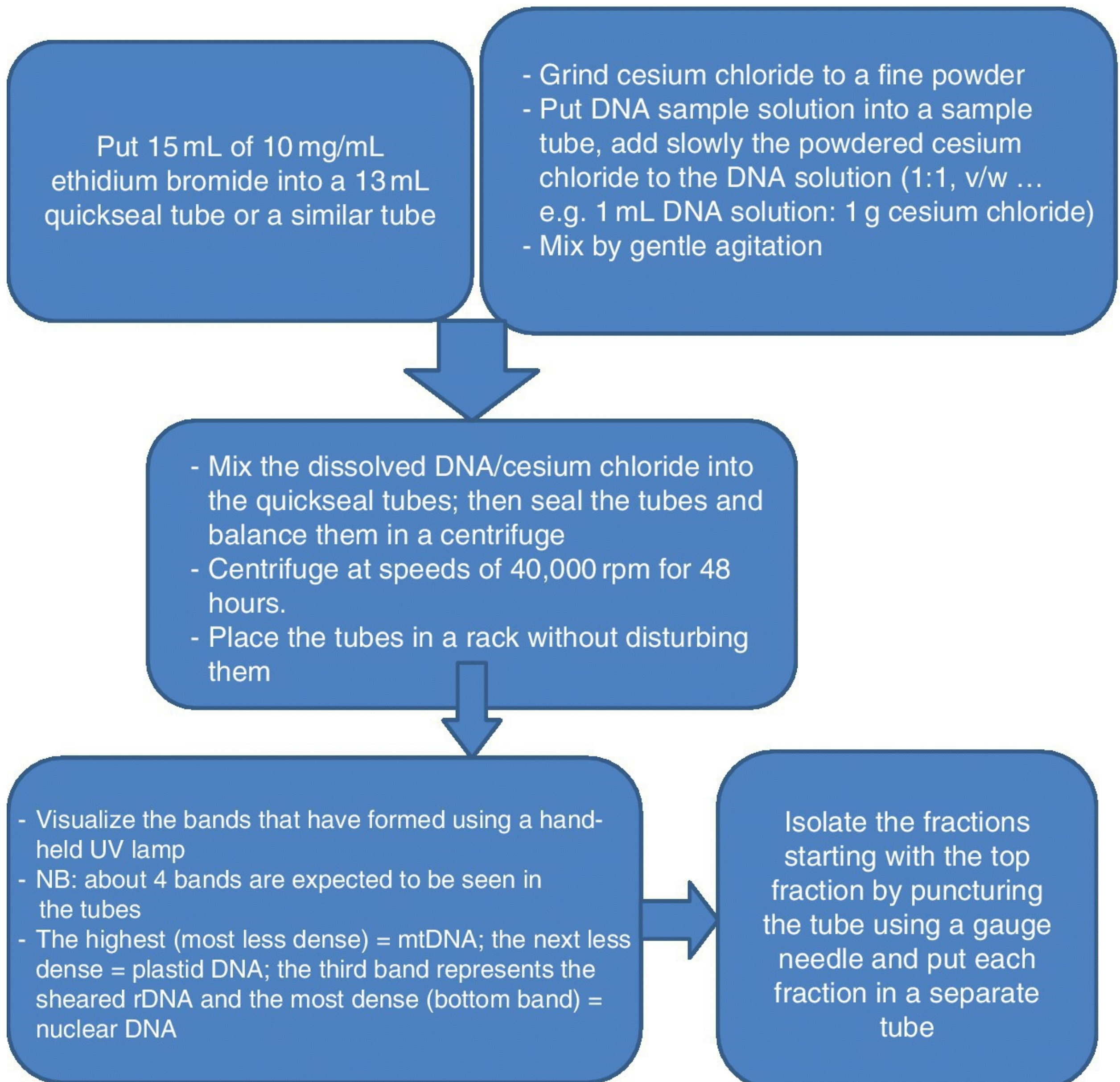
Ammonium acetate (100 microliter, 0.5 Molar) to resuspend the DNA pellet

- 70% ethanol (400 microliter)
- Mix thoroughly by repeated inversions
- Keep the tubes at room temperature for about 10 minutes

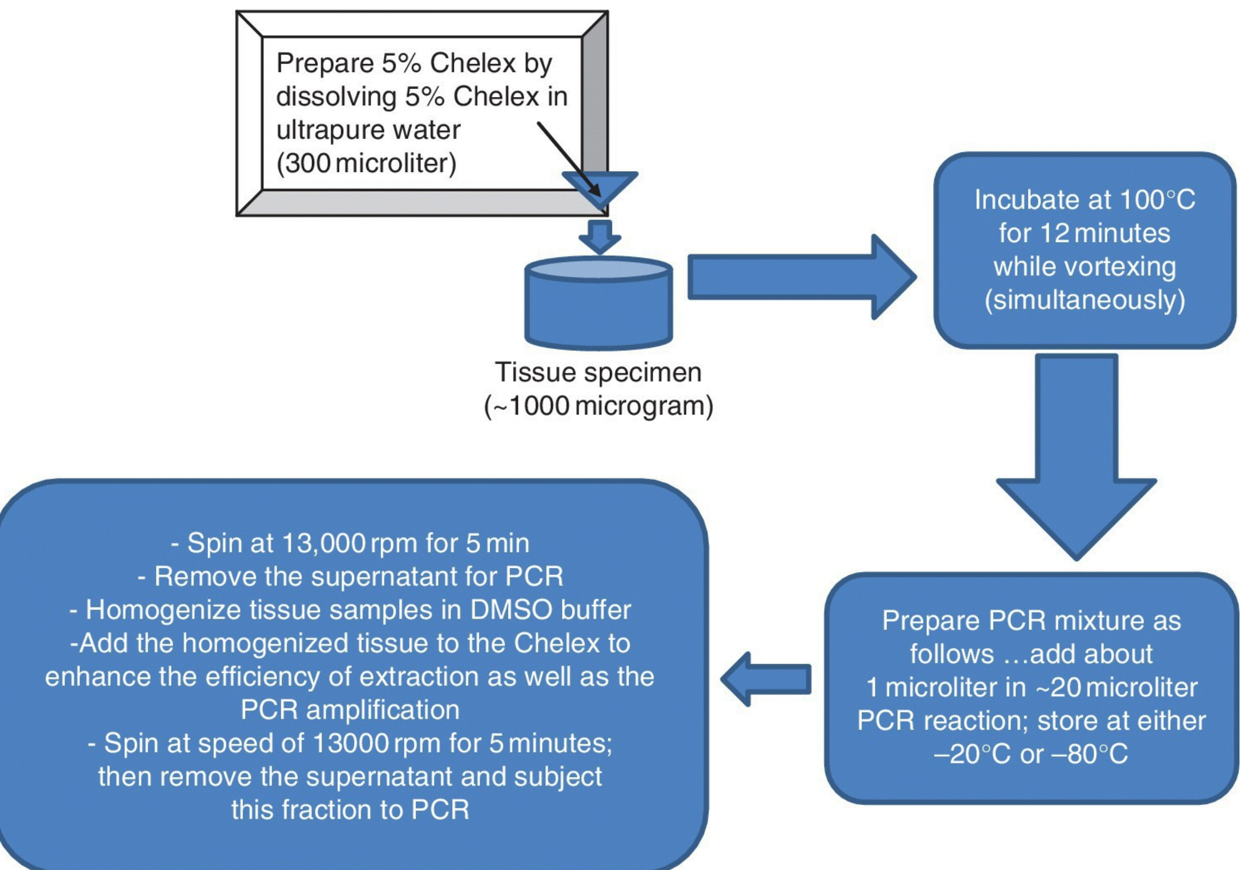
Tune the microfuge at maximum speed for 10 minutes to pellet the DNA

- Decant off the ethanol
- Air dry the DNA pellet for 1 hour
- Re-suspend the DNA pellet in 100 microliter of TE buffer

Scheme 11.3 An example of a cesium chloride gradient protocol for the separation of nuclear DNA and organellar DNA.



Scheme 11.4 The use of Chelex as chelating resins for vertebrate DNA extraction.



Scheme 11.5 Formalin-based procedure for the isolation of DNA from museum-preserved specimens.

Tissue specimen should be put in TE buffer first for an appropriate length of time to remove fixative material depending on which fixative was used… e.g. formalin directly or from ethanol preservation after formalin fixation

TE Buffer

Add 0.5 mL of STE buffer which contains 0.2% SDS; 250microliters of 10 molar ammonium acetate to each of the sample specimens in sample tubes/eppendorfs

Heat the sample in tubes using a heating block set at 55°C

Put tiny tissue sample specimen in each tube; use sterilized kontes (teflon eppendorf grinder) to grind it heat at 55°C for 1 hour

Centrifuge sample tubes for 5 minutes at 14,000 rpm to precipitate proteins and pellet debris. Then put the supernatant in a new vial and add 2 volumes of 99% ice-cold ethanol then mix by repeated inversions

-Incubate sample tubes overnight at –20°C and some at –80°C for an hour to precipitate nucleic acid
-Centrifuge sample tubes for 15 minutes at 4°C, 14,000 rpm
-Take out the supernatant, to this add 70% ethanol (1:1, v/v)

-Centrifuge at 4°C for 10 minutes at 14,000 rpm
Remove the ethanol then dry tubes
-Re-suspend the pellet in TE (50 microliter)
-Perform PCR as desired.

Add 100 microlitre of Triton X-100 to the tissue, then cover sample vials with ice (bury in ice) for about 5 minutes

Pellet the DNA and collect it from the supernatant after spinning sample vials/tubes at 2500 rpm. NB:- repeat spinning several times and pool the DNA material

- Add PCI (phenol-chloroform-isoamyl alcohol) solution to the DNA sample (1:1, v/v) then homogenize by repeated inversion of the sample tubes, then spin the sample tubes at speeds between 3000 to 5000 rpm for about 5 minutes and collect the upper layer which contains the DNA

- In case of a rare specimen; Add TE buffer:phenol (1:1, v/v) in order to do a back phenol phase extraction. Then mix by repeated inversions of the tubes and centrifuge at speeds of between 3000 to 5000 rpm, then pool the aqueous phases containing DNA from this and previous step
- To this aqueous phase, add chloroform (1:1, v/v) then mix and spin at same speeds (between 3000 to 5000 rpm)
- Collect the supernatant upper layer; add two volumes of ice-cold 99% ethanol and keep the tubes at 20°C for 10 minutes to precipitate the DNA
- Spin at maximum speed of micro centrifuge to collect the DNA; remove the ethanol and re-suspend the DNA pellet in 500 microliter TE buffer solution
- Add 300 microliters of 5 Molar ammonium acetate and 99% ethanol to the re-suspended DNA to reprecipitate the nucleic acid
- Place the solution at –20°C for 10 minutes and re-suspend the DNA pellet in TE buffer

Scheme 11.6 Procedures for the extraction of enriched cytoplasmic nucleic acid from animals.

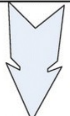
- Cut small portions of about 5 mm of the base of feather specimen (calamus)

- Prepare 5% Chelex in a 1 milliliter tube, and into it, add 250 microliter of 5% Chelex

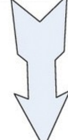
- Incubate the tubes at 100°C for 15 minutes, then vortex twice for about 15 seconds while incubating and then cool the sample solution to room temperature before spinning at maximum for 30 seconds

Scheme 11.7 Protocol for DNA extraction from bird's tissues and feathers.

Sample specimen
(an egg or tissue (fish/caviar))

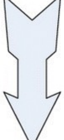


To the specimen, add
(i) Lifton buffer (200 μL); (ii) 20 mg/mL proteinase K solution (20 μL). Crush the egg with the aid of a pipette tip.
- Incubate at temperatures 55–65°C for one hour or more (to maximize the yield) while gentle rocking during the incubation. The yield of the extraction increases with the amount of incubation time.
NB: For caviar... the incubation should be longer, e.g., overnight.
NB: The tissue has to dissolve 100%, otherwise the incubation has to continue.



- Add 300 μL of phenol and 25 μL 5M potassium acetate, pH 4.8.
- Shake vigorously.
- Incubate at temperatures 55–65°C for 10 minutes while rocking.
- Spin 14,000 rpm for 5 min to separate the various phases.
NB: The less dense aqueous phase containing the nucleic acid material will be at the top, while the interface (whitish color) will contain denatured proteins and carbohydrates.
- Decant out the less dense top layer (aqueous phase) **without including the interface layer** and transfer to new tube but keep the remaining solution.

- Add chloroform (300 microliters), mix gently, incubate, and spin at 14,000 rpm for 5 minutes to separate the solution into phases.
- Decant the less dense top layer (aqueous phase) and transfer to a new tube.
- NB: **remember not to include the interface layer!!**
- Add 1 mL of 100% ethanol to the aqueous phase, mix by repeated inversions of the tube, incubate at –20°C for 30 min or more (NB: alternatively the tubes can be incubated overnight at –20°C in order to maximize the nucleic acid yield).



Spin the tubes at 14,000 rpm for 5 min, then decant out the supernatant, without losing the pellet (NB: the DNA pellet may not be visible).
- Re-suspend the DNA pellet in 200 μL of RNAse free, sterile water.
- Add (i) 500 μL of 100% ethanol and (ii) 25 μL of 7.5 M ammonium acetate and incubate the tubes at –20°C for 20 min.
- Spin the tubes at speeds of 14,000 rpm for 5 minutes, then decant off the supernatant, invert the tube, then dry completely before re-suspending the DNA pellet in 50 μL sterile RNAse free water.
- Dilute the DNA material obtained such that 1 μL of a 1:100 dilution to 25 μL or 50 μL total PCR reaction (this should only be done in cases of a high quality DNA).

Scheme 11.8 DNA extraction from fish tissues.

This protocol can take at least 3 hours and is suitable in cases where large sample size from individual samples need to be investigated for either systematic or population genetic investigations.

This protocol can take up to 72 hours. The other steps for obtaining high molecular weight nucleic acids using phenol-chloroform-based approaches have been omitted. If they are to be included, the time taken for the procedures will be even longer.

This procedure can take about 15 min. The chelating resins are known to denature proteins as well as remove that tend to be inhibitory, which known contaminants are. The procedures can also work well with different types of tissue matrices, including blood, liver, and muscle.

This procedure can take up to 24 hours. The specimen (a known amount), can either be minced meat tissue, ground meat tissue, powdered tissue, or dounced tissue and then a known volume of homogenized buffer is added. The preceding procedures are outlined in the scheme below.

This procedure is suitable for a fish specimen, including normal fish tissue, fin snips, and eggs.

The procedure can take up to 3 hours

Requirements:

- ultraviolet hood;
- sterilized razor blade;
- negative extraction controls should be included;
- buffer ATL, AL, AW1, AW2, AE (included in the kit); and
- proteinase K (included in the kit).

DNA Extraction Procedure

- a known amount of avian tissue specimen or feather is placed in a sterilized vial and then ATL buffer is added together with proteinase K (the ratio of these can be roughly 9:1, ATL Buffer:Proteinase K);
- the set-up is then incubated by either shaking or rotating in an incubator at 55°C until complete dissolution of the sample is achieved. **NB**: Depending on the situation, the Buffer ATL and Proteinase K may be scaled up, but with

proportional ratios without violating the original balance of the two;

- after complete dissolution, the setup can again be incubated at 65°C for about 15 minutes and 95–100% ethanol added to it with vortexing to thoroughly mix and homogenize before incubation at 4°C for 60 minutes; then spin the sample that is placed in a spin column at 8000 rpm for 1 minute and decant the filtrate;
- add AWI buffer (~500 microliter) and centrifuge at 8000 rpm for 1 minute and again decant the filtrate;
- repeat this step using Buffer AW2 and centrifuge at 8000 rpm for about 3 minutes, then discard the filtrate. Introduce preheated (70°C) Buffer AE (~50 microliter) into the sample contained in the spin column and incubate at room temperature for about 45 minutes, centrifuge at 8000 rpm for 3 minutes;
- store at 4°C if the nucleic acid is to be used immediately, or at –20°C if to be used later.

Sample Preparation Methods

In most cases, sample preparation is essential in the majority of analytical and molecular procedures. In isolation of microorganisms from cultures, normally culture dependent methods are used in order to obtain pure cultures of the same species before the identification using phenotypic or genotypic techniques. Some of these sample preparation methods for microbial isolation involve dilution in media such as isotonic buffers, for example phosphate-buffered saline (PBS), homogenization, and then plating in either adequate selective or differential culture media.

There are also culture independent methods that do not require any pre-microbial isolation procedures, because the sample/specimen matrix containing the microbial species under investigation will form the sample. The genomic DNA of the microorganisms present in the sample can be extracted using commercially available kits.

Molecular Markers in Food Forensics

Molecular markers refer to the genomic fragments resulting from PCR amplification of random segments of the DNA using a single primer of the target

nucleotide sequence. Molecular markers in food forensics play crucial roles in a number of areas, for example, in genetic fingerprinting of both plant and animal varieties, determination of similarities among various varieties, mapping of plant and animal genomes, as well as in the ascertaining of phylogeny among species. Molecular markers enable species and/or their products to be compared in a number of molecular techniques such as through the use of restriction fragments techniques, techniques that rely on the identification of isoenzymes (protein/gel electrophoresis), as well as those that utilize products of the polymerase chain reaction (PCR).

General PCR Procedure

In performing PCR analyses, the PCR mixture is normally prepared to contain an enzyme (PCR polymerase), nucleotide (dNTPs), PCR buffer, and appropriate forward and reverse primers, which may either be genus-or species-specific. The DNA extract is then added to this PCR mix and also for control, NA free water is added to the PCR mix.

The prepared samples (sample and control) are introduced to a PCR machine, and a PCR program is run using optimal conditions, depending on the type of DNA polymerase that was used in the experiment, as well as an optimal annealing temperature suitable for the set of primers that were used. When the program has run to completion, DNA loading buffer is added and then the amplified products are visualized in an agarose gel to which an ethidium bromide stain has been added.

PCR Procedure and Principle

The polymerase chain reaction employs two oligonucleotide primers to hybridize to opposite nucleic acid strands and therefore flank the target DNA sequence being amplified. The process uses enzymes such as Taq polymerase to act as catalysts to enable the elongation of the primers and repetitive series of cycles involving several steps such as: (i) template denaturation; (ii) primer annealing; and (iii) extension of the annealed primers with the ends of the fragment characterized by the 5’ ends of the primers, so that the primer extension products synthesized in a given cycle can also be used as templates in the next cycle.

The manipulation of PCR technology has resulted in an increased use and application to many other different molecular biology products. For example, when the PCR is merged with reverse transcription, it makes it possible to obtain

an extended technique RT-PCR, which can enable the analysis of RNA in addition to DNA. The use of short primers enables the generation of a genomic fingerprint, which is useful in the investigation of genetic information of organisms whose genomic sequences are completely unknown and this is the bottom line of molecular techniques, such as random amplified polymorphic DNA (RADP) and Differential Display. In some cases, molecular tags can be introduced into the PCR products. These molecular tags, which include digoxigenin (DIG), biotin-labeled dUTP, etc., make the PCR technique very useful for applications in medical diagnostics, since these labeled PCR products can serve as hybridization probes that can detect very small amounts of pathogens.

Genome Segmentation and Identification/Typing Methods: Molecular Techniques

Genome segmentation and identification/typing methods are molecular biology techniques that are useful in food forensic analyses. There are numerous genome segmentation/molecular techniques and approaches, with the majority being polymerase chain reaction (PCR)-based methods (classical and improved classical PCR techniques) that are known to be frequently used in food forensics qualification and quantification of food elements. These methods include amplified fragment length polymorphism (AFLP)-PCR; inter-sequence simple repeat (ISSR)-PCR; simple sequence repeats (SSR)-PCR; real-time (RT)-PCR, sequencing and blotting methods; DNA microarray; PCR-restriction fragment length polymorphism (RFLP); PCR-forensically informative nucleotide sequencing (FINS); DNA hybridization; PCR-random amplified polymorphic DNA (RAPD); real-time PCR; species-specific PCR primers; DNA sequencing; DNA microarray; and PCR-specific primers.

Amplified Fragment Length Polymorphism (AFLP)-PCR Methods in Food Forensics

The amplified fragment length polymorphism (AFLP)-PCR method is a technique based on the principle of selectively amplifying a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction endonucleases. It involves four steps, which involve:

1. Digestion: using mainly two different restriction endonucleases, whereby one of these enzymes is a 4-base cutter (e.g. MseI with sequence 5’TTAA3’) and the other is a 6-base cutter (e.g. EcoRI with sequence 5’GAATTC3’);
2. Adaptor ligation: whereby two different adaptors are used. It should be noted that adaptors refers to short double stranded DNA sequences with sticky ends, which are ligated to the digested fragments such that one adaptor complements to the Msel cut end, with the other to the EcoRI cut end;
3. Amplification process: whereby the DNA fragments with MseI-EcoRI ends are selected as the DNA template for amplification using two PCR primers, which are complementary to the two adaptors employed in the amplification process and these adaptors are labeled using either radioactive or fluorescence dye to facilitate the detection of DNA bands on the gel. Generally, the number of DNA bands detected by AFLP is normally very high and to reduce this number, it is customary to add certain selective bases (mainly 1-3 nucleotides) at the 3’-end of the PCR primers; and
4. Electrophoresis: for the separation of DNA fragments.

In AFLP, which is known to be highly polymorphic, DNA variation is detected based on either the presence or absence of DNA bands due to:

1. the presence or absence of restriction sites; and
2. additional bases (insertion) between two restriction sites that are too large.

Generally, the AFLP-PCR technique is used to identify and detect the genetic natural variation due to single nucleotide polymorphism (SNP) within the biological specimens (population). All organisms are built by small entities known as cells, which contain a biological molecule known as deoxyribonucleic acid (DNA) encoded with the blueprint for the growth characteristics, composition, and functions or characteristic information of all components that form that particular organism. The various body functions of an organism are encoded in a segment of the DNA known as a gene (e.g. information about the structure and sequence of a particular protein or enzyme, height or skin/hair/eye color, etc.). The sum of all genes in an organism is collectively known as a genome. However, organisms that belong to the species have their DNA identical to about 99.9% with 0.1% difference caused by natural variations within the species in the population, which is here referred to as SNP.

The SNP variations can be detected using AFLP-PCR technology, which can also locate points of polymorphism simultaneously in an organism’s genome. The AFLP-PCR technology can thus be used to authenticate the origin

The AFLP-PCR technology can thus be used to authenticate the origin, composition, adulteration, diversity, and fingerprinting in food forensics. AFLP-PCR can also be instrumental in detecting illnesses that are foodborne within a particular food chain. It can also find application in areas of genetically modified animals/organisms (GMA/GMOs). The PCR technique that is linked to AFLP plays an important role in amplifying the gene, because in most circumstances, the DNA material presented as the sample specimen is always in minute quantities and thus the need for amplification. During the AFLP-PCR process, a typical fingerprint pattern of various fragment sizes is observed on a gel. These fragments are then matched with an AFLP marker to establish either the presence or absence of the pattern of interest in a particular gene in the sample. In most cases, AFLP-PCR is performed using restriction enzymes and selective nucleotides, depending on the samples.

The AFLP-PCR procedures involve cutting the DNA of the samples at specific sites using two restriction enzymes, then this step is followed by ligating the double stranded nucleotide linkers that link the two DNA strand ends. Then the fragments are amplified using PCR before separation and identification using electrophoresis techniques. Then the gel is stained to enable the visualization of the DNA fragments, which can be observed as bands representing particular sizes of the DNA fragments.

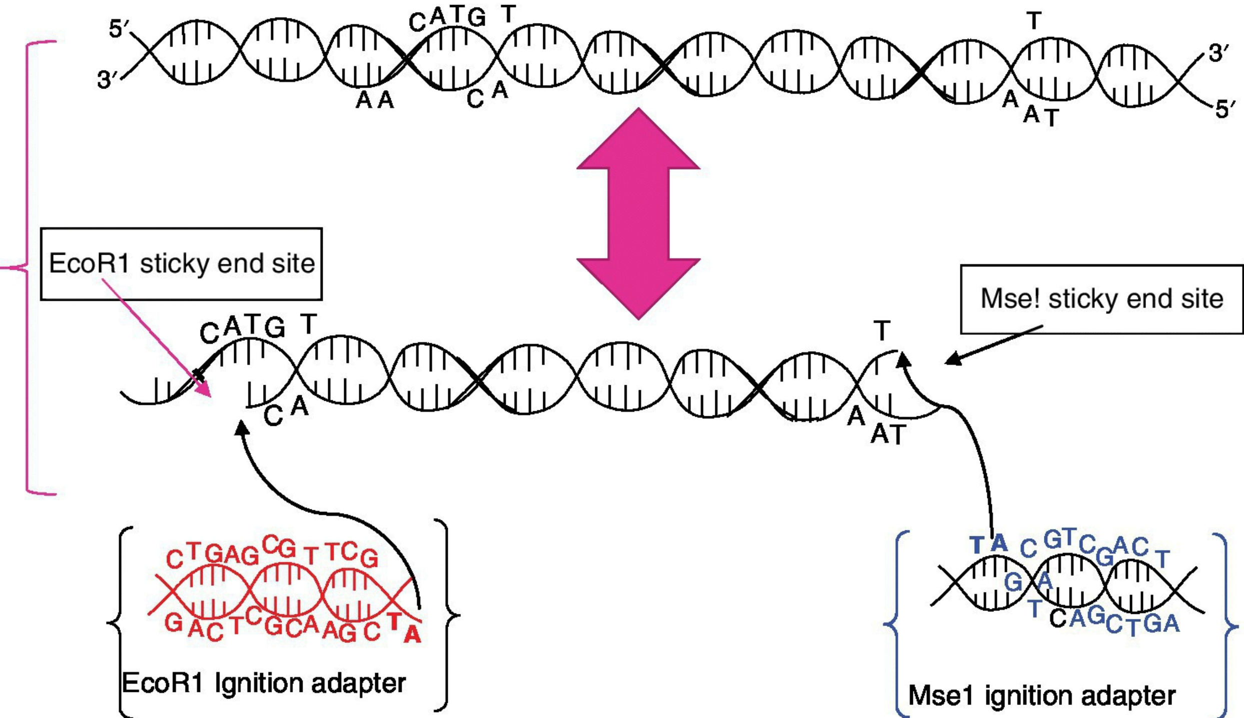
AFLP-PCR Technique

AFLP-PCR technique is attractive, mainly because there is a synergy between restriction fragment analysis of genetic materials, which is known to be highly reproducible with the capabilities of the PCR technique. The synergy has an advantage of enabling the random and simultaneous analysis of several loci within the genomic materials under study. The principles of AFLP take advantage of the selective nature of the PCR technique on the restriction fragments arising from the digestion of genomic DNA (Zabeau and Vos, 1993). In this method (Scheme 11.9), restriction enzymes (e.g. HindIII, MseI, EcoRI, etc.) are first used to digest small quantities of highly purified genomic DNA and the process is followed by the ligation of the doubly stranded oligonucleotide adaptors to the sticky ends of both ends (5′ and 3′ endings) DNA fragments that result from the digestive action of the restriction enzymes. These DNA fragments that have been ligated are then subjected to a double PCR amplification process under an excessively stringent environment, in a process that involves the use of a complementary set of: (i) primers to the adapter and restriction site sequence; and (ii) an added nucleotide at the 3′ sticky ending of

the DNA sequence. The last step will now be used to identify the extent and nature of polymorphisms by subjecting the amplified fragments to denaturing polyacrylamide gel electrophoresis (PAGE) or any other similar technique (Blears *et al*., 1998; Mueller and Wolfenbarger, 1999).

- Extract DNA sample from the tissue/specimen (The important requirements for the DNA needed for AFLP is that it must be very clean and of high molecular weight)

- Cut the DNA at specific sites using appropriate pairs of restriction enzymes, eg. **EcoRI/MseI**; HindIII/MseI; HgaI/MboII, etc. in order to specify the core sequence and the enzyme specific sequence.



- Perform PCR on the prior-chosen amplification using [two (2), repeat with four (4) …] selected nucleotides
- Employ capillary electrophoresis to separate DNA amplified fragments

Scheme 11.9 Summary of the AFLP-PCR procedure.

Summary of AFLP-PCR Process

- extract DNA sample from the tissue/specimen. The important requirements for the DNA needed for AFLP is that it must be very clean and of high

molecular weight;

- using the appropriate restriction enzyme, ligate the DNA core sequence adapters. In cases where the site to be cleaved is known, the site is denoted with a ^ mark, *e.g.* HgaI/MboII cleaves the sequence at (5/10) site. Then pre-amplify the sequence using correct primers and an appropriate nucleotide (N) of interest and the sequence will be as follows:

  5'ATGCANNNNN^3'

  3'TACGTNNNNNNNNNN^ 5';

- perform selective amplification using the same set of primers and increase (e.g. triple) the number of nucleotides from the previous step; and
- then separate using gel electrophoresis.

Gel Electrophoresis Separation of Biomolecules and PCR Products

Electrophoresis is a separation technique where its mechanism is based on the transport of charged analytes under the influence of an electric field. This technique is applied in molecular biology to separate biomolecules such as nucleic acids and proteins, since they are charged. Normally, nucleic acids are subject to electrophoresis in either neutral or basic buffers, in order to make them negatively charged (anions) where phosphate groups become negatively charged. Under acidic conditions, these polynucleotides become insoluble in water and thus cannot be separated using electrophoresis. In this modern age, the majority of electrophoresis methods for the separation of biomolecules (nucleic acids or proteins) tend to employ solid supports, for example polyacrylamide gel and agarose gel, which act as anticonvective agents to create an environment that will retain the integrity of separated sample products by lessening the convective transport and the diffusion phenomena, which will cause the appearance of sharp bands (zones) throughout the electrophoretic run. Moreover, these solid supports (gels) play an important role in acting like molecular sieves, which separate the products based on their molecular sizes.

Types of Electrophoresis Methods

For separation of biomolecules, two types of electrophoresis are known: (i) slab gel electrophoresis; and (ii) capillary electrophoresis (CE), which is further

subdivided into several modes, mainly capillary zone electrophoresis (CZE) that separates biomolecules on the basis of charge to mass ratio and is normally used for the separation of bases, nucleotides, nucleosides, investigation of damage to DNA, and also the analysis of small-sized oligonucleotides. Other CE modes are micellar electrokinetic capillary chromatography (MECC), which separates biomolecules based on charge to mass ratio by partitioning into micelles; capillary isotachophoresis (CITP), which separates on the basis of moving boundaries or displacement and is mainly useful as a preconcentration and enrichment method for CZE and MECC. Another CE mode is capillary isoelectric focusing (CIEF), in which separation is dependent on the isoelectric point of the analytes (used mainly for separation of proteins). There is also another CE mode known as capillary gel electrophoresis (CGE), which works as a molecular sieve and on the principles of reptation. CGE is mainly useful in the analysis of oligonucleotides, primers, probes, PCR products, ascertaining of point mutations, and for the sequencing of DNA, *etc*.

Slab Electrophoresis: (Gel Electrophoresis) – Polyacrylamide (PA) and Agarose

There are a number of variants of the slab gel techniques, which utilize different types of media, mainly polyacrylamide and agarose for the separation of proteins and nucleic acids. These variants include formats such as horizontal, vertical slab gels, and cylindrical rods (Andrews, 1986; Rickwood and Hames, 1983). Unlike in the case of capillary electrophoresis, where separation and detection processes are synchronized through real time analysis, the processes in many of the slab electrophoresis techniques are such that after electrophoresis of the proteins or nucleic acids, the gel matrix is soaked in the fluorescent dye solution (e.g. ethidium bromide solution), washed, and bombarded with ultra violet radiation in order to generate a photograph that shows the fluorescing DNA band patterns with their molecular weights (base pairs) identified with an appropriate marker. Apart from this fluorescence detection technique, autoradiography has also been used for the detection and identification of genetic materials for samples submitted for forensic crime investigations or samples collected from genetic materials that are suspected to be the cause of a certain disease, where specific sequences of the genetic materials in the sample can be identified in order to prove their identity. An example of autoradiography is a technique known as Southern blotting, which was invented by Southern in the mid-1970s (Southern, 1975), which makes use of DNA probes that are radio-labeled to target specific complementary DNA fragments in the sample presented for

target specific complementary DNA fragments in the sample presented for analysis or fingerprinting. Despite the fact that autoradiography slab gel techniques are unpopular, because they are time-consuming, they still have some attractive features, such as the possibilities for the simultaneous analyses of samples.

Types of Slab Gels Used in Electrophoresis of Biomolecules

The three most used gels in electrophoresis of proteins and nucleic acids are polyacrylamide gel (prepared by the cross-linking of acrylamide with *N*,*N*'-methylenebisacrylamide), agarose gel (a polysaccharide prepared algae extract known as agar-agar), and pulsed-field gel. The choice of the gel to be used is in most cases governed by the size of the biomolecule (nucleic acid or protein) to be analyzed. When dealing with relatively shorter or smaller fragments of nucleic acids or short peptides, then polyacrylamide gels becomes the convenient choice. In this case, sodium dodecyl sulfate (SDS) with polyacrylamide gel electrophoresis (PAGE) is used (SDS-PAGE). On the other hand, when the size of the nucleic acid is relatively large, a gel with larger pore size becomes the best choice and therefore agarose becomes the gel to be used in this case. During the gelling process, unlike polyacrylamide, agarose does not cross-link such that dilute 0.2% or more concentrated 0.8% agarose gels can be enough to make a gel set (stiffen) to enable the separation of nucleic acid materials of molecular weights from 150 million and 50 million respectively (Schwartz and Guttman, 2016). Both polyacrylamide and agarose gels are commonly done on DC electrophoresis fields and can separate nucleic acid fragments of size up to 30 kb (Schwartz and Guttman, 2016). When dealing with nucleic acid materials larger than 30 kb (e.g. chromosomal DNA, which has sizes measuring up to 6 Mb), alternating current (ac) electrophoresis fields are normally used and therefore pulsed-field gel electrophoresis (PFGE) is the preferred gel to use.

Capillary Electrophoresis (CE)

Unlike the slab gel electrophoresis technique, capillary electrophoresis (CE) employs two types of driving forces: (i) it is associated with the force causing the electrophoretic migration; and (ii) it uses force generated by the electro-osmotic flow (EOF) through the tiny capillaries.

Separation of Biomolecules Using Non

Separation of Biomolecules Using Non-electrophoresis Techniques

There are other techniques, apart from electrophoresis, that are used to separate biomolecules. These methods can be categorized based on the mode and principles of separation that are used. There are those techniques that take advantage of the chemical and biological properties of the biomolecules to be separated. Methods that fall into this category include immuno-adsoption-based methods in which nucleic acids can be separated using immunosorbent or silica adsorption that enables nucleic acid molecules to bind to the stationary phase (silica) surfaces, which also contain specified salts and are at optimized pH conditions. Another category of these non-electrophoresis techniques utilizes differences in the sizes of biomolecules to separate the analytes. These methods include centrifugation driven by centrifugal force, whereby differences in terms of the measure of retardation occurring during the transport process as electrophoresis is proceeding, creates an environment where there are differences in transport velocities of the analytes, thus effecting the separation. There are also those methods that take advantage of the electrical charge differences between biomolecules to effect their separations.

High performance liquid chromatography (with a variety of detectors, such as fluorescence, UV, etc.) can be used as an alternative to electrophoresis.

Application of AFLP-PCR in Food Forensics

There are many areas where AFLP may be applied. These areas include poisonous microorganisms that may be introduced into foods of both animal (through injections, feed, water, etc.) and plant origin (through spray, fertilizer/manure, watering, etc.)

Genotypic Detection and Identification Methods: AFLP-PCR

AFLP-PCR has been used to trace the presence of type C botulism found in bovine carcasses to the feed (silage) fed to the animals (Myllykoski *et al*., 2009). The technique was successfully used to identify type C bovine botulism after isolating *Clostridium botulinum* type C in bovine liver samples and performing the identification procedures, which employed the use of amplified fragment

length polymorphism (AFLP). In this work, the results proved that the microorganisms belonged to group III *C. botulinum* after diagnosis using AFLP-PCR. The results prompted the call to propose a measure to prevent the botulinum compounds, which are known to be neurotoxic in the animal feed, by introducing an acidification step during the production process for silage. Though this example in its presentation may not be typical of a forensic case, it may turn out to be so if the owners of the farms had been required by law to include the acidification of silage before feeding it to animals and if it had been proved that they deliberately bypassed this step for economic reasons or otherwise.

Identification of Toxigenic Strains in Animal Carcasses and Varieties in Plant Crops

There have been cases where meat carcasses were deliberately contaminated with toxic substances released by certain strains of toxigenic microorganisms, by either avoiding or deliberately omitting appropriate procedures that are meant to eliminate such microorganisms, with the intention of avoiding costs or to intentionally cause harm to consumers. These microbes may be derived from either water or animal feed, in which the steps to inactivate or kill pathogenic microorganisms are omitted, either to save on production costs or to intentionally cause damage to people’s health (intentional crime). When these animals are slaughtered, the meat products tend to contain the toxic compounds released by microorganisms and if they are not detected before consumption, may affect the health of consumers. This may turn out to be a legal case, as consumers are likely to sue meat vendors, butchery owners, owners of abattoirs, and animal farmers. This situation may necessitate and call for the tracing of the source of the problem, as well as the culprits.

Another example where the AFLP method was applied in plants, where leaves of certain cultivars of grapevines were artificially infected with the fungi species (oomycete *Plasmopara viticola*) to study the molecular compatibility *vis-à-vis* disease development (Polesani *et al*., 2008). The cDNA-AFLP was successfully used to selectively amplify more than 100 primer combinations, enabling the identification of thousands of transcript-derived fragments (TDFs) from the fungal infected leaves. After sequencing some of these fragments, about 82% were attributed to the grapevine cultivar, about 10% were correlated to the fungi species (*Phytophthora* spp.), while the rest, 10%, were attributed to orphan transcript-derived fragments.

PCR: Random Amplified Polymorphic DNA (RAPD)

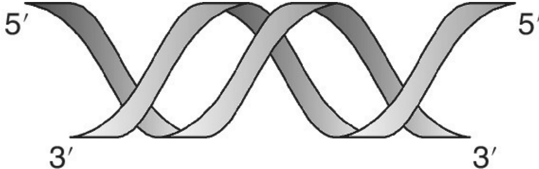
Random amplified polymorphic DNA (RAPD) is one of the molecular techniques based on the modification of the polymerase chain reaction (PCR) method. RAPD makes use of a single, short, and arbitrary oligonucleotide primer that has the ability to anneal and prime at multiple locations throughout the whole DNA to produce a wide range of amplification products that are typically a characteristic of the template DNA that was used.

This means that in RAPD, segments of amplified DNA are random and the primers will bind somewhere in the sequence, though there is some uncertainty as to where exactly the binding will take place. For this reason, no prior knowledge of the DNA sequence for the targeted gene is needed.

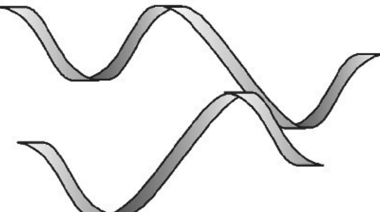
As opposed to other normal PCR analyses, procedures that may involve the RAPD of the whole species (especially plant parts such as leaves) do not necessitate prior knowledge of the gene sequence of the organism under investigation. RAPD analysis is useful for the comparison of plant types. In this technique, decamer oligonucleotide primers (i.e. in a multiple of 10 bp each) are added to the DNA extracted from the species under investigation and then the whole mixture is subjected to PCR for amplification. The attractive features of RAPD include the fact that it is sensitive, fast, and does not make use of radioactive probes. However, RAPD has some limitations due to the fact that they are dominant alleles and therefore it is important to have many closely related markers to get reliable comparisons. Moreover, in cases where there is a mismatch between the primers and the templates, PCR products may not be observed, which may complicate the interpretation of the results. Another limitation comes from the fact that it is difficult to make a clear-cut distinction between the amplified segments of the DNA and the heterozygous loci (a single copy) or homozygous (two copies), due to the fact that RAPD markers tend to be dominant. Moreover, with PCR being an enzymatic process, there is a possibility that the quality and quantity of both the template DNA, amount of PCR components, and the conditions of the PCR cycling, influence their products. This complicates the carrying out of the RAPD analyses, necessitating that the technique be done by qualified personnel in a well-established laboratory.

RAPD Principle

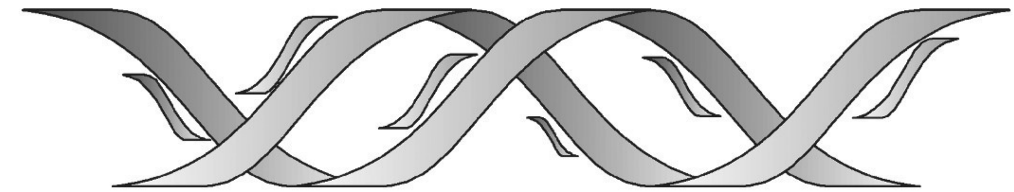
Figure 11.1a,b summarizes the procedures (in two cycles), which can show the principle in which the technique operates:



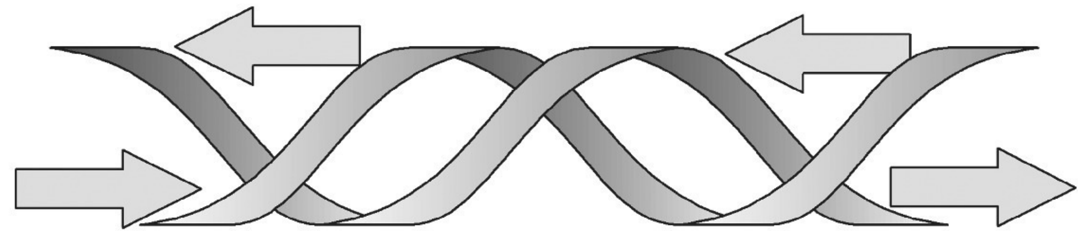
First step of the first cycle involves the denaturation of the dsDNA



Second step of the first cycle involve the annealing at less stringent conditions



Third step of the first cycle involves primer extension



Fourth step of the first cycle involves denaturation and annealing at less stringent conditions

Figure 11.1a Cycle one: Summary of the procedures (in two cycles), showing the principle in which the technique operates.

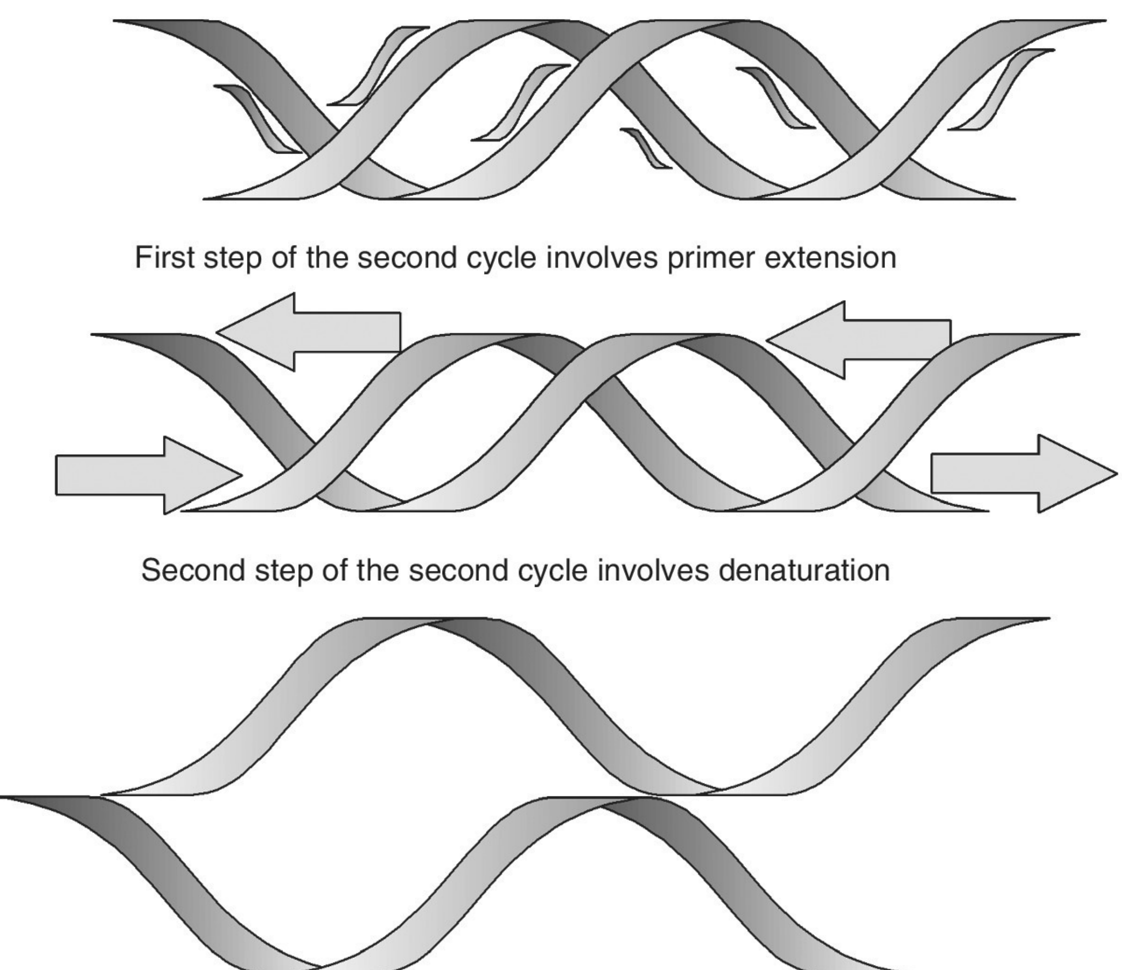


Figure 11.1b Cycle two: Summary of the procedures (in two cycles), showing the principle in which the technique operates.

Microsatellite Techniques

By definition, a microsatellite refers to segments of tandemly repetitive sequences of adjacent/allelic genes, for example (CA)n, which occurs at numerous eukaryotic genome locations. The general microsatellite procedures involve the PCR amplification of the region on the genome that contains the microsatellite using appropriate primers. The number of repeats on the allelic gene for the microsatellite under study will determine the size of the amplified DNA and the DNA fragments are then separated on a gel. There are several microsatellite-PCR techniques that are used in food forensic studies and they include inter-sequence simple repeat (ISSR)-PCR and simple sequence repeats (SSR)-PCR, *etc.*

Microsatellites sequences are also called by other names, such as short tandem repeats (STRs) or variable number of tandem repeats (VNTRs), as known by forensic geneticists who use the technique for forensic identification. Microsatellite is also known as simple sequence repeats (SSRs), mainly by plant geneticists who use the technique for DNA profiling, genetic linkage correlations, *etc.* Microsatellites occur mostly in gene introns (non-coding regions of the DNA, thus biologically silent), do not have any consequence with regard to the functioning of the gene, but are used merely as markers to identify loci and some specific chromosomes. They are also not associated with causing disease by themselves. Microsatellites are well known for their diversities, for example in some of these microsatellites, the repeated unit (e.g. CA) may occur three times, in others it may occur ten times, etc., but generally more than twice. Microsatellites are also known for undergoing high rates of mutations and rely mostly on the variations in the number of repeats for the allelles being studied rather than on the number of repeats for the microsatellite under study.

In a population, if an organism's DNA polymerase adds to the microsatellite, this will result in a larger copy of this repeated sequence, and will be passed on to subsequent generations for further replications, which will create the possibility for recombination over the repeated breeding cycles. This trend is meant to ensure that the variability of the repeated sequence in this particular population is maintained, making it characteristic for that population and distinct from all other populations that do not form part of the interbreeding.

There are a number of methods that are possible for use in the detection of microsatellites, but most studies have involved the use of polymerase chain reaction primers (PCR primers – Figure 11.2). These primers are designed such that they are highly specific and unique to one locus, such that just a single pair of PCR primers can be used for the identification of every individual in the same species, although they may result in products of varying sizes of the amplified products, depending on the different length microsatellites used.

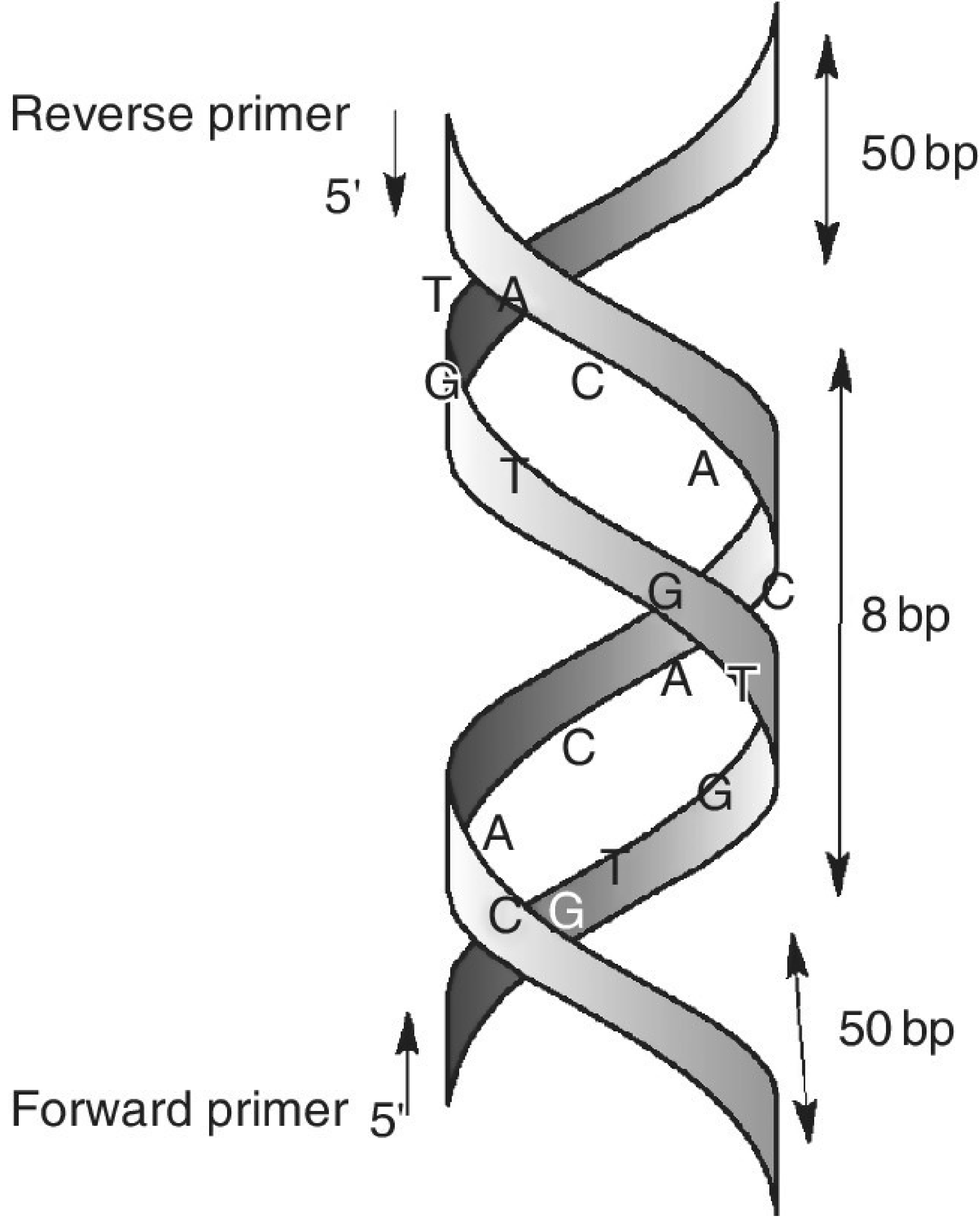


Figure 11.2 Illustration of the PCR method used as a detection method for microsatellites.

Inter-sequence Simple Repeat (ISSR)-PCR

Inter-sequence simple repeat (ISSR)-PCR has several synonymous variants as follows: anchored simple sequence repeats (ASSR); anchored microsatellite primed (AMP)-PCR; microsatellite primed (MP)-PCR, which is an unanchored primer; simple sequence repeat (SSR)-anchored PCR (Zietkiewicz *et al*., 1994); inter-sequence simple repeat amplification; random amplified microsatellites (RAMs); random amplified microsatellite polymorphisms (RAMPs); and single primer amplification reaction (SPAR).

Genome Fingerprinting by Simple Sequence Repeat (SSR)-anchored Polymerase Chain Reaction Amplification

The simple sequence repeat (SSR) technique combines the advantages of both AFLP and RADP and involves the use of the sequences of microsatellites as primer, together with PCR to create polymorphic markers for the loci present in the target genome (DNA) (Godwin *et al*., 1997). ISSR-PCR does not require any construction sequence information/data, because the chain polymerase reaction procedures are included in the procedure itself. This attribute, together with the fact that low amounts of the DNA template are required and also the ISSRs are always randomly distributed over the whole genome, are the main advantages of ISSR-PCR. However, the technique suffers from the fact that it may well be associated with phenomena related to non-homology of DNA fragments with similar size, because the technique is multi-locus. Another shortcoming is that ISSR has reproducibility problems, just as RAPD does.

However, despite these limitations, ISSR-PCR is highly useful in forensic investigations of genetic identity, strain identity, *etc.*

Forensically Informative Nucleotide Sequencing (FINS)/DNA/PCR Sequencing/Barcoding

In the forensically informative nucleotide sequencing/barcoding (FINS)/DNA/PCR technique, highly conserved species specific mitochondrial genes, such as 12S rRNA, Cytochrome b, and 16S rRNA are amplified using universal primers for the identification of species, using the biological material presented as forensic evidence.

Species-specific PCR Primers

Species specific primers are possible to design due to the availability of the information on the species genome sequences, also due to the possibility of identifying single base polymorphism. To be able to design such primers, one may have to employ stringent conditions to generate a fragment of the DNA that may then be visualized on agarose gel in the presence of the gene from a specific species. The advantage of this technique is that there is an assurance of getting the gene fragment amplified, unless there is a technical error. The limitations of this technique arise from the fact that prior knowledge of the specimen to be analyzed is essential and has to be available to enable the identification of the target species. Moreover, the technique requires the inclusion of controls to exclude the possibilities of false positive or false negative observations.

Multiplex PCR

Multiplex PCR is a variant of PCR-specific primer techniques, which is capable of amplification of several targets of interest simultaneously, by using more than one pair of primers in one set of the reaction (Michelini *et al*., 2007).

Conclusions

Molecular biology-based methods are very attractive in food forensic cases due to their specificity, as they target molecules such as nucleic acids, proteins which are specific for each individual organism. These methods provide more reliable evidence. However, they require highly skilled personnel and advanced equipment, expensive reagents, and a laboratory environment that is clean and sterile.

A combination with other analytical/bioanalytical methods for a concrete proof of the results may be mandatory.

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12
Application of Atomic and Molecular Spectroscopic Techniques in Food Forensics

The electromagnetic radiation methods in foods are very common. These methods can be used either as part of the food processing train or as analytical methods in providing evidence in food forensic cases. Both types of radiation (ionizing and non-ionizing) have found application in food forensic analyses and/or food processing procedures. Ionizing radiation includes gamma-rays, which can be emitted from sources including radioisotopes, for example 137Cs, high energy electrons, X-rays, etc. Non-ionizing radiations, on the other hand, include ultra violet-visible radiation, nuclear magnetic resonance (NMR), infrared, Raman, and microwaves, are also used in food forensics. In most cases, these methods complement each other to enable more reliable information to be deduced from the sample under investigation. Non-ionizing spectroscopic techniques that will be discussed as far as this topic is concerned include ultra violet-visible, infrared (near, mid and far/terahertz), Raman, and microwaves. Gamma rays and X-rays will be covered in the next chapter.

Introduction

Atomic spectrometry techniques of interest for this chapter are mainly atomic absorption spectrometry techniques and atomic emission spectrometry techniques.

Atomic Absorption Spectrometry (AAS)

Atomic absorption spectrometry (AAS) is a spectroscopic-based analytical technique for the determination of elements based on the absorption characteristics when their free atoms in gaseous state are exposed to optical radiation in the ultraviolet region. For AAS to be used for quantitative measurements, it requires the use of standards with known concentrations that will provide the means to come up with the possibility to correlate the analyte concentration with the corresponding measured absorbance according to Lambert–Beer's law.

AAS requires that elements be converted to gaseous atoms and for this reason, several techniques are available to provide atomization of the elements, which include flame (mainly air-acetylene flame and nitrous oxide-acetylene flame); and electrothermal (also known as graphite tube atomization). Other atomization methods include glow-discharge atomization, hydride atomization (for hydride formers such as arsenic, selenium, antimony) and cold-vapor atomization (for mercury species). Flame atomization techniques work best for analytes contained in liquids/solutions (dissolved samples), while electrothermal AAS can analyze any kind of sample (solid, liquid, and gaseous samples). ET AAS results in much lower detection limits as compared to FAAS.

Atomic Emission Spectrometry: Inductively Coupled Plasma Techniques

Inductively coupled plasma optical emission spectrometry (ICP-OE and ICP-MS) is another analytical technique used for the measurements of trace metals, which makes use of inductively coupled plasma for both atomization as well as for ionization of sample analytes to produce atoms and ions that when exposed to a specific electromagnetic radiation (light), will produce a signal that is characteristic and specific to that particular element. For quantitative measurements, standards are normally employed such that the intensity of this emission is directly proportional to the concentration of the element present in the sample. In addition to trace metal determination, ICP-MS can also be used for the determination of isotopic concentrations in food samples. ICP is more attractive than AAS-based methods, because it gives much lower detection limits, wide-range linearity, superior sensitivity, high efficiency, and it is also a multi-elemental technique capable of the simultaneous multi-element determination of the majority of elements from a single sample in one run.

Applications of Ion Chromatoraphy and Atomic Spectrometry in Food Forensics

Ion chromatography and atomic spectrometry techniques are widely used for mineral and trace element determination in food samples, as well as for the verification of authenticity and also for the indication of the geographical origin of foods.

The Relationship Between Mineral Composition in Foods, Fruit Bearing Plants, Flowers, Vegetables/Vegetable Derived Products, and Soil Structure

The geological set up of a place determines the type and composition of soils, as well as the quality and quantity of mineral elements. This, together with factors such as pH, mineralogical patterns, clay type, and composition and humic complexes of the soil, determines the bioavailability of the minerals, both major and trace (Alloway, 1995). Since plants that form the source of food and foodstuffs, including fruits, drinks derived from fruits, corn, etc., their mineral composition can be taken as a reflection of the element composition of the soils in which the plant grows. This fact can thus justify the relationship between mineral composition in the soils of a particular locality to the mineral composition in plants growing there and to the fruits, oils, *etc.* derived from those plants.

For this reason, plants grown in different geographical locations would be expected to exhibit different patterns and profiles of trace elements due to the differences in the chemistry of the soils, which is patterned by different climatic and weather agents, such as weathering of rocks that differs from place to place. It should be noted that different geographical locations may have similar precursors of rocks, which may result in similarities in the types of soils, thus mineral and trace element patterns; however, scientific and geological data suggest that it is highly unlikely for the trace element geochemistry and precursor-mineralogy in different geographical locations with similarities in rock types to be equivalent, due to the fact that each of these areas tend to be subjected to a wide variety of geological processes that will give uniqueness to each location (Kabata-Pendias and Pendias, 1984). Moreover, the chemistry of a particular place will tend to influence the uptake of minerals by plants different from another location, thus resulting in a unique uptake of trace elements and minerals by those plants (Kabata-Pendias and Pendias, 1984). Therefore, it is very scientific to link, discriminate, and classify different soils and crops (fruits and vegetables) that grow there, by using analytical techniques capable of single and multi-elements analysis such as AAS and ICP (Ariyama and Yasui, 2006).

Atomic Spectrometry (AAS, ICP-OES, ICP-MS)

Ground samples are subject to dissolution procedures and analysis for solution-based ICP-MS and ICP-OES, or AAS and IC. Similar dissolution and analysis procedures were used for all sample types investigated in the studies detailed in this chapter. However, pork samples were treated differently as these were dissolved without drying.

Dissolution can be done using nitric acid (appropriate volume per unit weight of the sample) and by leaving the sample in an oven at about 90 °C overnight. Add more nitric acid (a volume of about 10% of the original volume used for dissolution), put on hot plates at 160 °C for 2 hrs, then evaporate the nitric acid and the samples should be allowed to dry. Then digest samples using 4:1 nitric/perchloric acid mixture and place them on hot plates at 180 °C for 3 hrs. The temperature can be reduced to 100 °C and the samples are allowed to remain at this temperature overnight to allow sufficient time for the reaction (digestion to set free elements from the matrix). Then evaporate nitric/perchloric acid to dryness to obtain the required residue and to this solution add an appropriate volume of 20% nitric acid with gentle warming.

Alternatively, the nitric acid/hydrogen peroxide procedure can be adopted where the case is related to protein samples. The nitric acid/hydrogen peroxide can be used to digest the protein matrix, with the hydrogen peroxide playing the role of destroying fat residues in the samples.

NB: For complete procedures, the interested parties may consult relevant literature, which is available in many databases.

The sample solutions can then be analyzed by IC (for inorganic anions), AAS (FAAS or GFAAS), or ICP (OES/MS, solution or laser ablation for solid samples) techniques, depending on the technique that is available or depending on the parameters that are required.

Soils also contain inorganic anions that are characteristic of a particular geographical location in terms of types and ratios and this can be used to identify plants and their derived products with the types of soils from which they originate. The inorganic anions can be determined by many techniques, but the most common is ion chromatography (IC). Examples of the application of IC in ion analysis include the analysis of toxic ion species, such as azide, arsenite, arsenates, cyanide, sulfide, iodide, and bromide, etc., which are normally detected using various detectors, such as conductivity or UV detector (Annable and Sly, 1991 ; Bond *et al*., 1982 ; Gailer and Irgolic, 1994 ; Larsen *et al*., 1993 ; Rockline and Johnson, 1983 ; Rockline, 1991 ; Sheppard *et al*., 1992 ; Williams, 1983).

Sample Preparation for Elemental Composition Analysis in Foods

Type of samples for trace element composition analysis are plant materials (fruits, flowers, pollen, etc.), and animal products from animals that graze or feed on plants growing in the different geographical localities earmarked for provenance or authenticity investigation.

For fruits, whole fruit samples can be used, for plants, relevant parts, for example flowers, pollen, roots, stem, leaves, *etc.* can be collected and for soils, representative samples should be collected. For soil, or if water used for irrigation is required, then a representative sample (volume) must be collected from the area under investigation.

Sample Preparation (Typical Example of the Procedures)

- Soil samples: air-dry the bulky soil samples, grind to obtain a fine earth;
- Roots: ensure that the roots are cut from the plant with the soil tightly adhering to the root surface (rhizo soil); then separate by gently shaking and scraping; air-dry, grind to powder;
- Stems and leaves: hand-clean then oven-dry at 55 °C for 48 hrs. then grind to powder;
- Fruits: hand-clean, freeze, and then freeze-dry at –40 °C for 48 hrs, then grind to powder;
- Meat samples: no need for drying; and
- For all these samples (soils and plant materials), measure the moisture by weighing after drying at 105 °C for 16 hrs.

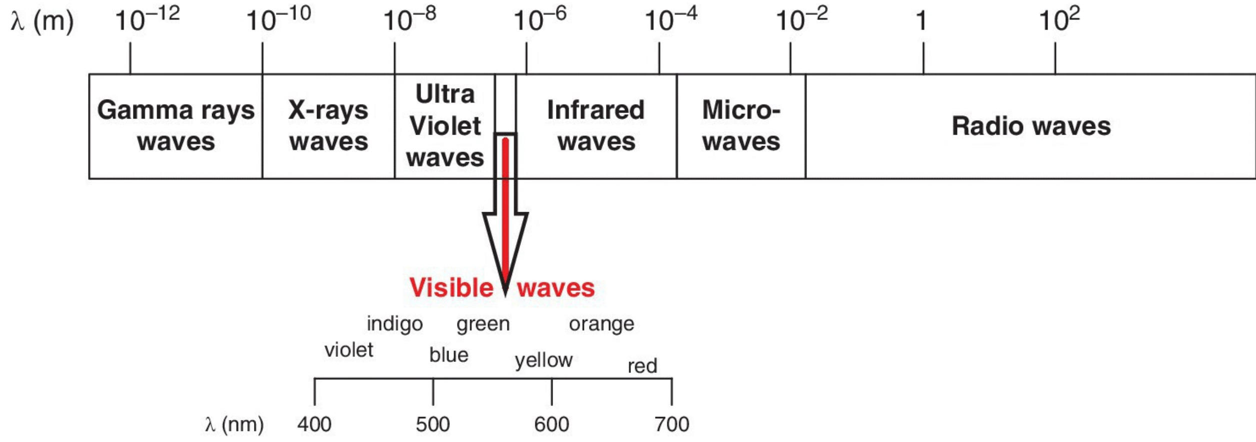


Figure 12.1 Positions of selected spectroscopic bands within the electromagnetic radiation.

Ultraviolet-visible (UV-Vis) Spectroscopic Methods for Food Forensics

The UV-Vis region of the electromagnetic radiation spectrum covers a wavelength range from 190 nm to about 800 nm. Irradiation of UV-Vis to sample molecules triggers the promotion of electrons to higher energy levels, which in turn provides information about the presence of conjugated p systems and the presence of double and triple bonds. UV-Vis is attractive for applications in food forensics due to its non-destructive properties, and the methods are known to involve simple operations, are economically viable, and can therefore be afforded by most laboratories. The principle for the detection is based on the ability of samples to be absorbed in a characteristic wavelength region of UV-Vis. Any sample with chromophores can be analyzed using this technique. Food samples that contain molecules that are either unsaturated, conjugated, or with aromaticity can be good candidates for UV-Vis analysis. UV-Vis can be used for a large number of samples daily to ascertain the concentrations of biomolecules such as proteins and DNA, oligonucleotide analysis, peroxide value determination, food color additives, authentication, and detection of illicit/surrogate food products.

For example, UV-Vis spectroscopy can be employed in the food industry to ascertain and verify the quality of edible oil products, based on the measure of a number of parameters including:

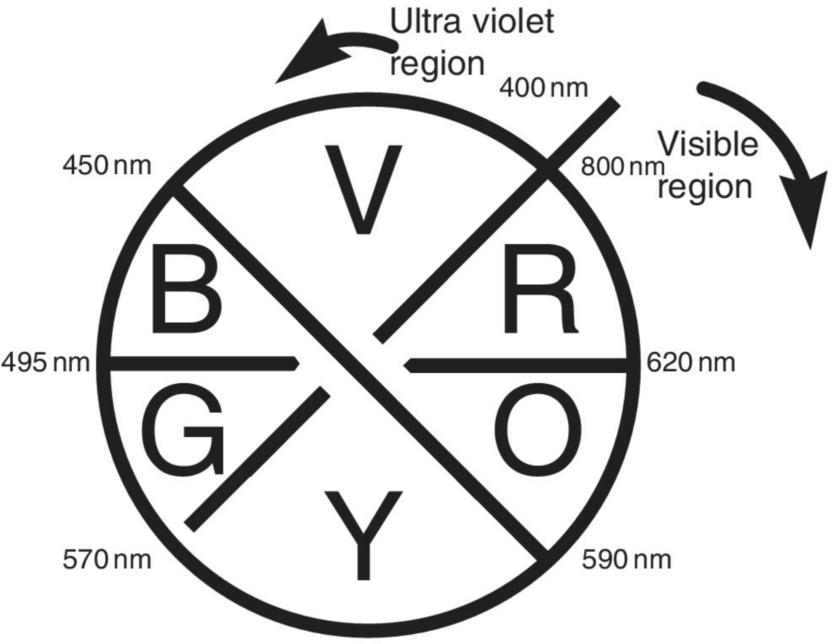
- the anisidine value that is related to the magnitude of fats oxidation by ascertaining the magnitude of aldehyde oxidation, which may occur during the oxidation of fat, and the anisidine value obtained can be used to assess the quality of edible oils. In measuring the anisidine value, UV-Vis spectroscopy measures the absorbance of the sample solution of fat foods that contain aldehydes that come from lipid oxidation (mainly dienals or alka-2-enas) after they react with p-anisidine. Since the anisidine value of edible oils is regulated (it should not exceed 8), the measure obtained from UV-Vis spectroscopy can be used in food forensic investigation in cases where there is no compliance;
- another parameter that can be monitored by UV-Vis spectroscopy is the total oxidation value, which can indicate the quality of edible oils in terms of total

fat deterioration;

- the peroxide value is another parameter that is used to ascertain the quality of edible oils after exposure to extreme physical conditions of temperature, oxygen, or daylight, which may cause generation/production of oxidation products of fatty acids, mainly peroxides, and hydroperoxides. The peroxide value is also regulated and the guidelines stipulate that the maximum permissible level in edible oils is 10 meq O_2 kg^{-1} for oil produced through cold press extraction and 5 meq O_2 kg^{-1} for a refined oil;
- another parameter of edible oil used to ascertain quality is general color, which is normally investigated by measuring the saturation of pigments, mainly chlorophyll and carotenoid pigments. It should be noted that carotenoid pigments are known to have antioxidant activity and thus are used as food additives and therefore foods with higher contents of carotenoids are classified as more nutritious than the ones with higher contents of chlorophylls. The UV-Vis measurement is normally carried out at wavelengths of 460 nm for carotenoid and 666 nm for chlorophyll.

Application of Spectroscopic Methods in the Determination of Food Colors

Food legislation and regulations always require the food processing industry, food suppliers, and distributors to strictly adhere to the labeling requirements regarding food ingredients, including food coloring agents (dyes). Under certain circumstances, food color may change, fade, or become diluted during food production. For example, when certain foods are exposed to oxygen or light for a prolonged period of time, there may be a change in food color concentration. For this reason, food manufacturers need to ensure that such changes do not take place, as this implies changes in the quality of the food. To test food colors, spectroscopic techniques (Figure 12.2) are very central.



NB1: V = Violet; B = Blue; G = Green; Y = Yellow; O = Orange; R = Red.

NB2: If specimen being analyzed absorbs electromagnetic radiation in the region of blue (between 450 nm–495 nm) it will appear as orange, etc.

Figure 12.2 UV-Vis in combination with HPLC and CE in food forensics.

Application of Molecular Spectroscopy Methods in Food Forensics: Fluorescence Spectroscopy

> *Fluorescence occurs when electrons have undergone excitation to higher energy levels and then returns to the ground state such that the emitted light has energy equal to the difference between energies of ground and excited states. The attractive features associated with fluorescence-based techniques include high sensitivity and specificity and due to these attributes, food analysis has exploited the characteristic advantages of fluorescence spectroscopy, especially in food forensic cases. The use of two kinds of spectra (excitation spectra and emission spectra) explains the reason for high specificity of fluorescence-based methods, but the high sensitivity of fluorescence-based techniques arises as a result of measuring radiation against absolute darkness.*

Fluorescence spectroscopy presents a number of attractive features as a technique with great potential in food analysis and food forensics. These attractive features include its high sensitivity and selectivity relative to other spectroscopic methods.

spectroscopic methods.

The advancements in technology that have application in fluorescence spectroscopy together with the increase in the use of chemometric techniques has accelerated the use of fluorescence spectroscopy in food research, including food forensics. Moreover, the use of fluorescence in food analysis and food forensics is plausible due to the fact that most foods contain intrinsic fluorophore moieities. Examples of foods with fluorophores include proteins that are made up of amino acids such as tryptophan, tyrosine, and phenylalanine residues. Fluorescence spectroscopy will generate spectral data and unique information about these aromatic amino acids and their respective chemical and biological environment in food or biological samples and such data can reveal the nature of the structure of proteins and how they interact with other proteins or other biomolecules (Herbert *et al*., 2000 ; Luykx *et al*., 2004). The uniqueness of the spectral data and the use of some specific fluorescence spectroscopic techniques such as front-face fluorescence spectroscopy can make possible the discrimination of protein-based foodstuffs based on their respective geographical origin (Dupuy *et al*., 2005 ; Karoui *et al*., 2004, 2005).

Fluorescence Coupled Techniques for Application in Food Forensics

Despite the fact that fluorescence-based techniques are very sensitive and specific, they cannot be used alone to provide a complete set of the quality data or measurements needed for all food samples.

It is therefore imperative to synchronize fluorescence spectroscopy (with other detection systems, *e.g.* spectrofluorimeter, electrophoresis equipped with laser-induced detector, high performance liquid chromatography fluorescence detector, fluorescence polarization (FP) immunoassay) and this makes it useful in providing appropriate measurements that may provide evidence in food forensic cases, such as food poisoning, for example issues related to toxic fungal metabolites poisoning (mycotoxins), which contain strong fluorophores, with the exception of G1 and G2, which do not exhibit fluorescence. Another application of fluorescence spectroscopy is in the authentication and adulteration of edible oils, such as olive oils, of which the market price is normally governed by the quality of olive oils. Unscrupulous distributors and suppliers tend to mix quality olives with those of inferior quality.

Nuclear magnetic resonance spectroscopy (NMR) makes use of radiofrequency waves of the electromagnetic radiation by bombarding these waves to atomic

nuclei with non-zero spins in a strong magnetic field (Ibañez and Cifuentes, 2001). NMR causes the excitation of the nucleus of atoms through radiofrequency irradiation, thus providing the information about molecular structure and atom connectivity of the sample under investigation. The presence of atoms in the surroundings does have an effect on the absorption of the atomic nuclei, as these atoms tend to cause minute but significant local modifications to the external magnetic field, thus enabling the analyst to obtain detailed characteristic data and information about the molecular structure of the food sample under investigation.

The physical principle on which NMR is based can be explained by the fact that some of the atomic nuclei have nuclear spin, for example hydrogen-1 (spin = 1/2), carbon-13 (spin = 1/2), nitrogen-15 (spin = 1/2), oxygen-17 (spin = 5/2), fluorine-19 (spin = 1/2), and phosphorous-31 (spin = 1/2) (Table 12.1) and this spinning charge creates a magnetic moment, such that these nuclei can be considered as tiny magnets. When these nuclei with spin are introduced into a magnetic field, they will tend to either line up with or against the field by either spinning clockwise or anti-clockwise. Depending on the strength of the magnetic field, normally the alignment with the magnetic field (assigned as α) is associated with lower energy than the alignment that is against the magnetic field (ß) (Figure 12.3).

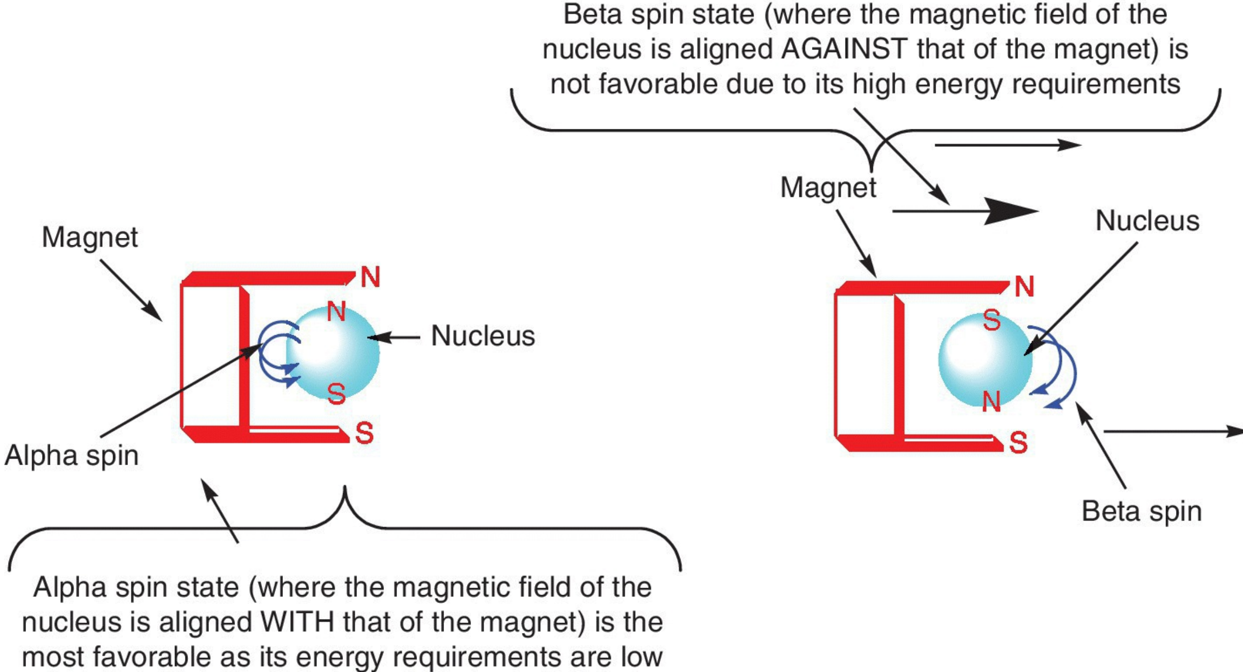


Figure 12.3 An illustration of alignment of nuclei with spin on a magnetic field.

Table 12.1 ½ Spin nuclei (Harris, 1983).

Nucleus	Chemical shift parameter range (ppm)
^{3}H	13
^{1}H	13
^{19}F	400
^{31}P	530
^{13}C	250
^{15}N	1700

There are several NMR experiments that are normally used concurrently to enable structure elucidation and these are proton NMR (^{1}H NMR), ^{13}C NMR, and its various experiments such as distortionless enhancement by polarization transfer (DEPT). The proton NMR spectra, for example, can generate information about the following data:

- Chemical shift data: this can provide information about what kinds of protons are present in the sample being analyzed;
- Integrals: provide the information about the ratio of each kind of proton in the sample; and
- ^{1}H–^{1}H coupling: provides information about protons that are near other protons.

On the other hand, the data from ^{13}C spectra can be useful in providing information about each carbon present in the sample, the type of carbons in the sample, the number of carbons in the sample, and the assignment of different types of carbons (primary, secondary, tertiary carbons… etc.), which can be done by performing DEPT 45, DEPT 90 or DEPT 135 experiments.

It should be noted that in case of nuclei that do not have spin, such as ^{12}C, they present no difference in terms of energy between alignments in a magnetic field, because they are not magnets and therefore it is impossible to analyze them using NMR spectroscopy.

Monakhova *et al.* (2011) recently reported the successful application of the UV-Vis spectroscopic method and ^{1}H NMR in the determination of surrogate alcohol products. Surrogate alcoholic products are contraband products, which are

legally banned as not fit for human consumption. However, such products are sold and consumed illegally and in such cases the consumer and seller are considered to be transgressing the law and thus subject to forensic investigation. because surrogate alcohols are known to have caused poisoning to consumers due to their composition, which may include disinfectants, medicinal alcohols, and perfumes (Monakhova *et al.*, 2011). In their report, they developed and validated spectroscopic-based techniques, mainly 1H NMR and UV-Vis for the determination of diethyl phthalate (DEP) and polyhexamethylene guanidine (PHMG), components that may be found in surrogate alcohol. The analysis using UV-VIS spectrophotometry was performed after derivatization with Eosin Y, while samples for ^{1}H NMR spectroscopy were analyzed after extraction with dimethylsulfoxide (DMSO).

Proton (^{1}H) NMR and ^{13}C NMR Spectroscopy for Food Fingerprinting and Food Provenance

Both proton (^{1}H) NMR and ^{13}C NMR in combination with chemometric methods have been employed in the investigation, fingerprinting, and characterization of various profiles of a food products and correlate the food chemical composition to their geographical origin. Generally, ^{1}H NMR has been widely used because it is more sensitive than ^{13}C, ^{31}P, ^{19}F, *etc.* and its application has been reported in food analysis, fingerprinting, and food provenance, for instance discrimination of various types of foods and food products such as olive oils into their respective regions of origin (Mannina *et al.*, 2001 ; Rezzi *et al.*, 2005). The discrimination of olive oil using ^{1}H NMR was possible, because phenolic extracts from different olives have differences in terms of ratios and abundancies for some of the characteristic phenolic compounds, as well as the ratio of the nuclei (^{1}H and ^{13}C) (Sacco *et al.*, 2000). In the recent past, the use of high resolution nuclear magnetic resonance (HR-NMR), in its mode known as “site-specific natural isotope fractionation” (SNIF), has been widely reported in the fingerprinting and identification of the geographical origin of foods and food products (Martin *et al.*, 1999 ; Ogrinc *et al.*, 2001). This implies that by determining the abundance of the natural isotopic ratios of the isotopes of carbon (C), hydrogen (H), or oxygen (O), it can provide the criterion for discriminating food products from different geographical conditions, as they are linked to climatic conditions in those geographical locations/countries. The identification by this NMR technique (SNIF) is based

on the fact that nuclei present in the components of these foods differ from those growing or found in one location to those in another locality/country, due to differences in climate in various places (Reid *et al.*, 2006 ; Remaud *et al.*, 1997). Apart from fats and oils, NMR spectroscopy in its various techniques has been reported in the analysis of many different types of foods such as fruits, beverages, vegetables, meat, and meat products. In these foods, the investigation covered chemical characterization, provenance, genetic modification, authentication, and quality control (Spyros and Dais, 2000, 2009).

These NMR techniques can be used to provide information about the time period at which the food was produced, by simply monitoring the disappearances of these nuclei through the isotopic ratio calculations, information about food composition, adulterations, geographical origin, and storage index (how long the food has been kept stored). For example, ^{1}H and ^{13}C NMR techniques can be used to ascertain quality control of foods such as olive oils by monitoring levels and ratio of glycerides. Virgin olive oils contain between 1 and 3% of total glycerides, while fresh olive oils contain 1,2-diglyceride. The diglyceride ratio in fresh olive oil to that in virgin oil will stand at 0.9 or above, because diglycerides become reduced with time during storage. Therefore, this can be used to give a measure for the ageing index for olive oils.

For solid and semi-solid types of foods, magic angle spinning (MAS) has been the NMR technique of choice, but it gives broad line spectra due to the immobility of its chemical constituents. This shortcoming can be minimized by turning the rotation of the sample at the magic angle (θ) to 54.7, which will generate narrow line signals similar to those obtained for liquid types of foods (Spyros and Dais, 2000, 2009). The NMR analysis of semi-solid foods is normally performed using high resolution magic angle spinning (HR-MAS) and cross-polarization MAS is used mainly for rigid solid foods such as grains.

Similar kinds of information can be obtained for other types of foods, such as beer (NMR can be used to provide information about the brewing site; the type of beer, whether lager or ale; whether barley or wheat malt was used in brewing; and ageing as well as storage effects). Other foods and foodstuffs such as vegetable oils, tea, coffee, wines, honey, *etc.* can be analyzed by NMR to provide forensic evidence.

Application of ^{19}F in Food Forensics

The use of ^{19}F nuclei NMR in food forensics has received a lot of attention and

popularity, mainly due to its high abundance in nature (99%), relatively high sensitivity when compared to other nuclei NMR used in the analysis of foods, and also the possibility of labeling with fluorine nucleus further increases its sensitivity, allowing the detection and quantification of the oil species, even the minor ones that enhance its sensitivity and the ability to detect analytes even at low concentrations, including by-products (Petrakis *et al*., 2008).

There are many biological molecules in foods, plants, or animals that are used as food sources and that are composed of fluorine-containing compounds of either amino acids, nucleosides, lipids, or sugars.

^{19}F NMR, like ^{1}H NMR, employs a similar approach in terms of obtaining NMR spectral data, except that it uses a variety of reference standards (Table 12.2) as opposed to ^{1}H NMR, which use mostly TMS (tetramethyl silane) as the reference standard, and is virtually the same as that involved in obtaining proton NMR data. Another marked difference between ^{1}H NMR and ^{19}F NMR can be observed from the fact that fluorine is surrounded by nine electrons vs. one for hydrogen and therefore one would expect that the range and magnitude of fluorine chemical shifts as well as sensitivity would be higher in ^{19}F NMR than in ^{1}H NMR (Tables 12.2–12.3). For example, in a given food amino acid moiety, where there is a replacement with a fluorinated analog, the fluorinated analog will tend to exhibit a spectrum that has s more resolved signal as compared to the same amino acid that did not have replacement of its protons with fluorine.

Table 12.2 ^{19}F NMR reference standards.

Compound	**Chemical formula**	**Chemical shift, δ (ppm) vs trichlorofluoro-methane, $CFCl_3$**
Trichlorofluoro-methane	$CFCl_3$	0.00
Trichloro acetic acid	CF_3COOH	–76.55
Hexafluorobenzene	C_6F_6	–164.90
Monofluorobenzene	C_6H_5F	–113.15
Trifluoro-chloro-methane	$CF_{-28.60}Cl$	–28.60
Elemental fluorine	F_2	+422.92
Monofluoro-	CH_2FCN	–251.00

Monofluoro acetonitrile	CH_2FCN	–251.00
Difluoro, tetracholoroethane	$CFCl_2CFCl_2$	–67.80
Trifluoro-toluene	$C_6H_5CF_3$	–63.72
Tetrafluorosilane	SiF_4	–163.30
Sulfur hexafluoride	SF_6	+57.42
Disulfuryl difluoride	$S_2O_5F_2$	+47.20
Hexafluoro acetone	$(CF_3)_2CO$	–84.6
Para-difluorobenzene	p-FC_6H_4F	–106.00
Boron trifluoride	BF_3	–131 to −133
Hydrogen fluoride (aq)	HF (aq)	–204.00
Carbon tetrafluoride	CF_4	–62.50
Aqueous fluorine	Aq F^- (KF)	–125.30

NB: (+) values refers to higher frequency (downfield shifts where there is lower shielding) and *vice versa* for (–) values.

Table 12.3 Fluorine chemical shifts in various solvents (Gerig, 2001).

Chemical functional group	Chemical shift relative to trichlorofluoromethane (ppm)	Chemical shift relative to trifluoroacetic acid (ppm)	Chemical shift relative to 1-fluorobenzene (ppm)
Substituted fluoromethane containing compounds	–210	–131	–96
Substituted difluoromethane containing	–140	–69	–26

containing compounds			
Mono substituted fluorobenzene containing compounds	–140	–60	–26
Substituted 2,2-difluoroethenone containing compounds	–125	–46	–11
Substituted 1,1,1-trifluoroethane containing compounds	–75	+4	+39
Mono substituted 2,2,2-trifluoroacetaldehyde containing compounds	–81	–2	+33
Sulfonyl fluoride containing compounds	+50	+129	+164

^{19}F NMR can be used to authenticate and identify nucleic acids in biomolecules in plants/animal that provide food sources, in case the nucleic acids contain fluorine or fluorinated compounds or if some of the bases or sugars in the nucleic acids contain fluorine. The same can be said for carbohydrates that are fluorinated. This technique can be employed in the identification of enzyme products that are essential in the formation of essential food molecules. For example, ^{19}F NMR can be used to characterize the regiochemistry of enzymes that play an important role in fatty acid biochemistry (Buist *et al*., 1996).

Phosphorus NMR and the Application of ^{31}P NMR Spectroscopy in Food Forensics

Phosphorus NMR is one of the NMR techniques known to be both non-destructive and non-invasive. This technique makes use of magnetic resonance

of a ^{31}P nucleus to identify the chemical species of ^{31}P that are present in the sample to be analyzed. Phosphorus NMR (^{31}P NMR) is attractive in that it can simultaneously identify all speciation forms of phosphorus present in the sample, for example phosphonates, orthophosphate, pyrophosphate, polyphosphate, orthophosphate monoesters, and orthophosphate die-sters. It has the advantage that all P species can be characterized simultaneously, without the need for complex purification and chromatographic separation procedures (Cade-Menun, 2004). ^{31}P is an isotope of phosphorus that has a spin 1/2 nucleus with about 100% natural abundance in nature. It has a good natural receptivity as compared to other isotopes, for example, ^{31}P is known to be 391 times larger than ^{13}C and the range of its chemical shifts extend from 500 to –200, with the chemical shift of 0.0 being assigned to 85% H_3PO_4, which is taken to be the reference 0 (Gorenstein, 1984 ; Quin and Verkade, 1994 ; Spyros and Dais, 2009).

^{31}P NMR spectroscopy has been useful as an NMR technique capable of supplementing ^{1}H and ^{13}C NMR spectroscopies, because in many cases proton and carbon-13 NMR spectroscopic techniques, when used in food analyses, have resulted in complex spectral patterns with overlapping ^{1}H NMR and also in the case of ^{13}C, the resulting spectra have produced long relaxation times, because ^{13}C nuclei is not that more sensitive and this makes it difficult to deconvolute and interpret the results.

Phosphorus in Foods

Both inorganic and organic phosphorus are known to be important components of the biological materials in both plants and animals (nucleic acids, proteins, adenosine triphosphate, cell membranes, etc.) and are actually part of what are known as essential elements that are involved in various metabolic processes necessary for life. Moreover, phosphorus forms part of skeletal structures (found in bones and teeth) in various complexes, for example as calcium phosphate, *etc.*

Another important aspect of phosphorus is that it is one ingredient that is normally incorporated in certain food additives, such as ammonium polyphos phosphatides in synthetic lecithins, where they are included to enhance emulsification and food texture. Also phosphates in their inorganic forms have been added in soft drinks as acidity regulators. In meat, inorganic phosphates are normally added to impart water holding capacity for meat and other food items such as cheese or bread.

Some researchers have, however, raised some concerns on the application of inorganic phosphates as food additives, although there has not been any solid scientific base to justify such concerns (Vartanian *et al*., 2007 ; Uribari and Calvo, 2003).

Assignment of Chemical Shifts for Food Components in ^{31}P NMR

The analysis of food samples that contain pentavalent phosphorus in the form of phosphate, results in a spectrum showing these pentavalent phosphorus species appearing in chemical shifts in the region between 20 and –40 ppm. Trivalent phosphorus needs to be derivatized before ^{31}P NMR analysis (Spyros and Dais, 2000) and have characteristic chemical shifts appearing in the region between 100 and 200 ppm. The derivatization methods and the choice of derivatizing reagents normally target the replacement of the labile hydrogen atoms in the chemistry of the functional groups, such as CHO, COOH, or OH. The examples of the derivatizing reagents may include 2-chloro-4,4,5,5-tetramethyldioxaphospholane (Jiang *et al*., 1995).

Application of ^{31}P NMR in Plant Based Food Samples

The majority of plant-based foodstuffs such as potato, sweet potato, maize, wheat, rice, oat, millet, etc., contain a high percentage of starch, because it is a major energy source on which plants rely as it is found in plant seeds, roots, and tubers. The human diet from plants harnesses this important polysaccharide in which phosphorus is present in different chemical forms, which are starch, phosphate monoester, phospholipids, and inorganic phosphate (Srichuwong and Jane, 2007 ; Tester *et al*., 2004). The different types and speciation forms can enable discrimination of foodstuffs from different geographical origins or starches from different types of foods, such as tubers (potatoes) vs. cereals (maize, wheat), *etc*. For example, starch from legume sources, such as green peas, lima beans, mung beans, and lentils, as well as waxy starches, have been reported to contain mainly phosphate monoesters, making them easier to discriminate from starches in other foodstuffs of plant origin (Lim *et al*., 1994).

Other plant originating foods, such as fruits and vegetables, have been reported

to be rich in inositol hexaphosphate, which is also known as phytic acid or phytate. However, for this type of phosphate to be able to be digested in the mammalian gut, some specialized enzymes known as phytases present in certain types of foods, fungi, yeast, and some bacterial species are necessary (Vohra and Satyanarayana, 2003). Inositol hexaphosphates are known to have many beneficial effects to humans, including the reported role they play as antioxidants (Wu *et al*., 1994) and they also have good anticarcinogenic activity (Harland and Morris, 1995). ^{31}P can be used to discriminate between phytate in raw foods vs. cooked/processed foods, or foods consumed in different cultural entities, or between leavened and unleavened bread, because there are lots of phytases in leavened bread products, making phosphate more bioavailable than is the case for unleavened bread (Spyros and Dais, 2009). The technique has also been used successfully in discriminating between different types of phosphorus groups found in different plant oils, such as phosphatidylcholine and lyso-phosphatidic acid, as well inorganic phosphorus (Bosco *et al*., 1997).

^{31}P NMR for Foods of Animal Origin

The quality of meat and meat products is normally highly dependent on their water holding capacity, which in turn influences the type and magnitude of metabolic reactions taking place in muscle (Bertram and Andersen, 2007). A number of these metabolic reactions lead to phosphorus-containing metabolites, which can be characterized using ^{31}P NMR as well as circular polarization – magic angle spinning (solid state NMR), which can generate data useful in the fingerprinting of the animal's identity in terms of species, age, geographical origin of the animal, level of phosphorylation, muscular type, or in the case of processed meat, storage conditions, levels and variation of soluble muscle metabolites such as adenosine triphosphate, phosphocreatine, phosphomonoesters, as well as inorganic phosphate (Bertram *et al*., 2004a, b ; Vogel *et al*., 1985). Moreover, Roberts *et al.* (1981) and Burt *et al.* (1976) have reported that ^{31}P NMR measurements for phosphorus-soluble metabolites such as adenosine triphosphate, phosphomonoesters, etc., which have stable pKa values, can be used reliably to ascertain the measure of intracellular pH of meat muscle by considering their chemical shifts.

^{31}P NMR can also be used to discriminate between the different quality classes of pork meat into their respective groups, which include normal pale soft exudative and dark firm dry, by evaluating the measure of the different types and

quantities of soluble phosphorus compounds, as well as a measure of intracellular pH and water-holding capacity. For example, pork meat that is characterized by high ultimate intracellular pH and high water holding capacity belongs to the dark film dry class, while the one that contains low water holding capacity falls under the pale soft exudative class (Honikel, 2004). Also pork meat that is characterized by high phosphomonoesters and low phosphocreatine concentrations implies that meat falls under the dark firm dry class, while the meat with high content of inorganic phosphorus and at the same time low concentration of phosphomonoesters, belongs to the pale soft exudative-prone pork meat.

One of the food products that is of animal origin is milk, an important food source of dietary phosphorus. Milk and other milk-derived products such as concentrated milk, whey, skimmed milk, casein, *etc.* contain different species of phosphorus in terms of types and ratios, such that ^{31}P NMR can be used to identify the presence of these phosphorus species, for example inorganic phosphates and phosphoserine residues, where the normal position of casein or glycerophosphorylcholine can be identified mainly in normal milk and skimmed milk (Spyros and Dais, 2009). ^{31}P NMR can also be used to authenticate milk products from various species by investigating the distribution of phosphorus compounds in milk samples, because the distribution pattern is species specific. Moreover, phospholipids contents in milk samples from different animal species tend to vary with the type of feed in terms of types, amounts, distribution pattern, and ratios, making it possible to categorize milk from different animals (Spyros and Dais, 2009). Sphingolipid content and composition in the milk samples from different animal species can also be analyzed using ^{31}P NMR to authenticate the milk or species from which it originated (Byrdwell and Perry, 2006).

Application of ^{31}P NMR for Seafood Forensics

^{31}P can be used to discriminate fish within and between species, by studying their phosphate metabolism and observing their responses in terms of muscle physiology, biochemical point of view, or their responses toward exposure to certain environmental stresses such as hypoxia, anoxia, *etc.* (VanDenThillart and VanWaarde, 1996 ; VanGinneken *et al.*, 1995 ; VanWaarde *et al.*, 1990). This technique can also be applicable in cases of determining the extent of freshness of fish and effects of heat during preservation and how different fish species can

be differentiated in terms of their responses to freshness after being preserved at a particular temperature (Chiba *et al*., 1991; Yokoyama *et al*., 1996a, b). ^{31}P NMR can also be used to characterize and discriminate fish species based on their phospholipid contents, as well as the degree of formation and ratios of phosphorylated metabolites in different fish species (Gradwell *et al*., 1998).

Application of ^{31}P NMR for Food Additives Forensics

A number of food additives contain phosphorus/phosphates in their chemical compositions. For example, phospholipid extracts (lecithins) from both animal and plant sources are incorporated in foodstuffs such as breads, baby foods, chocolates, etc., where they are used as emulsifiers or dispersing agents due to their unique surface-active properties (Diehl and Ockels, 1995 ; Diehl, 2001). ^{31}P NMR can be used to classify and identify various brands of these lecithin-containing foodstuffs, based on the phospholipid content, type/class, or give an emulsification signature/properties of lecithins for different types of foodstuffs where it is being used as an additive (Helmerich and Koehler, 2003). ^{31}P can also be used to identify lecithins obtained through different extraction procedures or from different geographical locations (Spyros and Dais, 2009).

Other food additives, especially those that play roles as stabilizers and gelling/foam agents contain peptide components (proteins) that impart textural properties to foods due to their characteristic surface-active functionalities (Weder and Belitz, 2003). There are naturally-occurring food stabilizer compounds, such as those found in milk and eggs, which are used in drinks and beverages such as beer; some are used in desserts, sweets, breads, and other baked products. ^{31}P NMR can be used to differentiate and authenticate protein-containing additives based on the stability, type, and quantity of phospholipids, phosphatidylcholine, and lysophosphatidylcholine, or how these molecules interact with other biomolecules such as albumin or fatty acids present (Chiba and Tada, 1989 ; Mine *et al*., 1992, 1993). ^{31}P NMR can also be used to verify whether these food additives have been phosphorylated, as well as the sites that have been phosphorylated (Li *et al*., 2003).

Other phosphate-containing food additives include leavening agents used in the bakery industry. The phosphate compounds used for this purpose include the oligophosphates (inorganic), cyclic phosphates, and linear phosphates, as well as

those that play important roles in the enhancement of water-holding capacity in meat and meat products, and as stabilizers in milk (both ultra-high temperature and sterilized brands) (Spyros and Dais, 2009). ^{31}P NMR can be used to identify at which point or step during the food processing the additive containing such a phosphate was added. It can also be used to track the stability of these food additives under various storage conditions (e.g. effect of temperature, freezing, etc.) (Belloque *et al*., 2000).

Where the presence of organophosphorus agrochemical residues in foodstuffs involve deliberate negligence or poisoning, ^{31}P NMR can be used for both qualitative and quantitative purposes to identify the phosphate-containing moieties for both the parent compounds and their metabolites.

Nitrogen NMR in Food Forensics

There are many nitrogen-containing analogs in foods, such as proteins, nucleic acid bases, *etc.* Therefore, nitrogen NMR should have a special place in terms of different types of applications in the science of foods.

^{14}N and ^{15}N NMR Techniques in Food Forensics

There are two NMR active isotopic nitrogen nuclei, which are ^{14}N and ^{15}N and of the two, the ^{15}N nucleus is characterized by poor or inadequate sensitivity, even though it yields sharp line signals as compared to ^{14}N, which is characterized by providing medium sensitivity and weak and broader line signals due to quadrupolar interactions. Both ^{14}N and ^{15}N share some common properties in that they both produce signals that make it possible for characteristic assignment to nitrogen found in various chemical/biological environments and that the two nitrogen isotopic nuclei have the same chemical shift ranges and that CH_3NO_2 (90% in deuterated chloroform (CDC_{l3})) and also liguid ammonia (NH_3, – under pressure) are used as chemical shift standards, with the latter being used more frequently, especially for ^{15}N. In order to convert ^{15}N chemical shifts to those obtained when CH_3NO_2 is used as a standard in ^{15}N measurements, 380.5 ppm should be subtracted, while 381.6 ppm should be subtracted for measurements involving ^{14}N.

The chemical shifts for nitrogen isotopic nuclei are very wide (0–900 ppm)

(Table 12.4).

Table 12.4 Approximate chemical shift values for nitrogen isotopic nuclei as found in different types of chemical environments when CH_3NO_2 is used as a standard.

Nitrogen compounds	**Approximate chemical shifts, ppm**
Amines	0 – 100
Ureas CH_4N_2O	20 – 140
Amides	30 – 170
Terminal azide	100 – 130
Nitriles R—C≡N	170 – 220
Indoles and pyrroles	180 – 300
Azide center, example	220 – 250
Diazo	200 – 450
[illegible]	[illegible]

Pyridines	220 – 380
Nitro	320 – 380
Polyheteroaromatics (compounds containing many aromatic heterocycles), eg those found in nucleic acids (DNA, RNA) and enzymes	250 – 550
Azo	520 – 580
Nitroso	550 – 900

Multidimensional NMR Techniques

Application of Molecular Spectroscopy in Food Forensics: Rotational-vibrational Spectroscopy

Rotational-vibrational spectroscopy covers that region in the electromagnetic spectrum that is useful in providing information for the identification of molecular species that are subject to exposure to rotational-vibrational radiation or molecular species produced as a result of surface reactions. The techniques that can be employed to deconvolute and study vibrational molecular surface data from either liquid, solid, or gas phase samples, include the infrared (IR), Raman, microwave, *etc.* Apart from these techniques, there are those techniques that can be employed to deconvolute data from the rotations/vibrations of molecules at interfaces and these include electron energy loss spectroscopy (EELS) and sum frequency generation spectroscopy (SFG).

Infrared Spectroscopy

Infrared techniques can be performed in a number of ways, using different principles depending on the sample characteristics (Cotton and Wilkinson, 1989 ; Hoffman, 1988). In the case of solid samples with high surface area, two IR techniques are normally suitable for use:

1. Transmission infrared spectroscopy (TIS): which can be used for solid samples that are infrared transparent; and
2. Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS): which is used for samples that are not transparent enough for TIS.

For low surface area samples, the following infrared techniques are normally used:

1. Reflection-absorption infrared spectroscopy (RAIRS): which is normally used for highly reflective samples; and
2. Multiple internal reflection spectroscopy (MIR): commonly known as attenuated total reflection (ATR).

Generally, the bombardment of infrared radiation triggers molecular vibrations which provides the most information about the presence or absence of certain functional groups. Infrared radiation is a form of thermal energy which, when exposed to molecules, induces molecular vibrations, especially for those molecules with covalent bonds, causing different types of vibrations such as bending, stretching, *etc.* (Table 12.5). However, with IR, specific types of bonds absorb or respond to IR radiation of specific wavelengths. Moreover, not all covalent bonds absorb in the IR region; it is only polar bonds that are capable of displaying bands at such wavelengths. The extent and magnitude of the dipole moment related to the polar molecular bond being analyzed will determine the intensity of the band.

Table 12.5 Example of some selected functional groups, their positions, shape and band strengths in IR spectra of bacteria.

Sources: Beekes *et al*., 2007 ; Helm *et al*., 1991a, b ; Maquelin *et al*., 2002 ; Mauer and Reuhs, 2010 ; Naumann, 2000 ; Naumann *et al*., 1991a,b; Agnieszka Nawrocka and Lamorska, http://dx.doi.org/10.5772/52722; Yu and Irudayaraj, 2005 .

Type of food component	Functional group	Molecular vibrations of the functional group	Wave number (cm^{-1})
Proteins	N─H	Amide (stretching) in proteins	3200
Fatty acids	C─H	$─CH_3$ (asymmetric stretching) in fatty acids	2955
Fatty acids	C─H	$>CH_2$ (asymmetric stretching) in fatty acids	2930
Amino acids	C─H	>C─H (stretching) of amino acids	2898

Amino acids	C—H	≥C—H (stretching) of amino acids	2898
Fatty acids	C—H	$—CH_3$ (symmetric stretching) in fatty acids	2870
Fatty acids	C—H	$>CH_2$ (symmetric stretching) in fatty acids	2850
Lipid esters	—CO—OR (ester group)	>C═O (stretching) of lipid esters	1740
Nucleic acids	—CO—OR (ester group)	>C═O (stretching) of lipid ester group in nucleic acids	1715
Carbonic acid	—CO—OR (ester group)	>C═O (stretching) of lipid ester group in carbonic acids	1715
Proteins		Amide I band components of proteins	1695–1675
Proteins		Amide I of alpha-helical structures of proteins	1655
Proteins		Amide I of b-pleated sheet structures of proteins	1637
Proteins		Amide II band of proteins	1550–1520
		Tyrosine band	1515
Lipid proteins	C—H	$>CH_2$ deformation in lipid proteins	1468
Proteins, carbohydrates, nucleic acids		C—O—H in-plane bending	1415
Amino acids and fatty acids		C═O symmetric stretching of COO— groups in amino acids and fatty acids	1400
Proteins		Amide III band components of proteins	1310–1240
Phospholipids		P═O asymmetric stretching of phosphoester in phospholipids	1240

Polysaccharides		C─O─C; C─O ring vibrations	1200–900
Phospholipids and nucleic acids		P═O (symmetric stretching) in nucleic acids (DNA/RNA) and phospholipids	1085
Fatty acids and proteins	C─H	>CH_2 (rocking) in fatty acids and proteins	720
		Fingerprint region	900–600

Vibrational spectroscopic methods are known to be very sensitive, non-destructive, and are also “first-pass” techniques that require either very minimal or completely no sample preparation procedures. These are attractive attributes in food forensic investigations, because in most cases specimens presented as evidential materials are low in quantities (concentrations and/or the target ingredient may be highly diluted or contained in the complex matrix of the sample). Infrared spectroscopy can provide spectral data information for all molecule dipole moments, such as those containing polar functional groups (e.g. hydroxyl (–OH) and carbonyl (C═O)), whereas homopolar molecules (N═N or C═C), which are not infrared active, are Raman active due to the fact that Raman spectroscopy is dependent on molecular polarizability effects.

Terahertz, on the other hand, uses the regime in the electromagnetic radiation that is found between the microwave and infrared frequencies. The common feature for the three spectroscopic techniques (infrared, microwave, and terahertz) is that they are all non-destructive and have the ability to penetrate non-conducting materials. Terahertz waves are non-ionizing radiations that cannot penetrate liquid, water, or metal, but can penetrate biological specimens such as tissue with low water content, for example fatty tissue. Terahertz radiation can be useful to provide information about the difference in the amount of water, as well as the density of a particular tissue.

Vibrational Spectroscopy in Food Forensics

There are three main techniques that fall under vibrational spectroscopy, which have been useful in criminal food forensics and these are infrared, Raman, and terahertz (THz) spectroscopy (Figure 12.4). These techniques are useful for forensic fingerprinting and identification of the forensic specimen materials presented as evidence, by probing the molecular structure of these evidential materials as characterized by the pattern of vibrational spectral data obtained, either in terms of the presence or absence of certain functional groups. The

spectral data emanating from these techniques can indicate the presence of a particular functional group that is a fingerprint of the suspected forensic material, by using the frequency at which the signal is occurring, shape, signal/band intensity, etc., or by comparison to a matched library pattern of reference spectra populated in a standard library database.

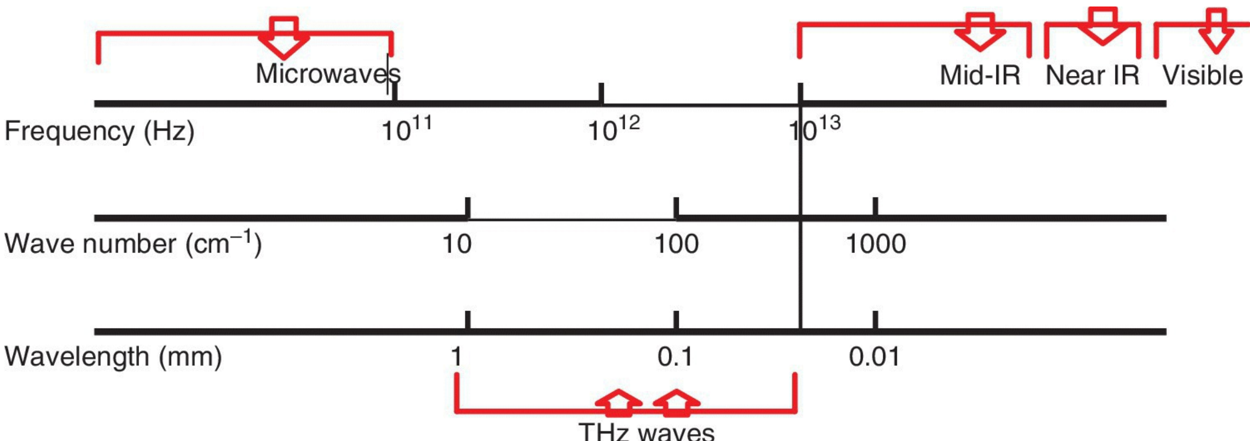


Figure 12.4 Infrared, terahertz and microwave vibrational spectroscopy waves.

The infrared part of the electromagnetic radiation spectrum has three regions, including the near infrared, mid infrared, and far infrared. These regions are located in the electromagnetic radiation in increasing wavelengths, such that the near infrared region covers wavelength bands 12 800–4000 cm^{-1}, the mid infrared covers 4000–400 cm^{-1}, and the far infrared covers wavelength bands 400 to ~10 cm^{-1}. On the other hand, the Raman spectral region covers the wavelength bands between ca. 400 and 5 cm^{-1} to ~4000 and 3800 cm^{-1}, which also encompasses much of the THz absorption wavelength band region. However, for food forensic specimens, the most plausible region will be the one where the normal modes of vibration of organic molecules normally occur and this region occurs in the mid infrared (4000–400 cm^{-1}) or for Raman, the region for organic molecules is 4000–400 cm^{-1}.

Unlike conventional spectroscopic methods, such as microwaves and X-rays, electromagnetic waves that fall within the region of THz radiation, or THz waves, are regarded as not only the modern but also the future technology for sensing and imaging. THz waves have been used in many scientific and engineering disciplines, such as the study of matter (solid, liquid, and gaseous) materials (De Lucia, 2003 ; Jacobsen *et al*., 1996 ; Woolard *et al*., 1999).

Other applications, which THz has been subjected to, include medical diagnosis,

health monitoring, environmental control, chemical and biological identification, and non-destructive evaluation (Arnone *et al*., 2000 ; Globus *et al*., 2003; Markelz *et al*., 2000 ; Walther *et al*., 2000; Woolard *et al*., 2000; Zelsmann and Mielke, 1991), as well as in food and agricultural products inspection.

The many applications of THz spectroscopy stem from the attractive and unique technical features THz waves possess for sensing and imaging applications. These attractive features include the fact that the magnitude of the energy levels of low photon energy, sub-millimeter wavelengths magnitude (in terahertz band measures between 1 and ~10 meV), is the same as the molecular transition. In addition to this, another important attribute is that large-scale molecular resonance is found in the THz band. As pointed out previously, THz radiation is non-ionizing and is well absorbed by polar molecules such as water. Since there are many biological samples (e.g. microorganisms) and chemicals that are active when subjected to THz radiation, this spectroscopic band may be useful in the identification and measurements of such materials. It should be noted that THz radiation waves possess the ability to penetrate various materials, including those used in food packaging with modest attenuation, making it possible for the inspection of food items in their packaging. THz radiations are capable of providing unique spectral signature information about the transitions between rotational states for the intermolecular and intramolecular interactions for even very complex (i.e. microorganisms) and polar molecules.

THz waves can also be used for the investigation of non-polar food components, non-polar liquids/fluids (which are transparent when subjected to THz radiation), and non-metallic materials such as food packaging made of plastic material, due to the fact that these materials are reflective and semi-transparent at THz magnitudes of between 0.2 and 5 THz. Water and ice behave differently when subjected to THz radiation, because water molecules absorb strongly in the THz range, its rotational motion arising due to its dipole properties, but ice, now a crystal, is transparent because the dipole motions in such a crystal molecule have been frozen.

Application of Vibrational Spectroscopy in Food Forensics

The application of vibrational spectroscopy in food forensics is made possible by the availability of a combined full set of spectral information from the molecular analysis of the food samples or specimens from a particular species. This

spectral information reveals the molecular composition, chemical composition, and structural composition, as well as information about molecular interactions for a distinctive target tissue. Such information is normally specific, unique, and characteristic to the target distinctive tissue, because cellular components tend to display a unique and characteristic spectral pattern in terms of the frequency of the vibrational spectrum where they absorb, and the intensity at that particular wavelength and bandwidth. Such spectral patterns, characteristic to a particular tissue, can then be useful in the identification and fingerprinting of a particular tissue in the food stuff or species under investigation.

Vibrational spectroscopy can be used to characterize the functional groups in a distinctive target tissue in food samples presented as evidence in food forensic cases. In addition to this, vibrational spectroscopy can be used to follow the biochemical changes associated with macromolecules (e.g. proteins, lipids, DNA, RNA, etc.) in terms of their levels and composition at various growth stages. Vibrational spectroscopy is also capable of monitoring physical changes in cellular or histological structural changes that involve biomolecules such as lipids, proteins, DNA, RNA, *etc.* However, for vibrational spectroscopy to be of use in food forensic issues, the band spectral data has to be correctly assigned in terms of the position of the absorption frequency, height of the peak signal, peak area, and peak bandwidth.

Infrared (IR) and Raman are known as complementary vibrational spectroscopic techniques that have been extensively applied to deduce molecular information about the sample being analyzed. The information obtained is about the vibrational states of the functional groups of the molecules in the sample, also the molecular imaging, molecular structure, and molecular composition of the whole sample being analyzed (i.e. the sample information is obtained without manipulation and the sample is analyzed as it is). Moreover, infrared and Raman can provide both qualitative and quantitative data about the sample’s chemical composition and concentration, as well as confirmation (Cakmak *et al.*, 2006 ; Chawla and Thomas, 2004 ; Dogan *et al.*, 2007 ; Nissen *et al.*, 2005). Despite the attractive features of these vibrational spectroscopic methods, they still possess some drawbacks. For example, the mid-infrared region of the IR spectroscopy, which is highly useful for the analysis of organic samples, suffers water absorption problems because many biological samples contain water, which then absorbs strongly at several wavelengths, such that the signal due to water molecules masks the vibrational signal due to functional groups from the molecules present in the sample of interest. One of the possible remedies for this problem is to replace water (H_2O) with heavy water (D_2O) during the sample

preparation procedures or analyze the sample in the form of a dry film. This may appear to be the solution to the problem, but it creates other different products. For example, by replacing H_2O with D_2O, the possibility of exchanging hydrogen for deuterium may occur and this will change the pattern of the vibration spectra for the sample. Or if dry film is used, it may imply that the sample integrity may be compromised. A more reliable solution will be to analyze the sample in its aqueous solution state and then subtract the spectral blank (buffer) vibrational signal from the real sample vibrational spectral signal.

With Raman spectroscopy, unlike IR, water does not present a problem, because the Raman spectrum for water is very weak, so cannot interfere or mask the spectra for the sample. However, the only limitation for Raman spectroscopic analysis lies in the fact that the technique itself generates spectra with low probability and therefore to get round this problem, the analyst must ensure that the sample amount (concentration of the analytes in the sample) is high, or otherwise high intensity laser sources have to be employed to enhance the vibrational signal intensity of the sample.

Monitoring of Foods in which Processing has Altered the Composition by Vibrational Spectroscopic Techniques

Some food processing may alter the composition of the final product. This will cause wrong labeling in terms of the presence of various ingredients and their levels and can result in a forensic case, especially if proven that there was unprofessionalism or an intentional or deliberate mishandling in some of the steps involved in food processing.

Application of Mid-infrared Spectroscopy

The mid-infrared part of the vibrational spectroscopy, which extends at wavelengths of approximately 4000–400 cm^{-1} (2.5–25 μm), may be used to monitor and ascertain the structural molecular rotational-vibrations of food samples, which are suspected to have undergone changes after processing. Processing is meant to improve the quality of food by adding some components at some specified conditions of temperature, pH, *etc.* For example, irradiation using various sources at an optimized irradiation dosage is normally practiced for disinfestation purposes (eliminate pathogens). For example, Dogan *et al.*

(2007) reported a study where they investigated the radiation-induced molecular changes in macromolecular components of hazelnut tissues (*Corylus avellana* L.). In their study, they used gamma-radiation from a cobalt-60 source to irradiate hazelnut at two different irradiation dosages, with one low dosage (1.5 kGy) and another higher dosage (10 kGy). The changes in terms of the mid-infrared position of the signal frequency, signal intensity, and intensity ratio of IR bands were then studied and the results showed that when the hazelnuts were irradiated with 1.5 kGy, the level of the total and unsaturated lipid as well as the ration of lipid to protein went up, but at a higher dose of 10 kGy, there was production of peroxide that was recorded and also the levels for unsaturated lipids seemed to go down. The infrared technique allows for the possibility to follow changes in the magnitude of concentration levels of biomolecules (functional groups of biomolecules) by monitoring the spectral signal intensities of the respective molecular functional groups.

Another observation that was recorded at higher dosage of irradiation was that structural changes in terms of protein composition became evident due to cross-linking and aggregation of proteins. Infrared can be used to track molecular structural changes, such as the magnitude of chemical/hydrogen bonding, the order and disorder of membrane lipids, etc., by monitoring the magnitude of the shift of spectral band positions that can be assigned using the wave number reading taken at the midpoint of the band wave (at 0.8 × height of the signal). However, for more conclusive results on the effect of irradiation on foodstuffs, IR results need to be complemented with chemometric methods, such as partial least square (PLS) and least squares support vector machines (LS-SVM).

In another study, Kizil *et al.* (2002) demonstrated that it was possible to employ mid-IR spectroscopy in combination with chemometric techniques, such as principal component analysis (PCA), to streamline the massive data obtained, as well as CVA and PLS models to probe and classify the effects of food irradiation on starch (Kizil *et al.*, 2002). Another report by Vlachos *et al.* (2006) detailed the application of mid-IR in combination with chemometrics to study the trend of corn oil oxidation after exposure to UV radiation (Vlachos *et al.*, 2006). The trend in the oxidation reactions and progress in this case can be easily followed by monitoring specific wavelengths associated with C–H stretching bonds, which occur in wavelengths of 2850–3100 cm^{-1} and also for ester's carbonyl carbon of triglycerides, which occur at around 1745 cm^{-1}.

These reports are among several such publications that show how useful mid-infrared spectroscopy is in providing explanations in food forensic cases associated with inaccurate food composition labeling, which may be a result of

associated with inaccurate food composition labeling, which may be a result of deliberate actions of improper food processing.

Application of Mid-infrared for Authenticity and Compliance Testing of Food Packaging

Currently, food industries, suppliers, and vendors are highly encouraged to move from the use of plastic food packaging to those that are highly biodegradable, such as those made of cellulose or hemicellulose materials. Normally, consumers and vendors have a tendency to warm foodstuffs using microwave ovens, the food wrapped in its packaging just before consuming. However, microwave energy may have a notable effect on these biodegradable packaging materials. Infrared has been employed to study the stability and effects of exposure of hemicellulose and cellulose-based food packaging to microwave radiation. After subjecting the hemicellulose materials to microwave energy, mid-infrared can be employed to probe the lauroylation of the hemicellulose and compare the spectrum profile of the native and lauroylated hemicellulose in terms of peak position shift, increase or decrease of certain peaks, the peak intensity, especially of hydroxyl functional groups that are expected to occur at wavelengths of 3413–3479 cm^{-1}. It should be noted that lauroylation may result in some peaks to disappear or decrease due to the formation of hydrogen bonding.

Application of Mid-infrared to Monitor the Effects of Processing on the Integrity of Food Components

Processes that are followed in food industries are regulated in terms of the conditions and the type of additives involved in the processing, in order to avoid production of undesirable products or loss of important nutritious ingredients. Care needs to be taken in carrying out of all steps during food processing, for example the optimal temperature or irradiation needed and skilled personnel to be involved. The ingredients may be altered during food processing, resulting in mislabeling that may trigger a forensic case.

Mid-infrared spectroscopy, in combination with chemometrics, can be a useful method to follow changes (both cellular structural and compositional) that may be associated with different types of treatment (e.g. high heat, low heat, etc.) during food processing. For example, plant-derived foodstuffs such as fruits and vegetables have their cellular structures enveloped by cell walls. The structure of these cell walls is highly influenced by the structure of various biomolecules in the cells, such as polysaccharides as well as the chemistry of the glycosidic

bonding types that characterize the building blocks for polysaccharides, which are mainly simple sugars (monosaccharide), for example, whether the glycosidic linkages are 1,2-; 1-3; 1,4-glycosidic bonds etc.; bond lengths of such glycosidic linkages; the types of monosaccharides that polymerize to form polysaccharides; the nature of polymerization, and whether it forms branched patterns or linear chains.

Many fruits and vegetables have their cell walls composed of pectin polysaccharides, hemicelluloses, and cellulose, which together play important functions in giving specific and unique structural and textural characteristics to these foods. These biomolecules provide a means of classifying and distinguishing between different species of foods, as they are characteristic to a particular species or variety. This is because different polysaccharides have different chemistries in terms of composition and structure of their monomers (monosaccharides), thus imparting different characteristics that are distinctively unique for each food item.

During food processing, especially where heat treatment is involved, heating facilitates some changes such as loss of rigidity (softening) where the cell wall becomes weakened because of the breaking of the glycosidic linkages, which imply that the depolymerization of polysaccharides is taking place. These changes, which arise due to heat treatment, can be followed by using infrared spectroscopy (mid-infrared). The mid-infrared technique can show the magnitude at which the side chains of the neutral sugar molecules have been altered during the heat treatment procedures. In such processing, the side chains of the neutral sugars are believed to interact chemically with other hemicellulose components such as xyloglucans and therefore mid-infrared will indicate the missing functional groups for both the xyloglucans and the side chains of the neutral sugars.

Also mid-infrared may be used to identify cases that involve mixed foods, where the ratios or types of components are deliberately mislabeled or not disclosed accordingly. For example, mid-infrared spectroscopy can be used to distinguish between pectic carbohydrates (polysaccharide), which contain low methoxylation from one with high levels of methoxyl composition or distinguish foods based on the extent of esterification. The distinction of these food properties and characteristics is possible by studying the spectral patterns obtained where wave number ranges of 1500–1800 cm^{-1} are normally correlated to carbonyl groups of esters and carbohydrates, while wave numbers between 850 and 1200 cm^{-1} are correlated to sugar components based on types and

compositional ratios, which provide a fingerprint pattern of a particular species. The fingerprint pattern of sugars can also be used as an indicator for a ripening stage of fruits or vegetables from a particular species. Mid-infrared spectroscopy can also be useful in the classification of the foodstuffs' polysaccharide, based on the extent of methoxylation or esterification.

Mid-infrared and Chemometric Methods

Generally, the spectral information generated by the infrared technique for food samples is normally highly complex, due to the fact that bio(macro)molecules that form food composition contain a diversity of chemistries and functional groups, with numerous possibilities of engaging in chemical interactions, which complicate further the spectral pattern generated. This makes the interpretation of these spectral data a difficult task. For the purpose of making it possible to simplify the interpretation of such data, multivariate chemometric methods have been employed due to their capabilities to compress massive data and reduce the complexity of information generated. Moreover, for more reliable conclusions and for validation of the results, mid-infrared spectral data are normally complemented with chemometric methods of analyses. These multivariate statistical methods are attractive, because of their capabilities to compress massive data obtained and can also be used to discriminate between different forms of monosaccharides, for example galactose and glucose or disaccharides based on their distinctive characteristic infrared spectral patterns.

The multivariate chemometric tools that have been employed to unpack the complex information generated by infrared spectroscopy, include principal component analysis (PCA), trimmed object project (TOP), outer product analysis (OPA), which is normally useful for unmasking spectral data information, and partial least squares (PLS), *etc.*

PCA and TOP are both exploratory multivariate chemometric techniques. PCA is a bilinear multivariate chemometric method that is useful in the compression and reduction of complex data and can be used to explain variability of properties for a small set of data (compressed) from a complex matrix. PLS, on the other hand, is used to perform predictions and calibrations that play an important role in explaining the relationship between the signal and the measured parameter, according to the appropriate vectoral regressions, in order to provide information about the sample and predict the magnitude of the parameter of interest for the sample under investigation.

Application of Near Infrared in Food Forensics

Unlike the mid-infrared spectral patterns that arise as a result of molecular vibrations at particular frequencies after exposure to mid-infrared radiations, near-infrared spectral information originates from the overtone in combination with molecular vibrations and for this reason, near-infrared spectra are complex, making interpretation somewhat of a challenge. Generally, near-infrared spectroscopic methods are suitable for applications in food forensic issues, both as at-line, on-line, and in-line techniques, especially for the authentication of food and agricultural products.

The attractive features of near-infrared spectroscopy include the fact that the technique is known to provide a rapid means of analysis; it is a proven economical approach and a non-destructive method, which is suitable and convenient for applications that need to establish the correct classification of foods or food quality. However, the application of near-infrared in the probing of the effects of irradiation on foods is not well reported.

A few instances where near-infrared spectroscopy was used in analysis of foods include a report by Barabassy *et al*. (1992), where this technique was employed in the investigation of the effects of gamma rays irradiation on paprika powder, which revealed that gamma rays caused significant changes in the structure of water and hydroxyls as a function of time. In another report, Seregely *et al*. (2006) employed a combination of techniques, which included near-infrared, chemosensor array, and chemometrics to monitor the effects of subjecting egg white to stresses, including high hydrostatic pressure (400 MPa), gamma rays (2 kGy), and low temperature pasteurization (4°C). This study revealed that irradiation and pressure caused significant changes in terms of the quantity of volatiles, whippability, and foam stability. These changes mean a lot to the quality of food in terms of taste, aroma, and other properties, including color.

It is possible to employ both mid-and near-infrared techniques to investigate food quality or as techniques that can enable proper classification of foods (Reid *et al*., 2005).

THz-based Techniques for Food Forensics

Terahertz spectroscopy can find applications in food science, especially in the identification of production area, food process monitoring, and virus and microbial testing in foods and also the analysis of the presence of agrochemicals in foods (Kawase, 2012). These waves are also useful in food forensics cases

in foods (Kawase, 2012). These waves are also useful in food forensics cases, which may involve deliberate food quality and standards violations, poisoning, adulterations, and presence of illegal or banned additives. The mode of action for THz radiation involves the interactions of the THz waves with biomolecules, whereby they initiate low-frequency molecular vibrations, which then promote molecular groupings through the formation of weak hydrogen bonds, van der Waals, and hydrophobic interactions.

There are several THz-based techniques that find application in food forensics, and these methods are discussed below:

THz-time Domain Spectroscopy (THz-TDS)

The instrumentation for THz-TDS involves the source for the ultrafast femtosecond laser. A splitter is also incorporated such that the laser is directed separately into two portions, with one directed to a pump beam (to illuminate the emitter) and another to a probe beam (to illuminate the receiver). In the set up, an optical delay line has to be included in the probe beam to control the variations of the difference in optical delay within the zero magnitude between the THz pulse that is incoming and the probe laser pulse at the detection system. A measure of the magnitude of the Fourier transforms and comparisons of these pulse shapes will provide information regarding the absorption and dispersion pattern of the sample. In order to obtain good results, the THz-TDS system has to be operated under room temperature in order to nullify signals arising from noise background noise, which would otherwise mask the THz-sample signal.

This technique has another advantage, as it offers the possibility to obtain the measurements of both absorption and refractive index of a sample and can also distinguish molecular species or mutants within the same species of organisms. It can also be used to differentiate different molecular conformations (Ferguson *et al.*, 2004 ; Markelz, 2002).

THz-TDS is therefore a suitable technique for the investigation of biological macromolecules that characterize food components such as proteins and tissues, because these molecules are characterized by low-frequency motions that can be found within the THz region. The THz signal for these molecules is produced such that it gives a distinct signature pattern for particular molecules analyzed.

THz-TDS can be used to provide evidential information regarding food materials wrapped in sealed packaging that are transparent to THz radiation. This is possible due to the high specificity of the transitions of the vibrational states that occur for materials that are used in the packaging, which are in most cases crystalline in nature and are triggered by the lattice modes that are unique to

crystalline in nature and are triggered by the lattice modes that are unique to particular crystalline materials and are also a fingerprint of the structure of the molecules. Different food materials will have a distinct fingerprint in the THz-TDS spectral range and therefore they can be recognized when this technique is applied.

THz Pulsed Imaging

THz pulsed imaging refers to an electromagnetic radiation-based technique that is capable of providing in-depth information about the measure of either transmission or reflectivity of the sample after it has been exposed to THz radiation. This technique causes the terahertz waves to penetrate dried samples, plastics, papers, and polar and non-polar organic sample specimens. For the principles of THz imaging for samples that display dielectric properties, their absorption characteristics are controlled by optical phonons as well as other sample properties such as the polarity and the measure of the sample's optical phonon resonance (Mittleman *et al*., 1996). This technique can provide crucial absorption spectral or sample's radiation scattering behavioral information and therefore by analyzing the resultant changes arising from terahertz pulses due to the interaction with samples, one is able to classify and group samples according to how they behave when exposed to THz waves.

THz Continuous Wave Imaging

As compared to THz pulsed imaging, the THz continuous wave imaging technique is inferior in terms of the quality and the depth of either the sample's frequency-domain information or the sample's time-domain information at any fixed-frequency source where the same detector has been used. The data that is generated by THz-continuous wave imaging can suit the intended purposes in all the sample's imaging applications (Karpowicz *et al*., 2005). Despite these limitations, this technique has several attractive features, including the fact that it is fast, compact, simple, and relatively economical as compared to pulsed THz (Karpowicz *et al*., 2005).

THz imaging techniques can be used to detect, fingerprint, and map the quantitative and qualitative composition of the food sample in terms of spatial distribution and concentration.

THz Wave and Molecular Fingerprinting

There is a direct link between the weak bonds that are responsible to make molecules active for THz spectroscopy and various chemical interactions that

molecules active for THz spectroscopy and various chemical interactions that enable the process of binding of substrates or inhibitors to enzymes, and the same bonding mechanisms are known to have a significant impact to the processes related to the transmission of genetic impulses.

This implies that biomolecules such as microbial cells, and their organelles, as well as nucleic acid materials possess optical characteristics that can make them active for THz spectroscopic analysis and can provide very useful fingerprint information about these biomolecules. The ability of THz waves to sense biomolecules, even in the liquid phase, opens the possibility for the fabrication of THz-based biosensors.

Food Forensics Application of Microwave Rotational Radiation

Microwave rotational spectroscopy makes use of microwave radiation as part of the electromagnetic radiation, to ascertain the energies of molecular rotational transitions for gaseous samples. Microwave radiations employs energy generated in the microwave region of electromagnetic radiation spectrum, to cause transitions in the rotational energy levels of molecules existing in the gas phase.

Principle of Microwave Spectrometry

When microwave radiation bombards the sample, depending on the orientation and relaxation, one of the following outcomes will occur:

- There may be field reductions caused by the dielectric properties of the sample molecules, because sample molecules are affected differently, depending on the magnitude of the dielectric constant (ε’);
- Alternating polarization phenomena of the sample molecules may be triggered and the sample molecules will be able to store a certain magnitude of the energy, which is then released slowly. The magnitude of the dielectric constant (ε’) is given a unity value when the microwave radiation passes through the vacuum and for dielectric materials this value is normally above unity;
- Heat loss due to phenomenon caused by the friction that occurs amongst molecules, which in turn causes a reduction of the magnitude of the signal of the wave amplitude and can be monitored by the magnitude of dielectric loss (ε”).

When either ε’ or ε” is plotted vs. the frequency at which the measurement has

When either ε' or ε'' is plotted vs. the frequency at which the measurement has been taken, it results in a spectrum (Walmsley and Loades, 2011).

It should be noted that IR spectroscopy can also be employed to monitor rotational transitions in molecules existing in the gas phase. However, unlike microwave spectroscopy, the rotational transitions in IR spectroscopy are coupled to the vibrational transitions. Raman spectroscopic techniques that are equipped with high sensitivity detectors can also be used to monitor rotational transitions, as they use UV-visible light scattering to ascertain the molecular energy levels (Harris and Bertolucci, 1978 ; Hollas, 2002). Microwave spectroscopy cannot be applied to study samples in liquid or solid states, because of the hindrance of intermolecular interactions. Moreover, compounds that are active in the microwave region are those that possess a permanent dipole moment (e.g. HCl, etc.) and thus possess the capability to absorb or emit radiation in the microwave transitions to produce a characteristic spectrum. Homonuclear molecular species, such as nitrogen (N_2) and oxygen (O_2), are inactive to microwave radiation and thus do not show any rotational spectrum, because the display transitions are not accompanied by any change in the dipole moment during the rotation. Moreover, diatomic molecules that are linear tend to be inactive to microwave radiation, due to the fact that their moment of inertia is small. Microwave spectroscopy can be useful in food forensics that contain volatile components, which are characteristic to that particular foodstuff. Examples of such components include those that impart characteristic aroma, flavor, or taste. In addition, microwave spectroscopy can be used to identify characteristic ions and radicals (reactive species, such as reactive oxygen/nitrogen species) in foodstuffs.

Another attractive feature of microwave radiation is that this radiation has the capability to penetrate materials that are characterized by a low dielectric constant, including glass and plastics. The limitations of microwave radiation in terms of its penetrability powers is that it cannot penetrate materials made of metallic substances because with these kind of materials, microwave radiation is reflected.

Different Microwave Techniques for Food Forensics

Non-contact Reflective Mode Microwaves

The non-contact reflective mode microwaves technique has been applauded and

described as the best suited for the measurements of the moisture content in cereals (Knöchel *et al*., 2001), measurement of water uptake, fat content, protein, salt, water, and phosphate in meat and meat products (Kent *et al*., 2001). For example, Jayanthy and Sankarranarayanan (2007) used the microwave technique to detect moisture content in spices, where the dielectric properties of spices were correlated to grain moisture content. The technique has also been used in the characterization of sugar content in yoghurt, measurement of protein, carbohydrates, and smaller organic molecules, which possess dipole moments (Bohigas, 2008).

Guided Microwave Spectrometry (GMS)

The guided microwave spectrometry (GMS) technique has been described as the most suited for ground meat and is attractive due to the fact that 100% of the sample is measured (Hildrum *et al*., 2006). This technique has been used in the indirect measurements of fat and oils, which are not microwave active, but since there is a relationship between fats/oils and water and proteins, which are microwave active, then it is possible to exploit this relationship and obtain measures of fats and oils. Cataldo *et al*. (2009) reported the use of microwave in the measurement of quality of vegetable oils. It should be noted that vegetable oils are controlled and regulated due to numerous adulteration cases. When using microwave spectroscopic techniques for oil measurements, one fact that is useful is that different magnitudes of dielectric moments can be associated with different oils. Also, there are certain characteristic frequency ranges that provide the possibility for the permittivity of different types of oils that can be selectively distinguished from others. This makes it possible to use microwave spectroscopy to fingerprint different types of oils and also their sources. Measurements of food samples using microwave spectroscopy are both quantitative and qualitative, because the signal intensities in the generated spectrum are related to the concentration of the food samples. The technique is advantageous in that it has the capability to penetrate droplets of up to several centimeters and is appropriate for heterogenous samples. It is fast, economical, and can provide multi-parameters in terms of measurements. The limitations of GMS are that the technique is associated with poor sensitivity when measuring particle size or when it is used to differentiate color. Also, it cannot work for frozen food samples or ice. It also does not work when salt is present, as salt occurs as an interferent.

Food Forensics Application of Multidimensional

Raman Spectroscopic Pattern Signatures

As discussed above, infrared as well as other methods have their limitations, both qualitatively and quantitatively, and so does Raman spectroscopic methods of analysis. Raman spectroscopic methods are based on the inelastic scattering of laser radiation when it is bombarded and thus interacting with vibrating molecules. Just as with the infrared spectroscopic technique, Raman spectroscopic methods are known to be non-destructive, require minimal or no sample preparation, and are fast. They are regarded as confirmatory methods for identification of analytes and are very sensitive. When standard Raman spectroscopic methods are employed for the identification of an unknown analyte, the confirmation is based on either the presence of certain characteristic peaks or a comparison of the sample that generated the spectra to other known/certified reference spectra or from known databases. However, most of food forensic samples have a complex composition present in a complex matrix, such that it is most likely that the matrix constituents may cause masking of the signals due to the analytes of interest or cause overlapping that will complicate the interpretation of the spectra. To overcome this bottleneck, multivariate chemometric methods have been complemented to Raman methods and in addition to this, multi-dimensional Raman spectroscopic approaches (those that are capable of providing a set of spectra that represent major unique variations of the sample) have also been employed to provide spectral pattern signatures that offer advantages that minimize the shortcomings of the traditional Raman methods. In this case, the possibilities of encountering false-negative or positive observations are greatly minimized (Sikirzhytskaya *et al*., 2012; Sikirzhytski *et al*., 2012; Virkler and Lednev, 2009, 2010a,b).

Chemometric methods form an important component to complement Raman methods, because food samples are composed of biological macromolecules such as nucleic acids, chromophores, amino acids, etc., which are known to result in a complex pattern of spectra, making it difficult to deconvolute. A mixture of functional groups in all these macromolecules will provide a complex and overlapping of spectra, covering many regions of the electromagnetic radiation, for example, UV, Vis, IR, and even NIR.

Conclusions

Spectroscopic methods (atomic and molecular) rely on the behavior of organic and inorganic molecules as they are bombarded by specific regions in the

electromagnetic radiation spectrum (light). Some molecules will absorb light from a certain region (e.g. UV-Vis) and they will therefore present a particular signal at that wavelength, and some will vibrate (IR), *etc.* Therefore, these methods are dependent on the property of the molecule. The instrumentation for spectroscopic analysis ranges from moderately expensive to very expensive (e.g. high resolution NMR) and the skills required for the analysis follows the same pattern, medium to highly skilled personnel.

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13
Application of Microscopy Techniques in Food Forensics

Microscopy methods are attractive in that they can provide information about the components of food and also evaluate foods for the possibility of food adulteration or fraud. Moreover, they open the possibility to study and investigate the existing relationship between the structure of food components and the physical or functional properties of foods, thus providing means of detecting possible adulteration or any other changes due to either omission or introduction of foreign substances into foods. When conventional microscopy techniques are employed in the analysis of foods, changes in the internal structures of food materials can occur due to the fragility of foods, which is a prohibitive property to the proper analysis using such techniques. To avoid this phenomenon, techniques such as rapid freezing of foods prior to microscopy analysis can be employed to preserve the integrity of foods in terms of their chemical and structural components.

Microscopy methods also play a crucial role in enlarging or magnifying minute structures to make the process of studying them easier. Forensic food microscopy methods are very important in food forensics, because they are not only useful in the identification of foreign substances in foods but also play a significant role in the whole process for the investigation of the food component's characteristics as well as facts that may be used to determine when and how the suspected fraudulent foreign substances were introduced into the food products. Microscopy methods may be used to determine whether the suspected fraudulent substance was processed together with the food product during the processing or whether it was introduced in post-production steps in the already finished food product. These methods can also be employed to ascertain whether particular vital procedures in food processing such as pasteurization have been done or performed at the appropriate temperature. Microscopy can be used to identify the match between materials used in a particular food industry, for example, plastics and metal utensils, etc.

In general, food-based microscopy techniques can provide information about food components and food quality, as well as food adulteration and the

presence of potential health hazard ingredients in food.

Forensic Food Microscopy Methods

There are a number of microscopy techniques that are used in food forensics and they include different types of light-and electron-based microscopy techniques. Examples of light microscopy include dissecting microscopy, optical microscopy, bright field microscopy, confocal laser scanning microscopy, fluorescence microscopy, *etc.* Electron microscopy examples include scanning electron microscopy (SEM) and transmission electron microscopy (TEM), *etc.* Other types of imaging scanning microscopy techniques used in food forensics are atomic force microscopy (AFM), X-ray microscopy, microscopy resonance imaging (MRI), positron emission tomography (PET) imaging, *etc.*

Light Microscopy Techniques

Food samples that are meant for light microscopy analysis normally have to be prepared by employing specific stains and staining techniques to differentiate the various internal microstructures, thus making their identification easier. After staining, the microstructures become more distinct and their morphological characteristics (shape, size and the mutual cell configuration, the presence of crystals, grains, or other elements) can be used for identification and classification. For example, hematoxylin-eosin (which imparts shades of red coloration) and toluidine blue (which imparts shades of blue coloration) have been used to distinctively reveal microstructures of foodstuffs of both animal and vegetable origin (Pospiech *et al*., 2011) According to Pospiech *et al*. (2011), light microscopy techniques can also reveal adulterations due to the presence of one type of tissue into another foodstuff, for example liver tissue in pâtés, salivary glands, head trimming products, natural guts as casings, presence of banned spices, food colors and other food additives.

There are also several staining techniques that are normally employed with food microscopy and they target specific analyses or tissues. For example, Calleja staining (to impart green coloration), blue Trichrome (to reveal re-processed food products), or using Picrosirius red (Flint and Pickering, 1984), seem to target collagen which forms part of the connective tissue. On the other hand, alizarin red can reveal the presence of bone fragments in foods and Sudan III can indicate the presence as well as the microstructures of adipose tissues.

In the case of plant-based foods, special staining methods based on polysaccharides have been devised. For example, starches can best be distinctively stained using Lugol-Calleja stain; also vegetable polysaccharides can be stained using PAS-Calleja stain (Valchař, 2005). For additives or spices such as black pepper, paprika, caraway seed, marjoram, coriander, allspice, etc., which can suitably be used for verification, purity and identity, can be stained pink by Schiff's reagent (Pospiech *et al*., 2011).

The detection of food adulteration or the presence of other health hazard ingredients in food using microscopy methods is possible due to the fact that the basic microstructures of both plant and animal origin foods are known and well documented. Therefore, as long as the microstructures are visible under a light microscope, it is possible to identify the additional or missing microstructures. Microscopy can detect and identify different types of tissues and food components that are characteristic to a certain type or class of foods and this strengthens the technique to be suitably used to detect adulterations or origin of the food.

In the case of the identification of plant-based components in foods such as the addition of starchy foods, flour. etc., staining can be applied to identify them due to the presence of specific structural features such as polysaccharides, aleurone cells in cereal seed coats, palisade cells included in soya, or reserve protein. Stains that can be used in these cases may include the Lugol-Calleja stain (which imparts dark purple to black to the starch) or the PAS-Calleja stain (which imparts a pink color to polysaccharides). The presence of fibers, such as those employed in food industries as additives and which are starch-based, can be verified by light microscopy based on their distinctive morphological characteristics. They tend to be disordered, lacking any definitive shape. For example, additives based on vegetable protein tend to possess the characteristic shapes of sponges (especially for wheat protein), and sickles or rings due to the presence of saccharides (Flint and Pickering, 1984). It is also important to note that in order to differentiate pure vegetable protein from other proteins, the product basic staining with toluidine blue has to be employed, as it will impart to the vegetable proteins different shades of blue coloration, for example, to wheat protein: light cyan; and to soy protein: dark blue, and this will contrast other microstructures present in the food product (Tremlová and Štarha, 2002; Tremlová *et al*., 2006). Moreover, other specific microstructures in plant-based foods, such as palisade cells and cup cells in soybean flour or textured soy protein, or aleurone cells in wheat flour, can be used to indicate any adulteration in food.

It should be noted that when microscopy is coupled to imaging techniques, it can be used for quantitative analyses.

Types of Light Microscopy Techniques for Food Forensics

There are different types of light microscopy that are available for food forensics and they are discussed briefly below.

Dissecting and Optical Microscopy

Dissecting microscopes with low-magnification may be useful in food forensics because they can be used to magnify food structures, thus enabling a better image. For example, they can magnify minute structures in food fibers, additives which are meant to spice the food, contaminants in foods, *etc.* Dissecting microscopes with high resolution can provide in-depth information as compared to the low resolution ones, for example, information about the changes on food components that may take place during food processing procedures and information about the cause of such changes.

Optical Microscopy

Like dissecting microscopes, the optical microscopy employs light and lenses to magnify small structures of cells (cell organelles, etc.) or small organisms (single-celled/acellular organisms). There are different types of optical microscopes that may have applications in food forensics and they are described below.

Polarizing Microscopy

Polarizing microscopy is a contrast-inducing microscopy technique mostly used in food forensics to investigate food structure. Polarizing microscopy light that vibrates in a single direction only (polarized light) is directed to bombard the sample specimen. The food sample specimen has to have anisotropic or birefringent structural components that are capable of rotating the light plane. Isotropic structures with only a single refractive index cannot rotate a plane-polarized light and thus are inactive to polarizing microscopy. For foods with active structures to rotate the plane-polarized light, a contrast will be obtained due to the fact that the emerging light beam from the sample will be altered, because once it has passed through the sample and rotated, it will be twisted

and/or somehow become extinguished to some extent.

Bright Field Microscopy

In bright field microscopy, species in a food sample that possess chromophores (functional groups having the ability to absorb light that passes through the sample) can be visualized when the sample is bombarded with light (from an incandescent source) and the difference in absorption can be recorded.

Fluorescence Microscopy

Fluorescence microscopy involves illuminating food samples using a specific electromagnetic band of wavelengths and the bands are absorbed by fluorophores in the food sample, causing the absorbing molecules to emit light bands of longer wavelengths. In this type of microscopy, the difference in excitation and emission wavelengths is computed to produce a signal with high contrast between sample and background.

Confocal Microscopy

In confocal microscopy, point illumination of the sample is done in various spots in the sample (scanning of the specimen) to enable the creation of an image of the whole sample. Confocal microscopy is thus useful in cases where an understanding of the performance of the food products as well as its ingredients during processing is needed, because it enables a real-time visualization of even complex microstructures of food samples. It is also the technique of choice for the simultaneous visualization of food in a process that incorporates the addition of specific dyes to enable the identification of the food components' microstructure in foods such as protein, fat and starch granules. In this case, this technique is suitable for the understanding of the distribution of the ingredients within the microstructure in various food products. Confocal microscopy can also be used to track changes in food microstructure that take place during food processing or even during food eating. This is due to the fact that food processing conditions have some control in the stability of the food products. The technique can also be useful to study the distribution and microstructures of food flavorings and biopolymer additives that enhance the flavor and texture of foods, such as emulsifying additives, stabilizing additives, carbohydrates, or thickeners to detect any possible adulteration or fraud.

Electron Microscopy

Electron microscopy is one of the techniques that has wide applications in many disciplines, including its use in the evaluation of the microstructure of food as well as other biological products. In electron microscopy, electron beams are used to irradiate the sample specimen and this prompts the need for appropriate sample preparation methods for each food type or for the type of information needed. Various electron microscopy techniques use different principles and are useful for particular analyses to reveal different kinds of information needed from the sample. These techniques include transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryo-SEM, cryo-TEM, and environmental scanning electron microscopy (ESEM).

In electron microscopy, unlike the light microscopy techniques, a beam of electrons (negatively charged particles that behave like radiation with very short wavelengths) is used to illuminate the sample instead of light. In this technique there is thus a requirement for high vacuum inside the microscope. Electron microscopy is more superior and advanced than light microscopy, as it is capable of providing a much higher magnification at far better resolution than with light microscopy. The main limitation of electron microscopy is that it is more expensive, both in terms of procurement of the machine, running costs and costs of sample analysis.

For the purpose of food forensics, SEM and TEM are the major electron microscopy modes mostly needed. The modern scanning electron microscopes can find applications in the investigation of fragile food samples that are hydrated, such as moulds (fungi and their spores), fruits and vegetables, without the need to dry or freeze the samples (Katsaras, 1994).

However, when using traditional electron microscopy, there are certain requirements that must be observed and these include the fact that the food samples in question should not release any gas or vapor when introduced into either the transmission or scanning electron microscope. Moreover, any food sample that contains water (hydrated) must be dried or frozen at a very low temperature (mainly at –100 °C) in order to eliminate any possibility of the food sample releasing gas or vapor. The exceptions to these include powdered foods samples such as maize/wheat flour (or any food flour), sugar and powdered milk.

Conventional Scanning Electron Microscopy (SEM)

Conventional scanning electron microscopy (SEM) is a technique that reveals the surface chemistry of the food sample specimen and can thus point to any deviations from the norm in the case of adulterations. Food samples (previously dried) are normally coated with carbon, gold or metal to dissipate charges and

dried) are normally coated with carbon, gold or metal to dissipate charges and provide a path for the electrons, thus enhancing electron absorption by the sample microstructures.

When a beam of electrons has been directed towards the sample specimen, the resultant scanned image that is generated is normally formed step by step and so it is not just a one-step process. In the first step, the primary electrons that are directed towards the sample are deflected by elastic scattering processes (Bogner *et al*., 2007). The use of SEM in food forensics is attractive due to the many advantages that SEM offers, which include its relatively simple sample preparation procedures, wide range of magnification, high depth of field and also the image that is generated is a representation of electronic data that enables both image analysis and quantification options to be performed simultaneously (Aguilera and Stanley, 1999; Fazaeli *et al*., 2012).

Cryo-scanning Electron Microscopy

Cryo-scanning electron microscopy (cryo-SEM) is one of the variants of SEM that is used to observe and investigate ice crystals in frozen samples, even without the need for thawing (Aguilera and Stanley, 1999; Fazaeli *et al*., 2012). Unlike conventional SEM, analysis involving cryo-SEM requires more sample preparation steps, such as coating with metal in order to facilitate and enhance the conductance of electrons (Preetz *et al*., 2010). The use of cryo-SEM is attractive in food forensics, as it is not associated with the potential presence of artefacts as in other SEM techniques (Preetz *et al*., 2010).

Environmental Scanning Electron Microscopy (ESEM)

The environmental scanning electron microscope (ESEM) is another variant of SEM techniques that are used in food forensics. It is attractive because it allows even wet samples to be analyzed, without any appreciable prior sample preparation such as drying or dehydration, but samples can be analyzed in their very original state. This technique is useful in the *in situ* dynamic testing of foods. For example, it can show ageing of foods to detect any mislabeling of expiry dates, changes of food components with time, *etc.*

Unlike conventional SEM, ESEM differs, requiring the presence of a gas in the sample specimen chamber such that samples are not viewed under high vacuum but rather under a diminished vacuum (Fazaeli *et al*., 2012). The presence of the gaseous environment around the sample is vital as it acts as an electrical charge conductor avoiding sample charging and therefore facilitates signal detection

and also acts as a conditioning medium, which will not allow the evaporation of liquids from the sample (Thiel and Toth, 2005).

Sample Preparation for Scanning Electron Microscopy (SEM)

(i) Preparation of Viscous Foods for Scanning Electron Microscopy

Due to the difficulties in sample preparation of viscous food specimens such as butter, stirred yogurt, different types and forms of creams, mayonnaise, *etc.* with tiny microstructures for SEM analysis, approaches that involve concentrating the individual specimens in agar gel capsules have been suggested by Salyaev (1968) as the solution to this limitation. The steps for sample preparation of viscous foods specimens for SEM analysis can be obtained from articles published by Sayaev (1968) and Kalib (1988), *etc.*

(ii) Preparation of Fat Foods for SEM Analysis

In certain foods such as milk, saturated fat that is present in milk fat globules tends to crystallize at refrigerator temperatures and does not interact with osmium tetroxide or stains. In order to distinguish it from unsaturated fats (when using SEM), some techniques need to be followed that from involve the use of osmium tetroxide, which is buffered by imidazole to make it possible to distinguish the two types of fats by light, color and also by the sharpness of crystalline outlines from unsaturated lipids. However, the sample has to be rapidly dehydrated in a graded ethanol series, and some solvents such as chloroform or n-hexane should not be used.

Conventional Transmission Electron Microscopy (TEM)

In conventional transmission electron microscopy (TEM), the operation has to take place under vacuum to prevent the deflection of electrons by gas molecules. When the sample has interacted with a beam of electrons and the electrons are scattered and thereby are creating a contrast, it then results in the generation of an image. The TEM technique is useful in the understanding of internal structures of food samples; tissues, *etc.* and thus it can be suitable to reveal changes in food samples that can provide evidence of the effects of the treatment processes or adulteration practices that may affect the structure of foods (Katsaras, 1994).

The general protocol for sample analysis using TEM starts with fixing the tissue

glutaraldehyde, dehydrating in alcohol, embedding in plastic by immersion in a solution and polymerized by heating, cut into ultrathin sections of approximately 50–10 nm with an ultramicrotome, and staining the sample with heavy metals for contrasting purposes. This technique is applicable for direct analysis of colloids in the vitrified frozen hydrated state and also in those instances where information about the internal structure of the colloidal system is needed (Kuntsche *et al*., 2011).

Sample Preparation Methods for TEM

The use of TEM for food samples involves a number of sample preparation techniques prior to the introduction of the sample to the instrument. These methods and techniques include:

Embedding the Sample Specimen in resins

In this method, the sample specimen of food is embedded in a particular resin, sectioned by means of a microtome, stained using heavy metal salts (e.g. metal salts of lead or osmium) and introduced into a TEM instrument for analysis.

Negative Staining

Alternatively, the sample can be subjected to negative staining, a process which involves mixing of very small amounts of food samples such as milk products (e.g. casein micelles, etc.) with solutions of heavy metal salts on thin electron transparent films and then drying and introducing into a microscope.

Metal Shadowing

Another TEM sample preparation procedure that is used for TEM analyses is known as metal shadowing, which involves drying the food samples on a translucent film and then shadowing with platinum vapor under vacuum. It is then introduced into a TEM instrument for analysis.

Freeze-fracturing and Freeze-etching

Another sample preparation technique for samples that are to be analyzed using TEM is freeze-fracturing and freeze-etching, which can be performed without the need for chemical fixation alteration or physical alterations (e.g. drying, embedding in a resin, expelling water/dehydration, etc.). This process, however, involves rapid freezing of the food sample and then freeze-fracture is at low temperatures below –110°C. The sample will then be thawed before analysis

with a TEM.

Preparation of Viscous Foods for TEM

Encapsulation techniques can be employed for food samples in both SEM and TEM. However, much smaller capsules are required for samples that are to be investigated using TEM as compared to the case with samples for SEM. For the latter technique, considerably smaller capsules would be required than for SEM. The details of this procedure can be obtained in other publications (Kaláb and Larocque, 1996; Veliky and Kaláb, 1990).

Sample Preparation of Protein Fat Foods for TEM

Fat foods that are to be analyzed using TEM have to be fixed using appropriate reagents. For example, proteins need to be fixed using glutaraldehyde, while post-fixation using osmium tetroxide followed by glutaraldehyde is suitable for unsaturated lipids present in foodstuffs. In this post-fixation process of unsaturated lipids in foods, there is a high possibility of the fatty acids unsaturation points having chemical reactions with osmium tetroxide (through the double bonds) and converting the fatty acids to saturated ones. To avoid this, the hydrolysis step is normally carried out immediately to get rid of the osmium tetroxide (Allan-Wojtas and Kaláb, 1984; Angermüller and Fahimi, 1982; Greyer, 1977).

Electron Energy Loss Spectrometry (EELS) and Electron Spectroscopic Imaging (ESI)

Electron energy loss spectroscopy (EELS) and electron spectroscopic imaging (ESI) are useful in food forensics for the investigation of nano-and microstructures for authenticity and detection of adulterations and contamination cases in foods. EELS can be used in conjunction with transmission electron microscopy (TEM-EELS).

Other Microscopy and Imaging Techniques in Food Forensics

Other imaging methods, apart from light and electron microscopy, which have found application in food forensics, include the scanning probe microscopy (SPM) techniques (including AFM and SPM), magnetic resonance imaging

(MRI) and acoustic microscopy.

Scanning Probe Microscopy (SPM) Techniques

Scanning probe microscopy (SPM) is one of the microscopy technique variants useful in creating the images of sample surfaces using a physical sharp probe that scans the surface of the specimen being investigated. SPM comprises of a range of techniques which all employ a scanning sharp probe in close proximity to the surface of the sample, to provide measurements of certain parameters in relation to the distance between the sample material and the probe. The SPM family of techniques include atomic force microscopy (AFM) and scanning tunnel microscopy (STM).

Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) is a technique that reveals the surface of the sample (micro-and nanostructures in foods, biological structures such as tissues, cells, bio-molecules) in three dimensions. This technique is based on the measurement of interactions between a sharp tip and the surface of the sample (Binnig *et al*., 1986) and in the process, the surface is scanned using a mechanical probe known as a cantilever and the deflection of this mechanical probe is detected by a focused and reflected light beam that strikes a position sensitive detector (PSD).

Unlike optical and electron microscopy techniques, which generate images in two dimensions as they cannot provide a vertical dimension (*Z*-direction) measurement for the sample (only height (for particles) and depth (holes, pits)) measurements, AFM can provide three dimensions (horizontal *X*-*Y* plane and the *Z*-vertical plane).

In food forensics, AFM is useful for the investigations of microscopic food components before, during and after food processing, to see whether the surface topography properties that influence textural characteristics of food change and the effect of processing on surface topography.

AFM Associated Imaging Modes

There are three main imaging modes used in AFM operations, which include contact mode, non-contact mode, and tapping mode.

In the contact imaging mode, electrostatic and/or surface tension forces from the adsorbed gas layer pull the scanning tip toward the surface and the whole

process takes place using a tip connected to the end of a cantilever. Since the operation under contact imaging mode applies a lot of force towards the sample, there is a high possibility of resulting in distorted or low-quality images, especially when dealing with soft samples (Power *et al*., 1998).

Non-contact AFM imaging mode has limitations in that it results in images of low resolution due to its mode of operation.

In tapping AFM mode, the cantilever oscillation amplitude and phase is measured while the feedback is maintained to keep the oscillation amplitude fixed such that the amplitude, phase and topography can be imaged simultaneously. Tapping AFM imaging mode is known to provide high resolution and is not associated with the application of destructive frictional forces to the samples, whether in air or fluid medium. The mode is appropriate for even very soft and fragile samples, as well as structural components of the membrane. Thus for food forensics, this will present the most suitable mode of operation, despite its limitation as being somehow slower when compared to a contact mode.

For example, AFM can be used to study polymer and polymer matrix as well as biological structures such as applications in distinguishing proteins and polymers in foods after different types of treatment and how the microstructures change (Gunning *et al*., 2004; Woodward *et al*., 2004); monitoring of how macromolecules change with different types of food manipulations (Yang *et al*., 2006); and analysis of gene location after fixing DNA in polymer-coated glass substrates (An *et al*., 2005; Nakao *et al*., 2002).

Potential Application of AFM in Nanofood Simulation, Optimization and Characterization

AFM has the potential for use in the simulation process of creating nanostructures from individual atoms or molecules in self-assembly procedures to create what is envisaged at the moment to be nanofoods. AFM can facilitate in the simulation, optimization and characterization of nanofoods to make the whole process more feasible, economical and practical.

Scanning Tunneling Microscopy (STM)

In the scanning tunneling microscopy (STM) technique, the current of electrons generated by atoms at the tip of the physical probe are tunneled to the surface of

the sample specimen to create an image of the sample's surface topography. One requirement for STM is that both sample and tip must be good conductors, a condition which prevents the analysis of many samples of biological origin.

Other Scanning Probe Microscopy Techniques

Magnetic Force Microscopy (MFM)

Magnetic force microscopy (MFM) is normally used to measure magnetic force associated with magnetic materials.

Scanning Capacitance Microscopy (SCM)

Scanning capacitance microscopy (SCM) is used to ascertain capacitance developed in the presence of tip near sample surface for conductors and solids.

Stylus Profilometer (SP)

Stylus profilometer (SP) is used to study and investigate surface properties for conductors, insulators, semiconductors and solids.

Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) is a technique that is based on nuclear resonance magnetic spectroscopy, which can be used to detect concentrations of nuclei with spins such as that of a proton, phosphorus, nitrogen, *etc.* This technique can thus be applied to investigate the measure of the distribution of water and lipids in foods.

X-ray Microscopy Non-destructive Methods

X-ray microscopy is a non-destructive technique that takes into account all the advantages of the magnifying power of optical microscopy and combines it with the penetrating power of X-rays, to generate high-quality two-dimensional images of minute microstructures. It also has advantages associated with the capabilities of 3-D tomography to generate high resolution 3-D images of the sample.

Conclusions

A number of microscopy-based techniques have the potential to provide

solutions to food forensics issues; however, care should be taken by the analysts to match correctly and appropriately these techniques to appropriate properties of the food sample composition that is being investigated, the nature of the sample and its matrix environment and other practical requirements. These techniques can be used successfully to investigate the basic structural properties of different kinds of foods and the differences within and between varieties, origin, the effects of processes such as different kinds of drying (hot air drying, spray drying, microwave, osmotic drying, freeze drying and superheated steam drying), freezing, high hydrostatic pressure, pulsed electric fields, and ultrasound on foods microstructure. These techniques can also be used to reveal the relationship between food processing parameters, conditions and morphological changes of the food components. However, in as much as there are a number of state-of-art microscopy related techniques that may be applied to provide solution to food forensic cases, this can be of value only if scientists or technicians operating these equipment/instruments are capable of manipulating the techniques and also capable or encoding the results generated (Ramírez and Aguilera, 2011).

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14
Application of Ionizing Radiations (X-rays/γ-Rays) Techniques in Food Forensics

X-ray Techniques in Food Forensics

X-rays or X-ray waves/radiation are positioned at the high energy/short wavelength part of the electromagnetic spectrum, being sandwiched between ultraviolet light and gamma rays. Their wavelengths extend to around 0.1 nm, a distance magnitude that is comparable to interatomic distances, and this makes it attractive to use X-rays to study atoms, interatomic bonds and atomic microstructures. In principle, X-ray analysis is capable of providing information in the form of the energy spectrum of the X-rays emitted by the sample, from which the composition in terms of atoms or elements can be identified, because the emitted X-ray is characteristic of that element or atom present in a particular chemical or biological environment (Heathcock and Gibson, 1990).

X-ray radiography technology is one of the techniques highly useful in food industries. For example, it is used to inspect grains like wheat for possible internal infestation that can be caused by certain insects. In this particular application, X-rays can classify stages of insect development simply by determining the insect morphology as well as the area occupied by the insect.

X-ray techniques, like all the other techniques discussed in previous chapters, are highly useful in food forensics due to ever-increasing demands by consumers as well as food regulatory authorities who are responsible for food safety, food quality, issues related to fraud, and intentional contamination of foods (European Commission, 2012).

There are different types of instruments or equipment that have been designed for X-ray inspection and analysis of food products in terms of identifying physical contaminations such as metal, glass, rubber, stone and some plastics (Graves *et al*., 1998). The application or choice of a specific X-ray technique is highly dependent on the nature of the food and the specific purpose of the analysis. The X-ray technique is attractive in food analyses due to the fact that it provides non-destructive imaging, making it very appropriate for analysis and inspecting packaged foods and processed foods, such as bottled drinks, canned

foods, and foods kept in jars and pouches (Haff and Toyofuku, 2008). Therefore this technique has found applications in in-line food production control, food verification and even in the grading of fruits, vegetables, grains and is suitable as a method for the detection of bones in chicken and fish (Haff and Toyofuku, 2008; Mery *et al*., 2011). For packaged or canned foods, X-rays can be used to monitor fill levels, packaging seal integrity, count and mass of the food product. X-ray techniques have also found application in the analysis to detect morphological changes that may occur in barrier packaging materials or structures, as such alterations have the potential to affect the packaging barrier properties, especially where the food needs to be treated to make it ready for preservation (López-Rubio *et al*., 2005). Some foods such as milk are known to contain crystalline complexes and can thus be analyzed and characterized using X-ray techniques (Holt and Hukins, 1991).

X-ray Techniques for Food Forensics

X-ray Microtomography (XMT)

X-ray microtomography (XMT) is a three-dimensional imaging X-ray technique that is useful in the analysis of various internal structural properties. Unlike other X-ray methods, XMT methods when applied directly to the sample are destructive and for this reason, before analysis using XMT, samples have to be prepared by cutting in order to expose a cross-sectional area that can be viewed.

The principle of XMT is based on the bombardment of the sample using polychromatic X-ray radiation that possesses high spatial coherence, such that a portion of the X-ray radiation that is not absorbed by the sample is collected by X-ray scintillators, which will result in producing visible light that can be captured and recorded by a charged-coupled device (CCD) camera. The sample being analyzed is then rotated between the fixed X-ray source and the detection system and the scans will be recorded to generate microtomograms.

One of the major applications of XMT in food forensics has been reported on the characterization of internal microstructures (void fraction and cell wall thickness) and porosity (pore structure and geometry, pore size distribution and interconnecting size of the pore aperture) for biopolymer starch extrudate foams. In food industries, there are different types of foamed food products such as bakery derived foods (breads, cakes, etc.), whipped cream, ice-cream, snacks, breakfast cereals and biodegradable packaging materials (Fang and Hanna, 2000; Trater *et al*., 2005; Wilkinson *et al*., 2000). Foam can be created through a

process that involves either starch, protein or cellulose extrusion, which ends up forming solid biopolymer food extrudate foams.

Food foaming (the incorporation of bubbles/air or another gas) is very important in the food industry due to the fact that it introduces zest and zip to beverages such as beer, champagne and soft drinks, as well as shaping and improving aroma in foods such as coffee, enabling the right texture for foods like ice cream, whipped cream, bread, milk, cereals, *etc.* (Niranjan, 1999). Moreover, the presence of these microstructure pores in biopolymer extrudates contribute immensely in shaping the physical properties of foods, including density and mechanical stability, which tends to improve food texture and other rheological properties (Wilkinson *et al*., 2000). If these physical attributes are not meeting the stipulated levels of the guidelines as per the regulations, it may constitute a food forensic case because properties such as the mechanical strength of food packaging during storage, transportation or containment can be the cause of food spoilage due to either microbial, environmental conditions (freezing, heating, etc.) or chemical attack and this property contributes greatly to the time measure of food shelf life. Therefore it affects the expiry time of the processed foods as well as the magnitude of other parameters such as aroma, taste, texture, *etc.*

In order to obtain the magnitude of pore size of starch exudates using XMT, a measure of volume fraction needs to be worked out and from this data, pore size of the extrudates can be calculated; likewise, to obtain a measure of aperture size, an area fraction has to be known. However, in order to obtain the magnitude for both pore size and aperture size collectively, a number of frequencies have to be established (Plews *et al*., 2009).

Ionizing Radiation in Food Preservation

In addition to the use of ionizing radiation in food processing, the same waves are also used in food processing steps that are aimed at minimizing storage losses by improving both antimicrobial and anti-parasitological properties of foodstuffs. However, when a high dosage of ionizing radiation beyond the stipulated limits is used, it may result in negative consequences to the integrity of food composition. Investigation of food samples presented in forensic cases using methods that employ X-ray radiation is attractive due to the advantages of X-ray properties related to its high penetration effects.

X-ray-(Micro) Computed Tomography (CT)

X-ray micro-CT is a technique that combines the advantages of X-ray microscopy and that of tomographical algorithms to obtain detailed information regarding the sample's structural size dimension to a magnitude measured in microns. The principle behind the technique is actually the differences in terms of X-ray attenuation measurements in terms of absorption and scattering, which occurs as a result of differences or variations in the density between or within the samples. The technique can be used to monitor density differences due to pore size differences and pore size distribution during food processing procedures, such as the effect of frying time and frying temperature on the structure of foods such as potato strips, meat emulsions, *etc.* (Einarsdottir *et al*., 2013). In this way, the technique can be used to relate the internal 3-D microstructure of food components and relate it to the magnitude of processing parameters and use the information for fingerprinting or detection of adulteration in foods as the processing parameters result in unique and specific microstructures at different treatments for different types of foods.

Moreover, unlike other conventional X-ray radiography, which is normally used for in-line inspection of food and which as such is incapable of providing 3-D information from the scanned sample, X-ray CT is capable of addressing such limitations (Luis *et al*., 2013). With X-ray computed tomography (CT), multiple projections are acquired from the sample of interest, which will then be combined mathematically to provide 3-D information about the sample. The major limitations of X-ray CT instrument include the fact that they are expensive; they are associated with long acquisition times and in addition, unlike the conventional X-ray techniques, X-ray CT does not permit the scanning of many samples, although some researchers have reported improvements in the technology to abate this limitation such that real time analysis is now possible (Haff and Toyofuku, 2008). Due to the X-ray CT capability of imaging 3-D structures of the sample, a door has been opened that allows for the determination of other important structural parameters, such as porosity of matrices, percent object volumes, the degree of anisotropy of food networks or analysis of binary composition (e.g. fat and protein in meat or different phases of bubble voids in bakery products) (Einarsdottir *et al*., 2013). Such information can be used to fingerprint food products or even be used to locate the geographical origin, production processes or adulteration.

X-ray Photoelectron Spectroscopy and its Potential Application to Food Forensics

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), is a surface-sensitive X-ray-based technique that relies on the principle that X-rays bombarding sample atoms tend to generate photoelectrons. In this technique, only those electrons that are generated at the top few atomic layers are the ones to be detected, making it possible for the surface atomic/elemental composition of the sample of interest to be established. An attractive feature of using XPS is that it provides data information for not only elemental/atomic but also chemical composition and chemical variability of the target sample. XPS can be used to analyze samples as received or samples that have been subjected to treatment such as clean-up procedures.

This technique can therefore be used as a fingerprint method for the identification of foods from either plant or animal origin, as it can profile the elemental composition of woods and plant parts and relate to their geographical origin or species. It can be used to provide a fingerprint of animal-derived foods from the data of elemental or chemical measurements of teeth and bone (Watts, 2010).

X-ray Diffraction (XRD)

The principle of X-ray diffraction is dependent on the dual wave/particle property of the X-ray waves to probe structural information, mainly of crystalline samples whereby target chemical/biological species present in the sample matrix are identified based on their diffraction pattern when subjected to X-ray bombardment. When the monochromatic X-ray incident beam is directed towards the sample, the target analyte molecules in the sample will tend to scatter the rays (diffraction phenomena). The size as well as the shape of the functional units of the sample will determine the direction of the diffractions, while the type and the atomic arrangement of the crystalline sample will determine the intensity of the diffraction. Generally, many different types of samples are not just single crystals but are actually composed of tiny crystals that are arranged randomly in different ways in terms of their orientations and this makes such materials polycrystalline aggregates.

This technique has been employed in many different applications related to food forensics, for example in the determination of the functional properties of oats, as measured by the physical properties of the lipids (Jayasinghe *et al.*, 1991). The same technique has been reported for the determination of the type A of starch in waxy and non-waxy barley (Song and Shin, 1991).

X-ray Fluorescence Techniques for Food Forensics

X-ray fluorescence is attractive as a technique for both quantitative and qualitative analysis, mainly for elemental analysis of samples where it can provide measurements related to composition, elemental concentration and identification of specific and trace elements. The principle of X-ray fluorescence is based on the fact that when elements in a sample are bombarded with X-rays, the fluorescent X-rays will be emitted from the sample at the magnitude of energy levels that are characteristic and unique to the elements in the sample. X-ray fluorescence finds application in the determination of metal cations in foods such as those of plant origin like vegetables and fruits, and since the technique is both qualitative and quantitative, it is possible to get the information as to whether levels of regulated metal cations in foodstuffs are in compliance with the guidelines (Nielson *et al*., 1991a,b).

X-ray fluorescence has also been used to analyze the presence of trace metal cations in foods of animal origin such as meat (both beef and chicken) (Tarafdar *et al*., 1991), and also in wheat and rice flour samples (Zhang *et al*., 1991). Since X-rays have the capability to penetrate deeply, even in thick samples, they have the advantage of providing a 100% measure of the whole food sample. For this reason, their application in the measurements of foods such as meat (for fat content, identifying unnatural substances in meat, etc.) is very reliable.

Total Reflection X-ray Fluorescence (TXRF)

This is a variant of the X-ray fluorescence technique that is useful for the measurements of minute amounts of samples. It is non-destructive, fast and sensitive to trace amounts of samples. In food forensic cases, this technique can be employed in the investigation of deliberate food poisoning.

The sample preparation involves spotting a small amount of sample solution (e.g. 1 µL) onto a silicon wafer, drying it and introducing the residue to TXRF. A control sample (uncontaminated sample specimen) should be prepared in the same way as the sample to be analyzed and introduced into TXRF of the spectra between the sample and control, which can be used to indicate the presence of the poisoning agents.

Summing-up X-ray Applications in Food

Forensics

In general, different types of X-ray techniques can find applications in diverse areas in food forensics. These areas include:

- Detection of contaminations in foods, such as presence of pieces of packaging materials in foods (e.g. metal pieces, glass pieces, foil/plastics/rubber pieces), mineral stones and calcified bones. In this application, X-ray techniques can provide in-depth information regarding shapes, sizes and composition (elemental and chemical) of contaminants. In this case, issues of adulterations, provenance, authenticity and mis-labeling can be revealed using X-ray techniques.
- Quality control monitoring and inspection, whereby X-ray techniques can provide information related to the identification of the missing components, fill level, mass measurements and seal integrity.

Application of Gamma Rays in Food Forensics: Radionuclide Food Poisoning

The presence of radionuclides in foods has been known for some time and the levels of occurrence vary depending on the type of food and the geographic location from where the food originates. Of the many radionuclides that have been implicated in food contamination, potassium-40 (^{40}K), radium-226 (^{226}Ra) and uranium-238 (^{238}U) and their respective associated progeny species have been the most encountered (World Health Organization and Food and Agricultural Organization, 2011). It should be noted that some of the radioactivity that normally occurs abundantly in nature, such as ^{40}K, has also been found in foodstuffs that are rich in potassium such as milk, bananas and meat in appreciable amounts, but other naturally occurring radioisotopes, which originate mainly from the decay of uranium and thorium, are usually found in foods in minute amounts (World Health Organization and Food and Agricultural Organization, 2011). Despite the occurrence of some of these radionuclides in minute amounts, when large amounts are released into the environment, they can significantly contaminate water, foods, sources of foods and feeds.

For example, radionuclides may be discharged and contaminate water bodies such as rivers, thus being absorbed in fish tissues and also vegetable plants in cases of irrigation activity in the area and get into the bodies of consumers who ingest and consume water, fish and plants from such areas. Moreover, in cases

where domesticated animals graze on contaminated grass and foliage or are fed with feed contaminated with radionuclides, then milk and meat will contain these radioactive species and affect consumers.

Radionuclides with short half-lives in most cases do not get incorporated into food structures, thus are not of major concern. Those which are considered a high risk when they contaminate foods include iodine-129 and iodine-131 (^{129}I and ^{131}I), strontium-89 and strontium-90 (^{89}Sr and ^{90}Sr), cesium-134 and cesium-137 (^{134}Cs and ^{137}Cs), plutonium-238, plutonium-239 and plutonium-240 (^{238}Pu, ^{239}Pu and ^{240}Pu), tritium (^{3}H), carbon-14 (^{14}C), technetium-99 (^{99}Tc), sulfur-35 (^{35}S), cobalt-60 (^{60}Co), ruthenium-103 and ruthenium-106 (^{103}Ru and ^{106}Ru), uranium-235 (^{235}U), cerium-103 (^{103}Ce), iridium-192 (^{192}Ir), and americium-241 (^{241}Am).

These radionuclides are normally classified into three groups, mainly gamma emitters, beta emitters and alpha emitters. Gamma emitters include ^{134}Cs, ^{137}Cs (^{137}mBa), ^{I31}I, while beta emitters include ^{89}Sr, ^{90}Sr and tritium (^{3}H), and the alpha emitters ^{238}Pu, ^{239}Pu, ^{240}Pu, ^{241}Am and ^{242}Cm.

Apart from food contamination due to natural sources and natural causes (accidents), targeted or deliberate radioactive/radionuclide food poisoning has been reported in many instances, such as those linked to politically motivated assassinations and even in criminally organized assassinations. Moreover, in cases of negligence and improper management of nuclear power plants or military hardware organizations that use nuclear power, there is the possibility to contaminate plant-based, animal-based and aquatic sources of foods with radionuclides. Many examples do exist where food poisoning using radionuclides has been used to target individuals for either political or economic reasons. It is not the aim of this chapter to mention specific instances of such cases; however, they are available in the public domain. The radionuclides that have been reported to be used in food poisoning include tritium (^{3}H), strontium-89 (^{89}Sr), strontium-90 (^{90}Sr), ^{131}I, cesium-134 (^{134}Cs) and cesium-137 (^{137}Cs) (*Handbook of Food Analysis, Nuclear Isotopes Tests, etc*., 2013). Of these radionuclides, ^{134}Cs, ^{137}Cs, ^{89}Sr and ^{90}Sr are known to have been found in poisoning cases involving water, milk, meat, fruits, mushrooms and potatoes, while tritium (^{3}H) has been reported mainly in water poisoning cases. Root vegetable plants can be susceptible to radionuclide contamination through soil–plant exchange mechanisms.

The presence of radionuclides in foods is regulated and the compliance is set such that levels of radionuclides in foods should not exceed the stipulated specific threshold guideline values (Food and Agriculture Organization, 1986; International Commission on Radiological Protection, 1984).

Sampling, Storage and Sample Preparation Methods for Food Specimens Suspected of Radionuclide Contamination

Sampling takes into consideration the type of food specimen under investigation and also the type of sample preparation. In addition to this, sampling of food samples for radionuclide analysis must ensure that an analytically acceptable sample representative from a relevant edible portion of interest or a prepared/reprocessed meal that was consumed and poisoned people, then each portion of the actual food that was consumed should be collected/sampled. For a meal dietary food sample, a total/entire meal has to be prepared in accordance to the recipe and the appropriate procedures for that particular meal and then portioned accordingly such that radionuclide analysis can be performed on any of the portions. There is one requirement which is crucial in sampling, which prompts the analysis to be conducted without too much delay after the sampling and if storage is necessary then it should also be as brief as possible and the samples should be thawed prior to the actual analysis.

After sampling (specimen collection), the storage considerations should take into account the stability and volatility of some of these radionuclides to ensure that they do not degrade or volatilize under the storage conditions. For samples that are to be analyzed for unstable and volatile radionuclides, special treatments such as freezing, refrigeration or addition of specific additives may be desirable in order to minimize analyte losses due to adsorption by the walls of containers. For this purpose, additives such as acids, complexing agents or some specific carrier solutions are normally involved.

Packaging Before Analysis

Food samples should be packaged in waterproof containers, in order to avoid the need for the addition of preservatives.

Sample Preparation Methods for Radionuclides

in Foods

The preparation step is crucial in the analysis of radionuclides in food, as it helps in removing or minimizing the effect of interfering matrix molecules and also enhances the detection limits of the analytical method for the target analytes.

Generally, there are at least two general steps that are normally followed during sample preparation procedures, for the food samples to be analyzed for radionuclides.

Blending and Homogenization of the Food Sample

Blending and homogenization of the food sample to ensure uniformity in terms of chemical and biological composition and the use of blenders or other food sample specific homogenizing equipment are normally employed. There are several techniques that are normally employed to carry out blending and homogenization of food samples that are meant for analysis for radionuclides. These techniques are used to blend liquid foods (juices, milk, etc.), semi-liquid foods and even solid foods including bones, animal skins, *etc.* The techniques include slurry blending, ashing and sodium carbonate fusion. In the slurry blending method, the food sample under investigation is mixed thoroughly using an appropriate homogenizing device to obtain slurry before the analysis using gamma spectrometry. Where the food sample has to be analyzed using radiochemical techniques, the slurry will have to be subjected to ashing before analysis. An example of a typical protocol for a slurry method will involve:

- Accurate weighing of the sample using an appropriate blender to homogenize the food sample until the slurry has been formed;
- Weighing the slurry, dispensing the slurry into smaller aliquot volumes and analyzing each of the weighed aliquots using appropriate techniques.

Ashing is normally performed in conjunction with blending, whereby the sample is dried and then ashed in a muffle furnace at high temperatures to form ash. The ash is then cooled, weighed, pulverized and portioned before analysis using appropriate techniques. Ashing can be used as a suitable sample preparation method in many instances, for example for a dietary meal, for raw foods, cooked foods and also in cases where one is dealing with intact fatty foods, in which case special care has to be observed such as cutting the meat samples containing intact fatty substances into smaller pieces before ashing, in order to ensure a proper homogeneity.

An example of a typical procedure for ashing can be as follows:

An example of a typical procedure for ashing can be as follows:

- Food sample under investigation is blended, aliquoted and the aliquots weighed before drying at 115°C in an electric oven for 2 days;
- The sample is subjected to a charring process by charring at 250°C in an electric oven for 4 hrs. The temperature can be increased to 315°C for another 4 hrs before it is further raised to 480°C where it is charred for a longer period of time (8 hrs). Then the charred sample is taken out of an oven for ashing at even higher temperatures (between 550 and 600°C) for about 16 hrs, then cooled, weighed and ground to obtain a fine powdered ash sample and this will be subjected to the chemical analysis (Nollet and Toldri, 2013).

Another technique is the sodium carbonate fusion homogenization method, which is mainly applicable for food samples with a high content of silica, something which necessitates a step involving fusing the food sample with sodium carbonate in order to facilitate dissolution. The method is based on the use of alkaline earth nitrate salt carriers such as barium nitrate or strontium nitrate, which are incorporated into the portions of food samples that have been subjected to ashing and this mixture is fused with sodium carbonate and sodium hydroxide to produce a melt which is poured into distilled water, washed and then precipitated using sodium hydroxide. This alkaline earth carbonate will then be analyzed for ^{89}Sr, ^{90}Sr and ^{140}Ba.

The protocol for the sodium carbonate homogenization method will thus involve the preparation of carrier reagent solutions such as barium nitrate (20 mg/mL), strontium nitrate (20 mg/mL), sodium carbonate anhydrous (^{3}N), and sodium hydroxide (0.1 N).

Dissolution of the Homogenized Food Samples

Dissolution of the homogenized food samples into appropriate solutions and techniques can be done by employing ashing of the food sample in a muffle furnace. It can also involve acid digestion of food samples, fusing of food samples, or a combination of fusing and acid digestion.

The criteria for the selection of a method/approach (out of the two mentioned above) is dictated by the volatility of the radionuclide to be analyzed. The volatility of radionuclides will dictate the choices for the temperature requirements in the sample preparation or the entire analytical procedure. In many cases, volatile radionuclides are analyzed directly using gamma spectrometry. Acid digestion is normally an attractive option for radionuclides

that are volatile at dry ashing temperatures. This implies that the analysis of such kinds of radionuclides is normally done chemically and not by gamma spectrometry.

One of the methods commonly employed to remove organic interfering molecules in food specimens that are suspected of being contaminated with radionuclide species is ashing, which is performed in either wet ashing mode or dry ashing mode. The wet ashing method is suitable for small-sized samples and also for samples suspected to contain volatile radionuclides, while the dry ashing method is an option of choice when dealing with large-sized/many samples. The dry ashing method is normally operated at high temperatures (up to 450°C). The wet ashing method involves an acid digestion step that employs acids such as sulfuric acid (H_2SO_4), nitric acid (HNO_3), and perchloric acid ($HClO_4$), and in some cases catalysts may need to be incorporated depending on the purpose of the analysis. Wet ashing requires high temperatures (up to 300°C) and like dry ashing, the process can be set up in a microwave oven.

Another sample preparation method, involves crushing of the sample in a ball mill or pulverizing (either chemically or mechanically) or suspending chemically/mechanically in order to ensure that the sample is properly homogenized. Microprecipitation using NdF_3 and also radiochemical separation are highly useful as sample preparation methods for radionuclides in foods.

Counting using proportional gas ionization containers is a sample preparation method of choice for the measurements radio strontium and yttrium, as well as tritium (^3H) and this method is compatible for detection using liquid scintillation counter.

Some radionuclides in foods, such as ^{89}Sr, ^{90}Sr, ^{134}Y, ^{131}I, ^{134}Cs, and ^{137}Cs, can be determined directly using beta instruments, especially if they are found in foods at concentrations above 100 Bq/Kg. However, in cases where low concentrations of transuranic radionuclides are found to be contaminating foods, then alpha spectrometry can be the method of choice for detection of such species.

Detection Techniques for Radionuclides in Foods

A number of techniques are available for the measurements of radionuclides in food samples. These include gamma measurement instruments, beta

measurement instruments and also alpha measurement instruments.

Radionuclide measurement using gamma spectrometry has its advantages in that the technique can directly measure photons/radiations emitted by radionuclides from the original food samples without the need for pretreatment steps such as separation. Gamma spectrometry is also capable of both qualitative identification and quantitative determination of radionuclides in foods.

Gamma-ray (γ-ray) spectrometry is advantageous in food forensics because it is fast thus allowing rapid analyses and it is known to be non-destructive. In this technique, the energy of incident gamma-rays is measured and by comparing the measured energy to that of the known energy of gamma-rays produced by known radioisotopes, it is possible to identify the emitting radionuclide in the food sample.

Among the detectors that are normally used by gamma spectrometer instruments for the measurement of low levels of gamma photons in foods is the solid scintillation detector and an example of this device is the sodium iodine-thallium-activated crystal (NaI/T) and also semiconductor detectors such as the high resolution germanium semiconductor detector (HRGe).

A scintillation detector is a device that makes use of scintillation, which refers to the process by which some materials (gaseous materials, liquid materials or solid materials) emit light when they are subjected to incident ionizing radiation. The procedure normally used is in the form of a single crystal of sodium iodide (NaI) that is usually doped with thallium to make NaI(Tl), which is then interfaced to a photomultiplier device in order to transform and amplify photons into an electrical signal via the photoelectric effect.

A semiconductor on the other hand produces a similar effect to a scintillation detector, which is mainly transforming and amplifying gamma rays into electrical signals. By employing a different mechanism, which exploits the energy gap that exists between the conduction band and the valence electron band, such that when a semiconductor is bombarded with gamma radiations, electrons in the valence band will be excited to the conduction band. This phenomenon will result in a change in the conductivity that can be detected and measured to generate an analytical signal. Typical examples of semiconductor detectors include germanium crystals doped with lithium, Ge(Li), and also high-purity germanium (HPGe).

Gamma spectrometry is also attractive in food forensics analysis of radionuclides, because it is suitable for the measurements of both plant as well as

animal derived foods. Other radionuclides such as uranium-235 (^{235}U) can best be measured using inductively coupled plasma mass spectrometry (ICP-MS) in combination with alpha spectroscopic techniques after sample pretreatment by separation and also by microprecipitation using NdF_3.

Detection of Radionuclides in Foods

Both alpha particle and beta particle detectors are used to detect gamma emitters in food samples. Alpha particle detectors are actually helium nuclei that possess positive charges and are known to be mono-energetic. These detectors are suitable for the detection of food samples that have thin dimensions, because this property enhances the ability of the system to reduce energy losses that occur due to the sample self-absorption phenomenon. If sample self-absorption takes place, the analysis suffers from reproducibility problems.

Liquid scintillation counter (alpha scintillation counter) is another alpha particle based detector that is mostly useful for the counting of weak beta emitting radionuclides, mainly tritium (^{3}H) and carbon-14 (^{14}C). This detector is attractive for use in food forensics samples suspected of radionuclide contamination, because it eliminates the effects due to sample self-absorption losses, thus enabling reproducibility of the counting.

Beta particles, also known as beta activity detectors, are the other detectors used for radionuclide detection in samples (including food samples). The beta particle refers to electrons plucked out of the nucleus due to the phenomenon that involves neutron decay, which results in the formation of protons and electrons. Since each radionuclide displays a unique decay pattern, it is thus possible to exploit this property to identify radionuclides in food samples with great certainty.

Conclusions

Ionization radiation in foods requires the availability of instrumentation and personnel well trained in handling ionizing radiation. The application of this technique in food forensics, especially in the developing world, may be rare due to economic reasons rather than the absence of incidences involving food poisoning due to radionuclides. However, as the trend of conflicts increases, it may be appropriate that even food forensic laboratories, both in developing and developed countries, be equipped with facilities that can provide evidence in

cases of food poisoning due to radionuclides.

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15
Application of Chromatographic Techniques in Food Forensics

In all analytical procedures, especially when there is a need to use the procedure for the detection of food adulteration, it is always desirable to have in place highly selective and sensitive methods and techniques that are simple and cost-effective. Chromatographic methods and techniques present such methods and are highly useful in many food forensics related cases.

Introduction

In the recent past, a number of scientific reports have reported on the extensive use of synthetic coloring agents in foods, food contamination with agrochemicals, use of banned hormones in animal husbandry and poultry farms, use of non-permitted additives, *etc.* The main types of food fraud that are normally reported frequently include the sale of food that is unfit for human consumption, such as those which are past their "use by" date, deliberate mis-description of the ingredients of the food products qualitatively and/or quantitatively, for instance, and the practice that involves substituting food products with cheaper and inferior alternatives. Other examples include instances where the whole food item is deliberately mislabeled, such as farmed salmon mislabeled and sold as wild salmon or basmati rice deliberately adulterated with cheaper varieties but falsely labeled as basmati rice, and in some instances even the geographic, plant or animal origin of foods is deliberately falsified.

Other examples have involved the use of banned Sudan dyes as food additive spices, melamine in milk powder, adulteration of horsemeat in beef, gelatine in chicken breast, mis-description of conventional foods/organic foods, seed oils in olive oil, cow's milk added to sheep's milk, milk dilution with water, brine in frozen meat, adulteration of fruit juices, wine and many other examples.

Due to the rampant practices of such food adulteration incidences, guidelines and regulations have been put in place by authorities, both in many countries (USEPA, FDA, etc.), as well as global institutions such as WHO, FAO, EU, etc., limiting the types, purity, uses, and quantities of food colors permitted in foods.

As a result the need has arisen to continuously monitor foods for permitted and non-permitted food additives and other contaminants, whether organic or inorganic in nature. To ensure compliance with these guidelines and regulations, techniques and methodology for both qualitative and quantitative analytical instrumental methods of determination have been developed, for example, methods based on chromatography, electrophoresis, mass spectrometry, and hyphenated techniques, involving mainly chromatography and mass spectrometry 9.10.

These analytical instrumental methods of analysis have been central in providing evidence in food forensic cases. These methods are based on modern technology with superior quality in terms of taking and processing measurement signals, which simplifies the process for determination as to whether a crime has been committed, and thus assists in the identification of the culprits.

Some of the modern analytical instrumentation with routine application in food forensics has already been introduced and discussed in the previous chapters. This chapter will concentrate on the separation techniques such as gas chromatography (GC); coupled traditional detectors, such as electron capture detector (ECD); flame ionization detector (FID) or thermal energy analyzer (TEA); capillary electrophoresis (CE) with various traditional detectors such as UV-Vis, fluorescence etc.; ion chromatography (IC) with conductivity detector; and high performance liquid chromatography (HPLC) with various traditional detectors such as UV-Vis, photo diode array, fluorescence, refractive index, *etc.* This chapter will also discuss hyphenated techniques that make use of chromatographic and mass spectrometry, which are useful in food forensics. These include liquid chromatography–mass spectrometry (LC/MS); capillary electrophoresis-mass spectrometry (CE-MS), and gas chromatography-mass spectrometry (GC-MS). Instrumental techniques employed in the analysis of ignitable liquids include gas chromatography coupled with a flame ionization detector (GC/FID) and/or a mass spectrometer (GC/MS). For the purpose of fingerprinting of food forensic samples, stable isotope ratio mass spectrometry (IRMS) will be discussed at length, due to its potential to fingerprint food samples of forensic interest.

Chromatography and Food Forensics

Chromatography is one of the analytical techniques based on differential migration of components in a mixture. Mixtures of solutes in a flowing mobile phase pass through a solid or liquid stationary phase where solutes that possess a

greater affinity for the mobile phase will move with the phase and elute faster than those that spend more time in the stationary phase. The most important phenomenon is that as the solutes move through the stationary phase, they describe different retention and are therefore separate in the process.

Generally, chromatography is used in analytical measurement procedures as a technique for separation and/or identification of components in a mixture. All chromatographic methods and techniques require two immiscible phases, such that one of these phases forms a static part (the stationary phase) and another phase forms a moving part (the mobile phase). The separation of components is possible due to the fact that different components in a mixture have different chemistries and thus they tend to adsorb onto a surface of the stationary phase or dissolve in a mobile phase differently.

Chromatographic techniques have been developed and established in the analysis of foods, for example in establishing of tolerance levels for chemical additives in foods, residues and contaminants in various food products. Chromatographic methods have been very central to the monitoring of the guidelines and regulations that have been enforced on food products such as food additives, coloring agents, sweeteners, etc., so that they do not exceed the stipulated levels. Different chromatographic methods have been developed for the qualitative and quantitative determination of components in food products and they have also been applied to ensure compliance with the regulatory requirements imposed on food products, including the whole food production chain, from the raw materials through the different production stages to end product.

There are two main chromatographic methods that will be discussed in this chapter and they are mainly liquid chromatography (LC or HPLC) and gas chromatography (GC). The criteria for the choice of whether to use LC or GC can be determined by the nature of the analytes; if they are volatile (use GC) or if non-volatile (use LC) (Table 15.1).

Table 15.1 Matching the analyte chemistry with the appropriate chromatographic stationary phase.

Analyte nature	Examples	GC	LC or HPLC
Volatile-hydrophilic	Volatile carboxylic acids, aldehydes, ketones, *etc.*	X	
Semi	Synthetic dyes, amino acids, water soluble	X (with	X

volatile-hydrophilic	vitamins	derivatization)	
Semi volatile-midpolar	(PG, OG, DG) phenols, (BHT, BHA, THBA) antioxidants, fatty acids, anabolics, PAHs in foods, organophosphorus pesticides in foods	X (with derivatization)	X
Volatile hydrophobic	C2/C6 hydrocarbons, essential oils	X	
Non volatile-hydrophobic	Phospholipids		X
Semi-volatile-hydrophobic	Fatty acid methyl esters	X	X
Semi volatile-hydrophobic	Aromatic esters, enzymes, aflatoxins	X	X
Non-volatile-hydrophilic	Sugars, sugar alcohol, inorganic ions		X
Semi-volatile-midpolar	Antibiotic in foods, fat soluble vitamins, natural food dyes, triglycerides	X (with derivatizaion)	X

GC cannot be used for the analysis of non-volatile compounds, such as inorganic salts, proteins, polysaccharides, nucleic acids, and other large molecular weight organic compounds, unless the analysis involves pyrolysis. HPLC, on the other hand, can be useful for the analysis of all types of organic compounds, independent of polarity or volatility.

High Performance Liquid Chromatography (HPLC) and Food Forensics

High performance liquid chromatography (HPLC) is one of the non-destructive techniques that can allow for the collection of fractions after separation for further analysis. Due to the complexity of the majority of food samples, the

analyte of interest is normally found in combination with many other chemical and biological species, which will certainly need to be excluded before chromatographic analysis. This necessitates the introduction of sample preparation steps to exclude all of the interfering species in food samples before chromatographic analysis. Among the sample preparation methods that are suitable, food samples include solid-phase extraction and supercritical fluid extraction (SFE).

HPLC as a technique is becoming increasingly attractive for many other applications due to the possibility of using different types of detectors, thus the enhancement of its selectivity for a wide range of chemistries for various samples. HPLC can be coupled to:

1. Bulky property detectors that include:
   - Refractive index (RI), which has a number of advantages, and being nearly universal, it gives a comparable response for different analytes and detects species with no chromophores.
   - This detector can be used for the measurements of sugars, polymers, amino acids, etc.,
   - Evaporative light scattering: Like RI, is also universal and detects species with no chromophores. This detector can be used for the measurements of sugars, polymers, amino acids, *etc.*
   - The conductivity detector. This is common for ion-exchange chromatography and can be used to measure anions such as fluorides, chlorides, *etc.*
2. Specific property detectors, for example:
   - UV-Vis absorption detectors: the analyte must possess chromophores (UV-Vis absorbing functional groups), for example, dyes in foods (e.g. E 102 Tartrazine, E104 Quinoline Yellow, E110 Sunset Yellow, E122 Carmoisine, E124 Ponceau 4R, E129 Allura Red, and E131 Patent Blue).

     NB1 Dyes are used in food forensics cases for the purpose of masking decay, to redye food, and to mask the effects of ageing. The regulation of colors and dyes requires the enforcement of the quality control criteria for traces of starting products and by-products as well. UV-Vis is also a suitable detector for antioxidants in foods (e.g. natural antioxidants: vitamin C, vitamin E; synthetic antioxidants: BHT/butylated hydroxytoluene, BHA/butylated hydroxyanisole,

TBHQ/mono-tert-butylhydroquinone, THBP/2,4,5-trihydroxybutyrophenone, PG/propyl gallate, OG/octyl gallate, DG/dodecyl gallate, Ionox-100 4-hydroxymethyl-2,6-di(tert-butyl)phenol, NDGA/nordihydroguaiaretic acid, TDPA 3,3'-thiodipropionic acid, ACP/ascorbyl-palmitate, etc.); preservatives in foods (e.g. benzoic acid, sorbic acid, propionic acid, methyl-, ethyl-, and propylesters of p-hydroxy benzoic acid (PHB-methyl, PHB-ethyl, and PHB-propyl)); artificial sweeteners in food products (e.g. acesulfam, aspartame, saccharin4) (Official Methods of Analysis, Arlington, VA, 1990; AOAC Official Method 979.08: Arlington, 1990; Official Method AOAC 981.13; Official Method AOAC 982.28; Official Method AOAC 977.23: 44'; Official Method AOAC 980.24, 1995).

NB2 Another group of foods that can be suitable for analysis with HPLC with a UV-Vis detector is flavoring agents used in food products such as lupulone and humulone (hop bittering compounds), and vanillin, naringenin and hesperidin (bittering compounds). It should be noted that there are three major classes of compounds that are used as flavoring agents in foods, which are essential oils, bitter compounds and pungency compounds:

For flavoring compounds that are not volatile or which are thermally stable, HPLC is the best choice and for those which are volatile, a GC can be an option:

- Fluorescence detectors: analyte must possess fluorophores;
- Electrochemical detectors: these detectors are based upon amperometry, polarography, coulometry, and conductometry – analytes must possess functional groups that can undergo either oxidation or reduction;

3. Element specific detectors: this is mainly a hyphenated technique where HPLC is coupled to inductively coupled plasma-MS – mainly for measurements of cations in food samples;
4. Derivatization detectors: in cases where an analyte is tagged with either a chromophore or fluorophore just before or after separation. There are a number of derivatization reagents that are used as precolumn fluorescence labeling reagents. These reagents have different characteristic excitation and emission wavelengths and are used for various functional groups present in

food samples as well as other types of samples. These reagents include o-phthalic anhydride (OPA) with characteristic excitation and emission wavelengths at 340 nm and 455 nm respectively, and are used to derivatize food samples containing primary amine and, thiols. Other fluorescence reagents include dansyl chloride (Dns-Cl) (350 nm_{ex}, 530 nme_{m}) and 9-fluorenylmethyl chloroformate (FMOC) (260 nm_{ex}; 305 nm_{em}), that are used for the derivatization of foods with primary and secondary primary functional groups; N-acridinyl melamide, which targets food samples with thiol groups and has characteristic wavelengths of 355 nm_{ex} and 465 nm_{em}; fluorescamine (390 nm_{ex}; 475 nm_{em}) is used to derivatize primary amines; while dansylhydrazine (Dans-H) (310 nm_{ex}; 350 nm_{em}) targets aldehydes and ketones; and naphthyl isocyanate (310 nm_{ex}; 350 nm_{em}) targets compounds with hydroxyl groups;

5. Indirect detectors: to detect the change in a constant signal as the analyte passes;
6. Hollow cathode detectors: can be used in the determination of molecular weights and stoichiometry composition of metalloproteins (e.g. cytochrome c, myoglobin, hemoglobin, transferrin, etc.) in food samples;
7. Mass selective detectors: these are universal.

Mobile Phases in HPLC

In chromatography, normally the mobile phase plays a significant role in controlling the retention and selection pattern of the analytes in the mixture. However, it is imperative for the analyst to have a proper choice for the mobile phases suitable for the separation of particular analyte species. The criteria for the choice of the mobile phase are based mainly on the solubility with the analyte. Other attributes of a good mobile phase will include low viscosity solvent; the mobile phase must be detector compatible and also column compatible (e.g. polystyrene stationary phases will match best with less polar solvents, while silica gel-based stationary phases/columns will match best with aqueous mobile phases (water) and also a wide range of other HPLC compatible solvents).

Stationary Phases for Separation of Analytes (HPLC Columns)

(HPLC Columns)

In HPLC stationary phases, column packing materials/sorbent play an important role in controlling the retention or selection of the analytes before the detection step. There are mainly two types of stationary phase: rigid cross-linked polymer gels and other polymer gels including resins, ion exchange materials, *etc.* These materials are classified on the basis of pore size, or range of pore sizes.

There are several mechanisms that are normally exploited to effect column separation in HPLC. These include the following:

Reversed Phase, Hydrophilic and Hydrophobic Interaction

NB: under this mode, separation of hydrophobic analytes, the stationary phase is nonpolar liquid immobilized and inert solid and polar mobile phases are employed.

In this mechanism, food components, additives, or adulterants in foods are discriminated based on their functional group chemistry (e.g. polar, neutral, ionic, etc.). For example, neutral food components, additives, or adulterants can be separated in a reversed-phase column where medium and more polar adulterants can be resolved/separated based on their differences in terms of hydrophobicity in a nonpolar stationary phase and a polar mobile phase. Examples of hydrophilic food components include amino acids, the majority of synthetic food dyes, sweeteners/sugars, sugar alcohols, water soluble vitamins (vitamins B and C), *etc.* Midpolar food components, additives, or adulterants may include fatty acids, flavonoids, natural food dyes, fat soluble vitamins (vitamins A, D, E K), *etc.* Fatty acid methyl esters, aromatic esters, *etc.* represents examples of hydrophobic food components.

Ion Exchange Chromatography (IEC) Interactions

In food forensics, ion exchange chromatography (IEC) can be used as a sample preparation technique for both inorganic (anions/cations) and biological (amino acids, sugars, etc.) ionic species in the food samples; for separation of inorganic and biological species in food samples; and also for purification of charged biological compounds such as amino acids, proteins, peptides, nucleic acids, *etc.*

In IEC, the analytes of interest interact with the stationary phase by charge–charge interaction, such that positively charged analytes are attracted and adsorb to negatively charged functional groups such as carboxylates, sulfates in cases

to negatively charged functional groups such as carboxylates, sulfates in cases where there is a cation exchanger stationary phase such as carboxymethyl (CM) cellulose, and positively charged functional groups such as tertiary or quaternary amines in cases where the stationary phase is an anion exchanger such as diethyl aminoethyl (DEAE) cellulose. The elution of the analyte of interest is normally performed using sequential elution strategy by using solvents with different pHs.

This chromatographic technique has been employed in food forensics, especially in the analysis, separation, and purification of charged species such as amino acids, peptides, *etc.*

Types of Stationary Phases Used in Ion Exchange Stationary (IEC)

Ion exchange stationary phases include:

- Cation-exchangers: have fixed negatively charged groups, used to separate positively-charged ions; and
- Anion-exchangers: have fixed positively-charged groups, used to separate negatively-charged ions.

Examples of cation exchanger functional groups include sulfonic acid ($-SOH^+$), which is a strong acid cation exchanger; carboxylic acid ($–COOH^+$), a weak cation exchanger; and carboxymethyl ($–CH_2COOH^+$), which is also a weak cation exchanger, *etc.*

Anion exchange functional groups include quaternary ammonium ($–CH_2N^+(CH_3)_3Cl–$), which is a strong base anion exchanger; and tertiary ammonium and diethylaminoethyl (DEAE), which are weak base anion exchangers (Figure 15.1).

(a) $–CH_2N^+(CH_3)_2–CH_2CH_2CH(Cl^-)$

(b) $–CH_2NH^+(CH_3)_2\ OH^-$

(c) $–CH_2CH_2NH^+(CH_2CH_3)_2\ OH^-$

Figure 15.1 Chemical structure of some anionic exchange groups used in ion

exchange chromatography: (a) quaternary ammonium; (b) tertiary ammonium; and (c) diethylaminoethyl (DEAE).

These ionic materials that make up the stationary phase in IEC can be packed onto several different types of support materials:

1. Cross-linked polystyrene resins: for use in the separation of inorganic ions and small organic ions;
2. Carbohydrate-based resins: for low-performance separation of biological molecules (dextran, agarose, cellulose, etc.); and
3. Silica-based supports: for high-performance separation of biological molecules.

Mobile Phases for IEC

Both strong and weak mobile phases are normally used in IEC. A strong mobile phase in IEC contains a high concentration of a competing ion for displacement of the sample ion from the stationary phase or a solvent that has a pH that decreases ionization of the analyte or stationary phase:

Cation exchange resin (K_{ex}):	$Tl^+ > Ag^+ > Cs^+ > Rb^+ > K^+ > NH_4^{\ +} > Na^+ > H^+ > Li^+$ $Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{\ 2}$
Anion exchange resin (K_{ex}):	$SO_4^{\ 2-} > C_2O_4^{\ 2-} > I^- > NO_3^{\ -} > Br^- > Cl^- > HCO_2^{\ -} > CH_3CO_2^{\ -} > OH^- > F^-$

Factors that affect mobile phase strength include mobile phase pH especially for weak acid or base analytes and weak acid or base stationary phases, mobile phase concentration of competing ion, and also the type of competing ion. For example, the stability and ionic character of proteins is highly dependent on the pH of the mobile phase used in IEC. At very low pH of the mobile phase (pH 4 or less), proteins exist as positively charged due to the protonation of amino groups and therefore in IEC they will be best attached to the cation exchangers. Under weak acidic conditions (pH above 5 to *5.5), most proteins are at their isoelectric points and actually most proteins are stable at pH ranges between 5.5 and* 8. At pH ranges above 6, most proteins are negatively charged due to the deprotonation of the carboxylic acid groups in their chemical structures and they thus attach to anion exchangers in IEC.

Affinity Chromatography (AC) Interactions

In food forensics, affinity chromatography (AC) has many applications, including purification of nucleic acids, enzymes, proteins, and peptides; isolation of cells and viruses, for specific analysis of components in biological samples in foods or sources of foods (plant and animal parts); and also for the study of biomolecular interactions, especially those occurring during food processing procedures.

AC separates components into a mixture of mechanisms controlled by the immobilized biological molecules (and related compounds), which serve as the stationary phases and also based on the selective, reversible interactions that characterize most of the biological systems. Affinity chromatography uses resins attached to ligands and the performance of AC is highly dependent on the sensitivity of most analytes towards ligands.

For enzymes and hormones, AC involves binding of an enzyme with its substrate or a hormone with its receptor, such that the procedure will ensure immobilization of one of a pair of interacting molecules onto a solid support. The immobilized molecule on the column is referred to as the affinity ligand.

There are two main types of affinity ligands used in AC and these are high-specificity ligands (compounds that bind to only one or a few very closely related molecules) and general or group specific ligands (molecules that bind to a family or class of related molecules). Examples of high specificity affinity ligands include antibodies in which antigens are retained; antigens in which antibodies are retained; inhibitors/substrates in which enzymes are retained; and nucleic acids where complementary nucleic acids are retained.

Examples of group specific ligands include lectins where glycoproteins, carbohydrates, and membrane proteins are retained; triazine dyes where NADH- or NADPH dependent enzymes are retained; phenylboronic acid where cis-diol containing compounds are retained; protein A/protein G where antibodies are retained; and metal chelates where metal-binding proteins and peptides are retained.

It should be noted that, due to the very selective nature of most biological interactions, the analytes of interest are in most cases retained with little interference from other components in the sample.

Mobile Phases in AC

In AC, just as in IEC, both strong and weak mobiles phases are used. A weak

In AC, just as in IEC, both strong and weak mobiles phases are used. A weak mobile phase is usually a solvent that mimics the pH, ionic strength, and polarity of the solute and ligand in their natural binding environment.

A strong mobile phase is a solvent that produces low retention for the solute-ligand interaction: by either decreasing its binding constant or by displacing the solute with the addition of an agent with competes for solute sites on the column.

Elution in AC

In AC, elution is governed by competition with soluble ligands or by disruption of interactions. There are mainly two elution approaches that are followed in AC:

1. Biospecific elution: whereby analytes are eluted by a mobile phase that contains a compound that competes with sample analytes for the ligand's active sites. This approach is known to be very gentle and it is useful mainly in the purification applications of active biological molecules. However, this approach is known to produce slow elution with broad solute peaks;
2. Non-specific elution: in this type of elution, the procedure is operated by changing the conditions in the column to disrupt the interactions between the sample analytes and immobilized ligand. The procedure is done mainly by changing the pH or ionic strength of the mobile phase. It is harsher than biospecific elution but its advantage is that it results in narrow peaks and faster run times and it is the option commonly used in analytical applications of AC.

Size Exclusion Chromatography (SEC)

In food forensics, the main applications for size exclusion chromatography (SEC) include the separation of biological molecules in food specimens (e.g. proteins from peptides), and also for molecular-weight determination. SEC separates molecules according to the differences in their molecular size/molecular weight. It is based on the use of a support material that has a certain range of pore sizes and on the different interactions of solutes with the flowing mobile phase, such that analytes travel through the support where small molecules can enter the pores, while large molecules cannot and therefore larger molecules in the sample elute before the smaller molecules. SEC does not have a "weak" or "strong" mobile phase, since retention is based only on size/shape of the analyte and the pore distribution of the support. This technique has different

names based on the nature of the mobile phase, whether it is aqueous or organic. It is known as gel filtration chromatography, but if an aqueous mobile phase is used it is gel permeation chromatography; if an organic mobile phase is used, it is usually tetrahydrofuran. It uses several types of detectors including refractive index (RI), evaporative light scattering detector (ELSD), dynamic light scattering (DLS), ultra-violet (UV), and has been used to assess many food forensic samples such as olive quality, mainly authenticity, and effects of storage (Jabeur *et al.*, 2015).

Gas Chromatography (GC) for Food Forensics

Generally, gas chromatography (GC) is useful in applications involving the analysis of nonpolar and semi-polar, volatile and semi-volatile compounds (Table 15.1). Other low-and mid-molecular weight compounds, which are not volatile enough can be analyzed using GC after derivatization with appropriate derivatization reagents. However, GC can be used without chemical derivatization for the analysis of sterols, oils, low chain fatty acids, aroma components and off flavors, and for contaminants in foods such as agrochemical residues, industrial pollutants in foods such as dyes, and drugs (e.g. antibiotics, hormones, veterinary drugs, etc.). Food components that require chemical derivatization before GC analysis are mainly polar compounds, such as amino acids, hydroxy(poly)carboxylic acids, fatty acids, phenolic compounds, sugars, vitamins, several veterinary drugs, herbicides, and “natural” chemical toxins.

Compositional analysis of foods is important for the verification of labeling of foods. Therefore GC plays an important role for the composition confirmation or monitoring of endogenous contaminants (e.g. aromatic hydrocarbons, heterocyclic amines, urethane, nitrosamines, etc.) in foods and also the analysis for volatiles and semi-volatiles in food components such as sterols, fatty acids, aroma compounds, flavoring compounds, *etc.*

GC is also used widely in the monitoring of transformation products in foods. Transformation products in foods refers mainly to compounds that are produced in foods due to chemical reactions, such as Maillard reactions, auto-oxidation reactions, products generated during industrial processes such as drying, smoking, thermal processing, and irradiation, other food processing procedures such as cooking, and spoilage that may occur during storage.

GC Derivatization Strategies for Food Forensics

Derivatization is a chemical process for modifying compounds with the intention of producing a different but related compound to the original, but which has properties that are suitable and compatible for analysis using a GC. The main objectives for derivatization include enabling indirect analysis of compounds that cannot be directly analyzed by GC due to lack of adequate volatility or stability; and to enhance or increase volatility of compounds with low volatility such as sugars whereby polar functional groups such as OH, NH and SH are converted because the process of derivatization targets hydrogen atoms bonded to O, S, N and P atoms. Derivatization serves also to increase detectability and stability of compounds such as steroids, *etc.* (Blau and King, 1979 ; Knapp, 1979 ; Regis 1998/99 *Chromatography Catalog*).

There are three main strategies used for GC derivatization and they include:

Silylation

Silylation is known to produce a readily volitizable derivative and is the most prevalent approach. Silylation produces silyl derivatives, which are more volatile, less stable, and more thermally stable. Silylation uses trimethylsilyl (TMS) containing groups (Table 15.2) to replace active hydrogens through a SN_2 nucleophilic mechanism. The efficiency of silylation is dependent on whether it involves a good leaving group. In other words, the silylation process is driven mainly by the presence of a good leaving group. Examples of good leaving groups include species with low basicity and those with the ability to stabilize a negative charge in the transitional state, as well as those with few or no π backbonding between the leaving group and silicon atom. However, the trend and ease of reactivity in silylation normally follows the order: alcohol > phenol > carboxyl > amine > amide > hydroxyl. For alcohols, the order is primary > secondary > tertiary. The reaction mechanism for silylation is depicted in Scheme 15.1, while the derivatizing reagents are shown in Table 15.2.

Table 15.2 Silylation reagents (Regis 1998/99 *Chromatography Catalog).*

Silylation reagent	Notes
Hexamethyldisilzane (HMDS)	HMDS is a weak donor, which targets hydrogens in easily silylated hydroxyl groups.
Trimethylchlorosilane (TMCS)	TMCS is also a weak donor but not commonly used as it is associated with the production of HCl which is undesirable.

Trimethylsilylimidazole (TMSI)	TMSI is a selective reagent which targets hydroxyls in wet sugars but does not target N containing compounds such as amines. However, for amino acids, TMSI can derivatize the carboxylic acid sites but not the amino (NH-) functional groups, which can be targeted using fluorinated reagents.
Bistrimethylsilylacetamide (BSA)	BSA is a strong silylating reagent with the acetamide being a good leaving group, thus making it widely used. However, BSA has limitations in that it is associated with the production of an undesirable by-product, TMS-acetamide, which produces peaks that mask the signals due to other volatile derivatives and it also oxidizes to produce silicon dioxide, a known foulant of FID detectors.
Bistrimethylsilyltrifluoroacetamide (BSTFA)	BSTFA has similarity to BSA in terms of how it reacts, but it involves trifluoroacetamide as a leaving group. This makes it react faster and the reaction goes to completion more feasibly than for BSA. Other advantages of BSTFA over BSA include the fact that BSTFA is highly volatile and generates by-products which are more volatile as compared to those produced by BSA, and BSTFA is not associated with by-products that have the tendency to foul detectors.
N-methyl-trimethylsilyltrifluoroacetamide (MSTFA)	MSTFA is known to be the most volatile of the TMS acetamides reagents, mostly applied in the analysis of volatile trace compounds.
Trimethylsilyldiethylamine (TMS-DEA)	TMS-DEA is mainly used to derivatize hindered compounds such as amino acids and carboxylic acids.
N-methyl-N-t	MTBSTFA involves mechanisms which

N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA)	MTBSTFA involves mechanisms which replace active hydrogen in sulfonic and phosphoric groups using t-BDMS groups, which are known to be more resistant to hydrolysis and have the advantage of being more stable as compared to other TMS derivatives.
Halo-methylsilyl derivatization reagents (BMDMCS, and CMDMCS)	These reagents are capable of generating both silylated and halogenated derivatives.

$$R{-}AH + R'{-}\underset{}{\overset{O\ \ Si(CH_3)_3}{\overset{|}{C}}}{=}H{-}Si(CH_3)_3 \longrightarrow R{-}A{-}Si(CH_3)_3 + R'{-}\overset{O\ \ Si(CH_3)_3}{\overset{|}{C}}{=}NH$$

Where R and R′ = alkyl chain

Scheme 15.1 General silylation chemical reaction (Knapp, 1979).

NB: silylation derivatives should not be analyzed using columns that are not compatible with silylating reagents, such as those composed of polyethylene glycol (e.g. carbowax) and those composed with free fatty acid stationary phases.

NB: For carbohydrates, HMDS and TMSI can be the best silylation reagents, while BSA, BSTFA, and MTBTFA can be used for alcohols and phenols. For amino acids, BSTFA and TMSI are most suitable, while BSA, BSTFA, and BSTFA/TMCS are more suitable for amides.

Acylation

Acylation is a strategy employed mainly to reduce the polarity of amino, hydroxyl, and thiol groups that are present in the sample to be analyzed using GC, and at the same time introduce halogenated functionalities and fluorinated functional groups to the compounds being derivatized. Unlike silylating reagents, the acylating reagents are normally used to target highly polar, multifunctional compounds, such as carbohydrates and amino acids in food samples. The acylation reagents include acyl anhydrides, acyl halides, and activated acyl amide reagents (Table 15.3). However, when using anhydrides and acyl halides, care should be taken as these reagents are associated with

harmful acid by-products that need to be excluded from the derivatives before introducing derivatives to a GC column. Activated amide reagents, such as MBTFA, do not generate acid by-products and when they are used there is no necessity to clean the derivatives before GC analysis (Regis 1998/99 *Chromatography Catalog*).

Table 15.3 Acylation reagents (Regis 1998/99, *Chromatography Catalog*).

Acylation reagent	**Notes**
Fluorinated Anhydrides: i. Trifluoroacetoic Anhydride (TFAA) ii. Pentafluoropropionic Anhydride(PFPA) iii. Heptafluorobutyric Anhydride · HFBA	These reagents target mainly alcohols, amines and phenols. However, they also generate the undesired acid by-product which should be removed before introducing the derivatized sample to a GC column, using either a stream of nitrogen or by incorporating bases, such as triethylamine, which will serve as acid receptors.
Fluoracylimidazoles: i. TFAI-Trifluoroacetylimidazole ii. PFPI-Pentafluoropropanylimidazole iii. HFBI-Heptafluorobutyrylimidazole ·	They are best suited for the derivatization of amines and hydroxy compounds. They generate imidazole as a by-product and as this is not an acid it eliminates the need for cleaning the derivative before introducing the sample to a GC column.
N-Methyl-bis(trifluoroacetamide) (MBTFA)	These reagents are best suited for the derivatization of primary and secondary amines and to a limited extent hydroxyl groups and thiols. They generate inert by-products which are non-acidic and this eliminates the need for cleaning the derivative before injection to a GC column.
Pentafluorobenzoyl Chloride (PFBCI)	Targets mainly phenols, alcohols and secondary amines.
Pentafluoropropanol (PFPOH)	Generally used in combination with PFPA to derivatize polyfunctional bio-organic compounds present in foods.

NB: For carbohydrates, MBTFA may be a suitable acylation reagent, for phenols and alcohols, MBTFA, PFPA, HFBA, and TFAA may be suitable. HFBI (+ silylation) can be the best acylation reagents for amino acids and HFBI for amides.

Alkylation

Alkylation, apart from being used to derivatize compounds, is also used as the initial derivatization step and other derivation procedures will then be implemented to impart the required functional group for the intended purpose. In other chemical processes, alkylation is used as a method of protection of certain active hydrogens.

The major role of alkylation is to reduce molecular polarity of compounds intended for GC analysis by replacing the active hydrogens in these compounds with alkyl groups through nucleophilic substitution mechanisms. The majority of compounds that can be derivatized through alkylation are those with acidic hydrogens (e.g. carboxylic acids – amino acids, *etc.* and phenols – antioxidants, etc.) to produce corresponding esters, ethers, alkyl amines, and alkyl amides. The selection of the best alkylating reagent can be the acidity of the active hydrogens in the sample, as these two properties have inverse relationships (i.e. if the acidity of the active hydrogen in the sample is low, choose the strong alkylating reagent). The harsher the reaction conditions or reagents, the more limited the selectivity and applicability of this method. When dealing with multiple functional groups, there may be the necessity to protect functional groups during the derivatization procedures (Regis 1998/99 *Chromatography Catalog*, p. 91). Table 15.4 summarizes reagents that are normally used for alkylation of compounds intended for GC analysis.

Table 15.4 Alkylation reagents.

Alkylation reagent	**Notes**
Dialkylacetals (e.g. DMF)	Results in the formation of a variety of esters.
Tetrabutylammonium hydroxide (TBH)	Used mostly for the derivatization of low molecular weight acidic compounds.
BF3 in methanol or butanol	Results in the formation of esters.
Pentafluorobenzyl bromide (PFBBr)	Suitable for the esterification of phenols, thiols and carboxylic acids.

NB: For carboxylic acids and amino acids, amines, alkylation reagents: DMF (dialkylacetals) and TBH.

Derivation of Chiral Compounds Present in Food Samples

The enantiopure derivatization reagents are used for chiral compounds and target a particular functional group to generate a corresponding diastereomer of each of the enantiomers.

Two strategies can be employed to discriminate enantiomers using chromatographic techniques and they include the process of eluting the compound on an optically active stationary phase, a procedure that involves the use of two types of reagents, mainly N-trifluoroacetyl-L-prolyl chloride (TPC), which is suitable for optically active amines, such as amphetamines, and ((-) menthylchloroformate (MCF), which is best suited for optically active alcohols. The second strategy involves the preparation of diastereomeric derivatives, which can be eluted using a non-chiral stationary phase.

GC Pyrolysis for Food Forensics: Analysis and Identification of Polymeric Components of Food Wrappings and Food Packaging

It should be noted that conventional analysis methods such as GC have the capability to analyze samples that require a very high temperature to volatilize, but only after an extensive and lengthy sample preparation procedure (e.g. gas chromatography coupled to mass spectrometry (GCMS) requires a high effort in sample preparation using Soxhlet, supercritical fluid extraction, microwave extraction, etc.). However, in food forensics, especially when the need for analysis of food wrapping (packaging) is required, these laborious sample preparation methods present serious shortcomings due to the fact that they will necessitate that the methods be adjusted frequently based on the type of polymer used in the wrappings (packaging), as different polymers have different solubilities in different solvents. To overcome these limitations presented by conventional/traditional GC methods, pyrolysis GC can be used as an alternative.

Other advantages for using pyrolysis GC include the fact that it presents the possibility for the analysis of even the smallest amount of samples. It is a technique in which the sample is subjected to a hot pyrolysis oven for fragmentation without the need for any sample preparation. The mixtures of

fragmentation without the need for any sample preparation. The mixtures of pyrolyzed components will then be directed to the GC column for separation. There are several techniques associated with pyrolysis GC and they include DoubleShot Pyrolysis GCMS, which takes place in steps with the first known as Evolved Gas analysis (EGA), at which the temperature needed to volatilize volatile compounds in the polymer material making food wrappings, such as additives, can be determined and/or optimized and also is the step at which the decomposition required to fragment the bulky polymer itself is determined. So the right temperature for every analysis problem can be found easily. This process (EGA) is performed by heating the sample using a linear type of temperature programming such that chromatographic separation is not involved, but only the detection system that can be performed by using a mass spectrometer.

Stable Isotope Ratio Mass Spectrometry (IRMS) for Food Forensics

With the exception of 12 elements, all others exist in a mixture of isotopes (i.e. atoms from the same element but with different number of neutrons in their nuclei), thus with different mass numbers. In each of the elements that exist in different isotopes, there is an isotope that exists in abundance (i.e. dominant light isotope), while other isotopes exist in minor percentages. For example, carbon has 3 isotopes, ^{12}C, ^{13}C and ^{14}C and the most abundant is ^{12}C. Other abundant isotopes are ^{14}N (^{15}N minor abundance), ^{16}O (^{17}O and ^{18}O minor), ^{32}S (^{33}S and ^{34}S minor), ^{1}H (^{2}H and ^{3}H minor), *etc.* The ratios of isotopes in each element can provide a specific signature that can be used for identification and tracing of the origin of the sample. Moreover, measurements of several stable isotope ratios of the same single element provide concrete evidence for fingerprinting of the food sample under investigation. Due to such attractive benefits of IRMS, the technique has been used for a number of applications in food quality control and evidence of authenticity of various food and beverage products such as flavors, fragrances, wine, fruit juice, honey, and vegetable oils (Augenstein and Liu, 2004; Boner and Forstel, 2002 ; Brand, 1996 ; Doner *et al*., 2004; Fink *et al*., 2004 ; Jamin *et al*., 2004; Kelly and Hoogewerff, 2004 ; Krueger and Krueger, 1983, 1985 ; Lofthouse *et al*., 2002 ; Meier-Augenstein, 1999 ; Meier and Koziet *et al*., 1993 ; Micromass Application Notes 502 and 503, 1999; Morrison and Fourel, 1999).

The principal requirements for analytes to be compatible for IRMS analysis

include the fact that they should be able to be volatilized, converted to a gaseous state and those gaseous samples should be isotopically representative of the original sample. The gaseous fraction of the sample can then be introduced into the IRMS system for analysis, where measurements of the continuous flow isotope ratios of $^2H/^1H$ (for H_2), $^{13}C/^{12}C$ (for CO_2), $^{15}N/^{14}N$ (for N_2), $^{34}S/^{32}S$ (for SO_2), and $^{18}O/^{16}O$ (for CO) are performed.

From the principles of IRMS, one can see that the technique is best suited for the analysis of the differences in terms of the isotopic ratios rather than a technique to provide the absolute isotopic ratios.

Conclusions

Separation methods involving the use of chromatographic techniques are available and used in many applications and in many laboratories. It may be advisable that each laboratory should have in their inventory these techniques and personnel trained on how to use and interpret data generated from chromatographic-based techniques.

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16
Application of Hyphenated Techniques in Food Forensics

Chromatography – Mass Spectrometry Hyphenated Techniques for Food Forensics

Mass spectroscopy, also called mass spectrometry, is a scientific method that analyzes a sample of material to determine its molecular makeup. By ionizing a sample, a scientist can cause it to separate into its individual ions. This allows him to analyze and categorize those ions to determine the sample's composition. Mass spectrometry has become a valuable tool in forensic science, where it can provide clues from the barest traces left by a suspect. Mass spectrometry in combination with chromatography (a technique which can separate mixtures) can provide a powerful means to confirm the presence of adulterants in foods or identity/authenticity of foods.

Hyphenated techniques can be used for targeted analysis for pre-defined components in food sample, whereby foods can be analyzed by either liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS), with further confirmation of the structures of the components using nuclear magnetic resonance spectroscopy (NMR). Targeted analysis can provide the means to resolve food forensic cases involving deliberate contamination of foods using banned food coloring agents, such as Sudan dyes normally incorporated into spices such as chilli as a flavor enhancer, in which case LC-API-MS/MS can be employed (http://www.scientistlive.com/content/14486). LC-API-MS/MS can also be used to verify the labeling presented on food labels regarding additives such as the authenticity of meat bindings, composition, quantities and ratios of all the ingredients and whether they comply to standards, regulations and guidelines regarding ethical and religious norms.

Additives such as meat binding (glue) are permitted to be used for meat binding of off-cuts and meat trimmings; however, the same meat bindings have been reported in fraud cases where offenders have tried to incorporate them for the purpose of deliberately increasing the weight of the meat content stated on the labeling (Grundy *et al*., 2007, 2008). Some other illegal practices have involved

the use of blood plasma fibrin protein together with thrombin to bind meat, a practice which may involve mixing blood thrombin fibrin protein from different animals (e.g. bovine, porcine, etc.). Hyphenated techniques such as LC-API-MS/MS can point to the exact origin of the blood or the issue of mixing species (Grundy *et al*., 2007, 2008). LC-API-MS/MS can differentiate the fibrin protein-thrombin blood clots used in the binding procedures, because of the specificity of the peptides that are released during the blood clotting process related to the binding agent technology used. The fibrio-peptides from different species vary and are species specific in terms of their molecular masses, bearing in mind that in LC-API-MS/MS one can generate fragments up to MS^3 or more to ensure that even similar peptides will have differences in terms of the fragments in a certain MS-MS experiment.

LC-MS/MS in combination with NMR can also be employed to verify cases of adulteration in other foods and food products, such as monofloral-honey due to the presence of unique and very specific biomarkers in the nectar of plant flowers that the bees used. Examples of the biomarkers which are unique and specific to the nectar of some plant species include kynuric acid found specifically in chestnut honey. Manuka honey, which is well-known for its health beneficial bioactive compounds, has a specific and unique nectar obtained from the *Leptospermum scoparium* plant species (Donarski *et al*., 2010).

Generally, all compounds that can be analyzed using HPLC or GC can also be analyzed by LC-MS and GC-MS respectively.

GC-MS for Food Forensics

GC-MS has been instrumental in providing evidence in many food forensic cases. For example, there have been a number of deliberate food poisoning cases using toxins such as pesticides (Ochiai *et al*., 2005), dioxins and phthalate acid ester plasticizers (Shen, 2005), scopolamine (Chu *et al*., 2006), and veterinary drugs and growth promoters in animal husbandry (Ramos *et al*., 2003).

These deliberate food poisoning scandals have even prompted import bans by some countries. The monitoring and analysis of such molecules has been made possible by such techniques as GC-MS. GC–MS is an appropriate and perfect analytical tool for forensic analysis of molecules like dioxins, pesticides, *etc.* in foods because of its simplicity, sensitivity and effectiveness in separating and identifying components.

In GC-MS hyphenation, the GC separates components of the sample mixtures in

the chromatography column based on their differences in terms of chemical properties such as boiling points, molecular weights, *etc*. The MS ionizes the individual separated species on the basis of their mass-to-charge ratio, which will be discriminated in the mass analyzer part of the MS and then detected by the MS detector.

Compound Specific Isotope Analysis (CSIA) and On-line Combustion Gas Chromatography Coupled to Stable Isotope Ratio Mass Spectrometry (GC-C-IRMS) for Food Forensics

The technique, known as compound-specific isotope analysis (CSIA) using isotope ratio mass spectrometry (IRMS) coupled to on-line combustion (C) – gas chromatography (GC), is one of the newly introduced techniques that have also found applications in food forensics. The attractive feature of this technique can be realized in its application related to the authenticity of foods as well as food provenance issues. This is due to the fact that CSIA-GC-C-IRMS has the capability to determine and analyze isotope distribution at natural abundance levels with great accuracy and high precision.

Sample Preparation, Derivatization and Isotopic Calibration for CSIA-GC-C-IRMS in Food Forensics

There are important considerations that are crucial when preparing samples for CSIA-GC-C-IRMS, which include that all steps involved during sampling and derivation must be thoroughly optimized to ensure that there are no phenomena associated or related to isotopic fractionation of the analyte of interest. Another consideration is for the incorporation of an internal standard, which takes care of the process of the fractionation of the analyte of interest. An ideal internal standard is the one that should not require any derivatization procedure and it must have a known isotopic composition. Moreover, an ideal internal standard must be proven to be highly chemically stable, must be of high purity, and be soluble in high purity solvents. It must have low vapor pressure at standard temperatures and pressures. It must be environmentally rare, must not co-elute with the analyte of interest in the chromatographic column, and must be compatible to both LC and GC techniques.

compatible to both LC and GC techniques.

In cases where derivatization steps for the sample are involved in IRMS procedures, account should be taken of the associated effect, because derivatization tends to add ^{13}C tracer dilution, which therefore requires a corrective step to compensate for changes related to ^{13}C. Moreover, apart from derivatization having effects on the changes of ^{13}C, it also has serious effects on the GC separation and volatilization of CO_2 and N_2 species. For example, derivation procedures that make use of silylation, especially where apolar derivatization reagents (trimethylsilyl-TMS, ter-butyldimethylsilyl-t-BDMS) are used, also results in the potential to jeopardize a proper resolution of the mixture in the GC column. In addition, such derivatives are known to be associated with high carbon loads, which have the potential to cause incomplete combustion, thus affecting the accuracy of the isotopic analysis. Other derivatization reagents, such as those in the class of trifluoroacetates (TFAs), are also not recommended because they are associated with non-quantitative sample conversion phenomena. Heptafluorobutylated (HFB) derivatization reagents are also problematic, as they tend to form refractory (stable) fluorine derivatives, which when they react with copper and nickel negatively affect the efficiency of combustion for CuO/NiO. Another reason for avoiding the use of HFB derivatization reagent is that fluorine tends to foul the combustion catalyst platinum (Meier-Augenstein, 1997).

CSIA-GC-C-IRMS for Adulteration Tests, Authenticity and Adulteration of Foods

This hyphenated technique can be useful to trace the origin and authenticity of a variety of foodstuffs such as sweeteners, flavors, flavor enhancers and fragrances among others, and its advantage is that it enables both the identification and isotopic ratios (Berneuther *et al*., 1990). For example, the authenticity of fruit juices such as apricot and peach has been investigated using CSIA-GC-C-IRMS by means of measuring $^{13}C/^{12}C$ ratios of food grade flavor ingredients, mainly γ-decalactone (Figure 16.1) and then using d^{13}C to determine the authenticity (Berneuther *et al*., 1990).

Figure 16.1 Chemical structure of γ-decalactone.

Together with $\delta^{13}C$ or $\delta^{15}N$ measurements in various foods, the enantioselectivity measurements versus measurements of GC-C-IRMS for the γ-decalactone has been proved to provide further evidence of the authenticity test for fruit juices (Mosandl *et al*., 1990) (Equation 16.1):

$$\delta[‰] = \left(\frac{R_{(Sample)}}{R_{(\text{Reference})}} - 1 \right) * 1000 \tag{16.1}$$

GC-C-IRMS can also be used to provide evidence in cases of food adulteration; for example, cases involving addition of sugar or vitamin C to fruit juices and wines using low-quality grade corn syrup. CSIA-GC-C-IRMS can in such cases be used to provide measurements of $\delta^{13}C$ of either glucose or bulky carbon, which can point to the availability of corn syrup glucose. Also, the presence of biogenetic biomolecules, such as L-ascorbic acid, L-malic acid and L-tartaric acid in wines, whey and fruit juices, is key for the application of the CSIA-GC-C-IRMS technique to point to cases of possible adulteration or authenticity of these foodstuffs and beverages (Gensler *et al*., 1995 ; Jamin *et al*., 1997 ; Weber *et al*., 1997a, b). In these cases, measurements of $\delta^{13}C$ can be taken and correlated to their corresponding sugar molecules. In the same way glycerol, which is normally sourced from natural sources and incorporated into wines, can be differentiated with artificial glycerol adulterated in wine products (Weber *et al*., 1997). CSIA-GC-C-IRMS can also be instrumental in resolving food forensic cases related to vegetable oil fraud and adulteration such as partial or total substitution of high-quality oil for low-quality ones. In such cases, $\delta^{13}C$ values of fatty acids can be evaluated to show the possibility of adulteration or provide evidence of the true geographical origin of the vegetable oil.

Ion Chromatography (IC) and Atomic Spectrometry Methods in Food Forensics: Verification of Food Authenticity, Adulteration, Provenance and Isotopic Fingerprinting of Foods

The inorganic anions, minerals and trace element content in foods can be used in forensic cases to provide evidence for food authenticity and indication of the

forensic cases to provide evidence for food authenticity and indication of the geographical origin of that particular foodstuff.

Ion Chromatography and Food Forensics

Ion chromatography is an analytical technique that is used to separate ions (mainly inorganic anions such as NO_3^{-}, NO_2^{-}, SO_4^{2-}, PO_4^{3-}, SiO^{-}, F^{-}, Cl^{-}, etc.) and polar molecules based on their affinity to the ion exchange stationary phases. The ions of interest are separated from other ions in the samples based on their affinity to the column stationary phases and are normally detected using a conductivity detector (Lopez-Ruizm, 2000; Tamisier-Karolak *et al*., 1999).

Conclusions

Hyphenated techniques that involve mass spectrometry are normally taken as confirmatory methods due to the fact that they are very sensitive and selective. Therefore, for a confirmation beyond reasonable doubt of authenticity of foods, evidence of adulteration, etc., these methods are normally the ones that are considered mandatory.

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17
Application of Electromigration Driven Techniques in Food Forensics

Indicative-species Targeted in Electromigration Methods:

- Hydroxymethylfurfural (HMF), lactoglobulins, casein, furosine – milk;
- Organic acids (benzoic, ascorbic, sorbic, erythorbic, citric, isocitric, malic, tartaric, fumaric, etc.), polyphenols, phenolic amines, flavonoids, DL-amino acids, aspartame, saccharine, acesulfame, alitame, BHT, BHA, gallate esters, erythrosine, fast green, SF yellow, light green, amaranth, sunset yellow, new coccine, tatrazine, – food additives (e.g. sweeteners, antioxidants, colorants), preservatives, food antioxidants, fruit juices and citrus fruits authenticity;
- Gliadins, glutenins, storage proteins, Glu-1 genes – differentiation of cereal (e.g. wheat, oats, rye, barley, rice, maize, etc.) cultivars;
- Cations and anionic profiles – fruit juices and beverage authenticity;
- DNA fragments – GMO foods;
- Hemoglobin, myoglobin, actin, myosin, MRM, lysozyme – meat;
- Sarcoplasmic proteins – fish.

Electromigration techniques employ external voltage sources of electrokinetic methods to run the separation process to discriminate components of mixtures. Examples of these methods include electrophoresis and electro-osmosis, which refers mainly to the volumetric liquid that is flowing in a capillary and which is driven by an electrical field. For a complete (100%) electrophoretic separation to take place, there have to be significant differences in terms of the velocities of the migrating charged species, which move under the electric field that is the driving force. Just like all other analytical separation methods and techniques, there are a number of parameters that control or influence the efficiency of the electromigration procedures that may be associated with the resolution of the mixtures and need to be optimized. Factors with the tendency to control separation of the mixtures in the electromigration driven techniques include electrode polarization, applied voltage, temperature, capillary, background electrolyte, and various additives. For example, a number of factors are known

to be responsible in causing the differences in the velocities of the migrating species and include electrophoretic mobility, whereby ionic species separate based on their differences in terms of the charge-to-size ratio. It should be noted that the magnitude of the electrophoretic mobility in a particular buffer is characteristic to that buffer system and thus it has a constant value that is unique to that particular ionic species in that particular buffer system. It follows therefore that when buffer properties and characteristics are carefully optimized, it makes it possible to control the resolution (separation) of the migrating ionic species, because it tends to greatly influence their electrophoretic mobility.

Another factor that influences the separation of the migrating ionic species is the electro-osmotic flow (EOF), which actually refers to the motion of the electrolyte (buffer system) that is inside the capillary tube where silanol groups are exposed. The EOF is highly influenced by the differences in potentials and is triggered by the electric charges. It is also dependent on both the ionization properties of the migrating ionic species that are present inside the capillary tube as well as the adsorption pattern of the migrating ionic species. The pH of the separation buffer also has the potential to cause ionization of the silanol groups inside the capillary tube, thus creating negative charges inside the capillary tube. The silanol ionization effect creates a double layer at the interface between the electrolyte and the inner walls of the capillary tube. The double layer is made of a static/permanent layer and a weak mobile diffusion layer. The migrating ionic species presenting the weakly-bound diffusion layer have the flexibility to become involved in the EOF due to their susceptibility to exchange phenomena with the ionic species present in the electrolyte.

At the interface of the two layers (diffusion and static/permanent), an electrokinetic potential known as the Zeta potential (ζ) develops. The magnitude of ζ is directly proportional to the charge density, which is also dependent on the pH of the separating buffer. Because of the dependence of ζ to pH, it follows that it has direct control over the EOF, such that when the experimental conditions are highly alkaline (at high pH) where the silanol groups are completely ionized, the EOF is quickest and vice versa under acidic conditions (low pH) where there is minimal dissociation of the silanol functional groups. The magnitude of ζ is also highly dependent on the ionic strength of the electrolyte and is actually inversely proportional to it.

During the optimization process, the performance of these parameters is measured by factors such as migration time, efficiency, selectivity and resolution. Unlike in chromatographic techniques, where the separation is

effected in the stationary phase of the column where the flowing phase is forced through using mechanical pumps, in electromigration driven processes, the separation takes place in a buffer, also known as either the electrolyte or background electrolyte, separation buffer or separation electrolyte. The buffer can either be an aqueous solution or it can be a solution based on pure organic solvents or their mixtures (Divall, 1985 ; Kvasnicka, 2000 ; Landers, 2007 ; Righetti *et al*., 1997 ; Vanhoenacker *et al*., 2001).

Capillary Electrophoresis and Food Authenticity in Forensics

Capillary electrophoresis has many potential applications in food forensics. especially in cases related to the authentication of foods. As indicated above, capillary electrophoresis is one of the electromigration (electrokinetic) analytical techniques employed mainly in the separation of compounds in a mixture based on their differences in terms of electrophoretic mobility, phase partitioning, ionic potential (pI), molecular size, *etc*. There are several variants (modes) of CE, which are all based on similar principles, but operate in different configurations and they are thus used for the analysis of a wide variety of analytes ranging from simple inorganic ions, small organic molecules, peptides, proteins, nucleic acids to viruses, microbes, and particles (Everaerts *et al*., 1976 ; Foret *et al*., 1993 ; Righetti *et al*., 1997 ; Vanhoenacker *et al*., 2001). These variants of CE techniques include capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), on-line coupling of CITP and CZE, capillary gel electrophoresis (CGE), and capillary electrochromatography (CEC). For food authenticity testing, fingerprinting, provenance, etc., there are several variants of CE that are normally employed and they include free solution capillary electrophoresis (FSCE) and micellar electrokinetic chromatography (MEKC), which can be used for DNA analysis, for identification and fingerprinting of foods; the same methods can be used for the analysis of proteins as biomarkers for foods.

Free Solution Capillary Electrophoresis (FSCE) and Food Forensics

Free solution capillary electrophoresis (FSCE) provides the possibility of simultaneous separation of both positively and negatively charged species, especially when experimental conditions such as electrophoretic mobility and

electro-osmotic mobility have been optimized. Generally, the order of migration in FSCE is such that ionic species with higher positively charged density and those with smaller radius tend to migrate first.

Despite the advantages of FSCE, the technique is limited when it comes to separating species with either similar (same) charge-to-mass ratio such as DNA fragments and sodium dodecyl sulfate complexes. Moreover, with FSCE there are challenges in terms of separating uncharged species. FSCE is also unsuitable for the separation of species that possess high positive electrical charge density, because such species have a tendency to adhere to the inner walls of the capillary tube. Moreover, like all capillary electrophoresis modes, the technique is not suitable for trace analysis.

Micellar Electrokinetic Chromatography (MEKC)

Micellar electrokinetic chromatography (MEKC) is highly suitable for the separation of non-charged species, although this does not mean that it cannot be used entirely for the separation of charged species. The principle of MEKC is that it incorporates surfactants (usually sodium dodecyl sulfate, SDS) to the separation buffer. The amount of surfactant added is such that it is sufficient to form micelles. The formation of micelles is crucial because they act as another stable phase (actually a pseudo-stationary phase) where neutral species interact at their optimal specific partition coefficient, and because SDS micelles are negatively charged, they migrate towards the anode (positive electrode).

There are other CE techniques such as agarose gel electrophoresis (AGE) that can be used for authenticity tests in DNA analysis, but these methods are known to suffer from low sensitivity, insufficient resolution and also make use of highly toxic and teratogenic ethidium bromide compounds to enable visualization of the amplicons, and so this makes this technique environmentally and user unfriendly. A good thing is that the bottleneck related to AGE can be addressed by coupling them to molecular biology methods such as a polymerase chain reaction (PCR) (Garcia-Cañas *et al*., 1994a,b, 2002).

In the case of protein analysis, normally polyacrylamide gel electrophoresis (PAGE) slabs have traditionally been used for the analysis of protein composition of foods and beverages. Capillary electrophoresis methods are not popular for protein separation because of the challenges that are normally encountered when these techniques (CE) are used for the separation of proteins. These problems arise from the fact that biopolymers such as proteins tend to

adsorb on the walls of the fused capillary tubes, thus making the technique less attractive for such applications.

The adsorption of biopolymer analytes onto the fused capillary tube walls is caused by the electrostatic interactions that occur between the positively charged biopolymer residues (i.e. protein residues) and the negatively charged groups of the silanol inside the capillary walls. However, a good thing is that these problems can be circumvented by the incorporation of either highly alkaline or acidic buffers and/or specific polymeric additive materials of high salt concentration in order to protect the negatively charged groups of silanol. Another alternative way to counter the electrostatic phenomena that occur between these oppositely charged species is to coat the inner walls of the capillary with specific adsorbents or to chemically modify the inner walls of the fused capillary tube (Clifuentes, 2006 ; Frazier and Papadopoulou, 2003).

Capillary electrophoresis methods have also been highly useful in the analysis of chiral compounds present in foods, such as amino acids (D and L amino acids). In order to enhance the separation of these chiral compounds in foods, specific chiral selectors, mainly cyclodextrins and their derivatives, have to be included in the separation buffer. In some cases (where necessary), some functional groups in D-and L-amino acids present in foods presented as evidence in food forensics related cases, may need to be derivatized using agents such as fluorescein isothiocyanate (FITC) in order to boost the strength of the fluorophores to obtain more sensitivity and for a better separation of such chiral amino acids, *etc.*

Considerations During the Application of Electromigration Techniques in Food Forensics

The application of electromigration methods in food forensics targets specific indicative target species that are electrophoretically active, which (indicative species) vary from one type of food to another. These molecules include specific protein classes such as gliadin, glutenins (for cereals like wheat), some organic acids, amino acids, antioxidants (for preservatives), *etc.* Electromigration methods also require optimization of the buffer systems (composition and type) and the type of capillary (coated or uncoated), the length of the capillary, temperature, voltage and the detection wavelengths in the case where fluorescence or UV-Vis are used as detectors.

Different food types may require specific electromigration techniques and

experimental conditions will also depend on all these factors (food type, the technique and the target indicative analyte species). This is evidenced by a number of reports that are found in the literature. Morales and Jimènez-Pèrez (2001) reported the application of MEKC to investigate the heat load of milk where hydroxymethylfurfural (HMF) was the target-indicative molecule. In this report, an uncoated fused silica capillary was used with the buffer system composed of 50 mM phosphate buffer (pH 2.4) and 100 mM SDS, the voltage was set at 20 kV, while the wavelength for the UV detector that was used was set at 280 nm.

The use of MEKC for the detection of food additives and preservatives has been widely reported. For example, Pant and Trenerry (1995) employed this technique making use of organic acids, mainly benzoic acid and sorbic acids as analyte indicative species. The separation buffer for this was composed of 20 mM sodium phosphate buffer and 50 mM SDS in an uncoated fused silica capillary. A UV detector was used and the detection wavelength was set at 230 nm, while the detection voltage was set at 25 kV.

The application of MEKC-LF in food forensics has also been reported in instances involving the geographical origin of orange juice, whereby DL-amino acids were analyzed as target analyte indicative species (Simo *et al.*, 2002). In this report, they used uncoated fused silica capillary and the separation buffer was made up of 100 mM sodium tetraborate, 30 mM SDS (pH 9.4) and 20 mM beta cyclodextrin (beta CD). The detection wavelengths (LIF) were set at 488 nm (excitation) and 520 nm (emission).

The application of MEKC for the investigation of antioxidants in foods has been reported by Boyce and Spicket (1997). In this application, uncoated fused silica was used with 20 mM sodium borate, 50 mM sodium cholate, 15 mM SDS and 10% methanol, at 2°C and 18 kV. The wavelength for the UV detector was set at 254 nm and also at 214 nm. In other reports, sweeteners and preservatives were detected using MEKC, whereby aspartame, saccharine, acesulfame, alitame, benzoic acid and sorbic acid were used as target indicative molecules (Thompson *et al.*, 1995), while in a report by Frazier *et al.* (2000), in which they determined sweeteners, coloring compounds and preservatives, they used caffeine, aspartame, brilliant blue, green S, benzoic acid, sorbic acid, saccharine, sunset yellow, acesultame-K, quinolone yellow, carmoisine, ponceau 4R and black PN as target analyte indicative species. In a report by Thompson *et al.* (1995), uncoated fused silica capillary was used (at 25°C) and the buffer system was made up of 100 mM sodium borate, 50 mM sodium deoxycholate and 10

mM potassium dihydrogen phosphate, at a volatage of 20 kV and UV wavelength detection set at 220 nm. The conditions in a report by Frazier *et al*. (2000) involved the use of uncoated fused silica and the composition of the separating buffer was 20 mM sodium hydrogen carbonate and 2 mM SDS (pH 9.2), the voltage used was 20 kV and the detection wavelength ranged between 190 and 600 nm.

Another electromigration technique, known as capillary zone electrophoresis (CZE), has been widely used in the differentiation of cultivars. A number of reports have exploited the advantages of CZE in food forensic identification of cultivars and their differentiation within and between species. In cultivar differentiation, the technique (CZE) targets various analyte-indicative species, for example gliadin proteins and glutenin proteins (Bietz and Schmalzried, 1995), which was used as indicative analyte species in the experiment for the differentiation of wheat cultivars. In this report, the buffer system that was used was composed of boric acid (30–60 mM), sodium hydroxide (pH8–9), acetonitrile (0–40%) and SDS (0.1–10%). Uncoated fused silica capillary was used (3–60 mm length; diameter 0.05–0.07 mm). Temperature varied between 20 and 50°C at a constant voltage of 10 kV. The detection wavelength was 200 nm.

The glutenins used as analyte indicative species are actually a group of protein aggregates comprised of both high-molecular-mass (HMW) and low-molecular-mass (LMW) subunits with molar masses that are characterized by a variety of intermolecular linkages and interactions such as disulfide linkages, hydrophobic bonding, *etc.* In wheat products and in the bakery industry, glutenins play an important role in strengthening and providing the needed elasticity of the dough (Belitz *et al*., 2004). Gliadins, on the other hand, are a class within gluten proteins that are found in a number of cereals such as wheat, oats, rye, *etc.* and they play an important role in making the bread rise during the baking process.

Structurally, gliadins exist as monomeric entities and based on their chemistry, they can be subdivided into alpha-gliadins, beta-gliadins, gamma-gliadins and omega-gliadins. This chemistry can be the basis of differentiating cereal species using electromigration methods, as the proteins are ionic in nature, thus active under electrophoresis conditions. Genetical differences can also be exploited to differentiate cereals using electromigration methods. There are several genes that code for omega-and gamma-gliadins in cereals, mainly wheat. These genes are located at the Gli-1 loci found on the short arms of group-1 chromosomes. In the case of alpha-and beta-gliadins, the genes that encode them are found on the short arms of group-6 chromosomes. In the case of low molecular weight glutenin protein aggregates, they are encoded by genes located at the Glu-3 loci,

which are linked to the Gli-1 loci. The most important feature that is used to differentiate species comes from the fact that each gene located at the Glu-1 locus consists of genes that encode for two different types of high molecular weight glutenins and they are the x-type and y-type. The permutations at this locus provide for the polymorphism that provides the possibility for a number of different alleles, thus the criteria for differentiating species in cereals. Some, for example, wheat, lack the y-type genes at the Glu-A1 locus and therefore this difference can be exploited to classify or differentiate wheat from other cereals or the criteria for the quality of the dough.

Lookhart and Bean (1996) also reported the use of CZE for differentiation of wheat cultivars using wheat gladins and wheat reduced gluteins as specific analyte indicative species. The difference with Bietz and Schmalzried (1996) was the experimental conditions, such that Lookhart and Bean (1996) used 100 mM sodium phosphate (pH 2.5), a non-ionic surfactant hydroxypropylmethylcellulose (HPMC, 0.05%) and methanol/acetonitrile/2-propanol/ethylene glycol (EG) at concentrations ranging between 0 and 20%. Uncoated fused capillary was used at a voltage of 15 kV and the detector wavelength was set at 200 nm. In another report, Bean and Lookhart (2000) employed CZE for the study of cultivar differentiation for cereals, mainly wheat, rye, oats, barley and rice, in which cereal storage proteins were specific analyte indicative species. The buffer system used was composed of 50 mM iminodiacetic acid together with acetonitrile (20%), HPMC (0.05%) and uncoated fused silica capillary at temperatures of 45°C and voltage of 30 kV. The detector wavelength was set at 200 nm.

CZE has also been used in food forensic milk differentiation, where casein proteins were used as specific analyte indicator species (Molina *et al.*, 1999). In this study, hydrophilic coated fused silica capillary was used, with a buffer system made up of 320 mM citric acid, 20 mM sodium citrate (HPMC + 6 M urea, 0.05%), a pH of 8.6, and detector wavelength set at 214 nm and 25 kV.

The CZE fish species differentiation study, using sarcoplasmic proteins patterns as target indicative species, was reported by Gallardo *et al.* (1995). Uncoated fused silica capillary was used together with a buffer system made up of sodium phosphate (pH 2.4) at concentrations between 75 and 100 mM, voltage between 10 and 20 kV, while the wavelength for the UV detector was set at 214 nm.

Hyphenated Techniques Involving Capillary Electrophoresis

Electrophoresis

A combination of CZE and reversed phase high performance liquid chromatography has been reported for applications in food forensics related to the fingerprinting of wheat cultivars, as well as for the differentiation of genetically closely related wheat cultivar lines (Bean and Lookhart, 1997). In their report, Bean and Lookhart (1997) used the CZE of gliadins and glutens as the analyte indicative species. The buffer system was composed of 0.1 M phosphate buffer (pH 2.5), 20% acetonitrile, 0.05% HMPC, the voltage was tuned at 12.5 kV and the temperature was set at 45 °C, while the detection wavelength was set at 200 nm. Uncoated fused silica was used for all the experiments.

In another report, Salmanowicz and Moczulski (2004) employed a combination of CZE and PCR in the study to investigate the selection of the quality wheat genotypes that may result in the best quality breads. In this work, 89 mM (Tris base and boric acid), 2 mM EDTA, and 1% hydroxyethyl cellulose (HEC) were used as a buffer system (pH 8.5), at 10 kV. Uncoated fused silica with a reversed LIF detector was used. The analyte indicative species were the DNA fragments obtained from Glu-1 gene high molecular weight (HMW) glutenin subunits encoded by the Glu-1 genes. Other molecules that have been reported as analyte-indicative species in cases of food authenticity or fingerprint tests include organic acids in fruits or fruit juices. Examples of these organic acids include citric, isocitric, malic, tartaric, fumaric acid, *etc.* The associated electromigration techniques that have been used for these organic acids include CZE (Saavenra *et al.*, 2001), where orange juice authenticity was being tested. In this report, the buffer system was composed of 200 mM sodium phosphate (pH 7.5), at a voltage of 14 kV, polyacrylamide coated fused capillary was used and the detection wavelength was set at 200 nm.

Capillary isotachophoresis (CITP) in combination with CZE has been reported by Kvasnička *et al.* (2002) and also by Kvasnička and Voldřich (2000). In the report by Kvasnička *et al*. (2002), citrus juice authenticity was tested in which conductometric detection was employed. The buffer system was composed of:

- *Leading electrolyte*: 6 mM HCl, 3–8 mM BisTrisPropan (BTP), 2 mM $CaCl_2$ and 0.05% hydroxypropyl methylcellulose (HPMC);
- *Terminating electrolyte*: 5 mM morpholinethanesulfonic acid (MES) and 1 mM BTP.

In a previous report by Kvasnička and Voldřich (2000), the authenticity of apple juice and also the addition of synthetic malic acid was tested, in which case

malic acid was used as analyte indicative species.

Other food components that have been used as analyte indicative components in citrus juice authenticity tests include amino acids, polyphenols, phenolic amines, flavonoids and ascorbic acid (vitamin C) (Cancalon and Bryan, 1993). The same indicative species have also been reported by the same authors for use in the detection of pulp wash additions to citrus juices. In the work by Cancalon and Bryan (1993), CZE was used and the electrolyte system was composed of sodium phosphate (50 mM, pH 6.8), and/or borax-boric acid (50 mM, pH 7.6–9.2) and/or 50 mM borax-sodium hydroxide (pH 10). Uncoated fused silica capillary was used and the detection wavelength was set between 200 and 500 nm.

Inorganic cations and anions have also been reported as CZE target analyte indicative species for orange juice authenticity (Jezek and Suhaj, 2001 ; Weston *et al.*, 1992). Weston *et al.* (1992) used potassium, sodium, calcium and magnesium as analyte indicative species, while Jezek and Suhaj (2001) used the anionic profile of the orange juice. Ammonium species (NH_4^-), potassium, sodium, calcium and magnesium were used in another work by Kvasnička (2000) for citrus juice authenticity testing using the CITP technique.

Capillary gel electrophoresis (CGE)–LIF (laser induced fluorescence) in combination with PCR (polymerase chain reaction), utilizing DNA fragments of maize has been reported by Garcia-Canas *et al*. (2004), for the detection of GMO in maize flour. The detection was performed using an LIF Ar with laser. The excitation wavelength was 488 nm, while the emission wavelength was set at 520 nm.

Another electromigration hyphenation technique, SDS-CE, was reported in the application directed toward cultivar differentiation in wheat, where wheat glutenins and Glu-1 HMW-glutenin subunits of wheat were targeted as analyte indicative molecules (Bean and Lookhart, 1999 ; Lookhart and Bean, 1996). In another report by Day and Brown (2001), results on the use of SDS-CE in the detection of mechanically recovered/reclaimed meat (MRM) were published, where hemoglobin, myoglobin, actin and myosin were identified as analyte indicative species. MRM is also synonymously known as either mechanically separated meat (MSM) or mechanically deboned meat (MDM). It is refers to a paste-like meat product obtained by forcing either pureed or ground meat using high pressure through a sieve to separate the bone from the edible meat tissue.

Other target analyte indicative molecules, such as carbohydrates and organic

acids, have been reported in the CZE studies on juice composition (Soga and Serwe, 2000). In this report, the supporting electrolyte was made up of 20 mM, 6-pyridinedicarboxylic acid, 0.5 mM cetyltrimethylammonium hydroxide and sodium hydroxide (pH 12.1). Uncoated fused silica was used and the voltage was set at –12 kV (reverse EOF), while the indirect detection was done at 350 nm.

In another report, lactoglobulins were used as target analyte indicative species in a study involving differentiation of milk (Cartoni *et al*., 1999 ; Herrero-Martinez *et al*., 2000). In the report by Cartoni *et al*. (1999), a methyl deactivated fused silica capillary was used and the electrolyte was made up of sodium borate (50–120 mM, pH 9.2), temperature and voltage were set at 25°C and 4–6 kV respectively, while the wavelength for the UV detector was set at 200 nm. The study by Herrero-Martinez *et al*. (2000) utilized uncoated fused silica capillary and the buffer system composed of 50 mM iminodiacetic acid, 0.1% HMPC (Tween 20 + 6 M urea, 0.1–10%), and pH 3.1. The voltage and the detection wavelength were 10–15 kV and 214 nm respectively.

Lysozyme has also been reported for use as a target analyte indicative molecule in the CE-MS (capillary electrophoresis coupled to mass spectrometry) study of meat differentiation, where ethylpyrrolidine methacrylate-N,N-dimethylacrylamide coated silica capillary was used. The electrolyte used in this work was made up of 75 mM ammonium acetate/acetic acid (pH 5.5), and the voltage used was 25 kV. The mass range selected was between m/z = 800 – 2200 (the target mass was m/z = 1500).

Vallejo-Cordoba *et al.* (2004) reported the use of furosine (Figure 17.1) (epsilon-N-(2-furoyl-methyl)-L-lysine 2HCl) as target analyte indicative molecules in the CZE study of the quality of dairy products (Vallejo-Cordoba *et al*., 2004), while the use of caseinomacropeptide (CMP) as the target analyte indicative molecule in the study of food adulteration involving the addition of whey rennet was reported by Cherkaoui *et al.* (1997) and erythrosine, fast green, SF yellow, light green, amaranth, sunset yellow, new coccine and tatrazine were reported as target analyte indicative molecules in the detection of food additives, mainly sweeteners, colorants and preservatives (Razee *et al*., 1995).

Figure 17.1 Chemical structure of furosine.

Protein-bound 3-methylhistidine as an analyte indicative molecule has been reported in the CITP study of lean-meat content (Kvasnička, 1999). In this study, the electrolyte that was used had the following composition:

- Leading electrolyte: 5 mM ammonium hydroxide, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES).
- Terminating buffer: 10 mM 6-aminocaproic acid, 5 mM acetic acid.
- A conductometric detector was used for the detection of the analytes of interest.

In another report, Chu *et al*. (1993) did a study based on the MEKC detection of collagen in muscle tissues, where phenylthiohydantions of hydroxyproline were used as the target analyte indicative molecules. The composition of the separating buffer reported in this study included 50 mM sodium malonate, 75 mM SDS, pH 5, voltage of 15 kV and the detection wavelength was set at 254 nm.

Isoflavones, mainly daidzein and genistein, were reported as target analyte indicative species in the CZE study that involved the detection of soy and lupin proteins in meat products (Melienthin and Galensa, 1999). Uncoated fused silica capillary was used in this study and the experimental temperature was set at 25 °C with the electrolyte being 200 mM boric acid and sodium hydroxide, pH 8.6. The voltage used ranged between 25 and 30 kV and two detection wavelength lines (260 nm and 270 nm) were used.

Food forensic studies involving the testing of wine quality have been widely reported. For example, Pazourek *et al*. (2000) reported their CZE study on the differentiation of Canary Islands wine products, where the profiles of polyphenols fingerprinting were tested. They used an uncoated fused silica capillary and the composition of the electrolyte included 25 mM sodium

tetraborate, pH 9.5 and the voltage used was 20 kV at 25 °C and the detection wavelength was 305 nm.

In another development, a combination of CITP and CZE was reported in the study of wine quality utilizing phenolic compounds such as gallic and caffeine, as well as vanillic acids such as rutin, quercetin and myricetin as analyte indicative species (Hamounovà *et al*., 2004). The leading electrolyte used was composed of 10 mM HCl, Tris, 0.2% hydroxyethyl cellulose (HEC) and pH 7.2, while the termination electrolyte composition included 50 mM boric acid, barium hydroxide and pH 8.2.

The background electrolyte (BGE) was composed of 25 mM (*N*-Tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid) (TAPS) – a zwitterionic buffer/(3-(*N*-morpholino)propanesulfonic acid) (MOPS), which is a buffer that is a structural analog to MES; 50 mM Tris, 15–40 mM boric acid and catechins.; 5 mM beta-cyclodetrin, 20% methanol, 0.2% HEC, and pH 8.5–8.7. The detection wavelength was set at 254 nm.

Another variant of the CZE technique (CZE on Chip) was employed in the study of wine quality, in which chlorogenic, vanillic, gentisic and ferulic acids were targeted as analyte indicative species (Scampicchio *et al*., 2004). The glass microchip CE separation channel was employed in this study, with the electrolyte being composed of 15 mM sodium borate, pH 9.5, the electrolyte injection voltage was 1.5 kV, separation voltage was set at 2 kV and the amperometric detection potential was +1.0 V.

In a report by Gu *et al*. (2000), MEKC was used in the study of wine quality, whereby cis/trans-resveratrol was used as an analyte indicative species. Uncoated fused silica capillary was used, the experimental temperature was set at 25 °C and the separation buffer was made up of 25 mM sodium phosphate, 25 mM sodium borate, 75 mM SDS, pH 9 and the detection wavelength was 310 nm.

Nunez *et al*. (2000) used CZE in the study which investigated the differentiation of Spanish red wines utilizing inorganic species, mainly potassium (K), sodium (Na), calcium (Ca), manganese (Mn) and lithium (Li) as analyte indicative species. Uncoated fused silica capillary was used with the buffer system composed of 5 mM Cia-Pack UV-Cat1 buffer, 6.5 mM alfa-hydroxyisobutyric acid, 2 mM 18-crown-6-ether, voltage was set at 22 kV and the indirect UV detection at wavelength of 214 nm was used.

Conclusions

Conclusions

Electromigration methods are normally used in certain molecules that are mostly charged, such as proteins, *etc.* Therefore, for such molecules, the analyst may opt to use them. They are powerful and reliable.

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18
Application of Thermal Methods in Food Forensics

Thermal methods of analysis provide a physical property measure of food samples as a function of temperature at the same time as when the food sample is being subjected to a controlled temperature program regime. The changes that are monitored include mass/weight (loss or gain), density, transition temperatures/energy, heat capacity, phase transitions, visco-elasticity/gelation, modulus, rheology, and dimensional properties. The magnitude of the change associated with physical properties is essential, as it provides information about the quality, authenticity, or possible adulteration of the food involved in the tests.

Introduction

Thermal methods of analysis are highly useful in food forensic analyses, because most foods are susceptible to variations in their physical parameters that eventually alter the chemistry of food constituents that are associated with the quality of foods, including texture, taste, aroma, stability, and taste. Moreover, parameters such as temperature may change during processing (cooking, freezing, pasteurization, etc.), transportation and storage, and will affect food properties such as mass/weight, density, rheology, and heat capacity. This may alter the composition and ratio of ingredients and thus lead to wrong labeling. Moreover, chemical reactions that occur during various stages and steps of food processing, preparation, storage, and consumption, such as hydrolysis and redox reactions, may be triggered in foods, thus causing changes in the food's physical properties in terms of, for example, evaporation, melting, crystallization, aggregation, or gelation. Thus, these changes will affect the overall natural properties of the food on the one hand, but may also be used to indicate where there is a misnomer in terms of food quality/composition.

The Relationship Between Temperature and Food Properties

There is a direct relationship between the physical changes of food components and the variations of the temperature during processing, preparation, or storage of foods. The physical changes that are related to temperature variation include density, phase transition, and gelation. Normally, solid food materials have a higher magnitude of density than liquid foods. When solid foods change phase into the liquid phase through melting processes or when liquid foods crystallize into a solid state, the change in density that accompanies such a phase transition is significant and therefore the variation of density with temperature. For pure materials that normally do not involve any phase transition processes, for example melting (solid-to-liquid), crystallization (liquid-to-solid), evaporation (liquid-to-gas), condensation (gas-to-liquid), sublimation (solid-to-gas), and glass transitions (glassy-to-rubbery), their densities tend to decrease with increase in temperature due to the fact that the space between atoms in such materials increases when the atoms absorb energy, thus increasing their kinetic energy causing them to move randomly and vigorously. For materials that undergo phase transition, there is a drastic variation that is observed with density as the temperature varies. On the other hand, the mass of materials does not have any relationship with the variation in temperature as long as processes such as condensation or evaporation do not take place. In cases where the volume is dependent on temperature, then there will be an inverse relationship between density and volume.

Phase transition brings about a change in the overall properties of foods and thus techniques capable of measuring physical properties such as molecular structure, molecular mobility, density, rheology, and heat capacity of a material that change with phase transition, need to be employed to monitor such changes.

Gelation, on the other hand, is a property that enables food materials to form gels under certain conditions of temperature. Gel structures refer to the three-dimensional assemblage of biopolymer aggregates or colloids that have attracted and gathered water around them. Gels may have different types of appearances such as opaque or they may be transparent, depending on the composition of their aggregates. The composition of aggregates also determines other properties of gels, such as stability and rheology. Certain foods are known where gelling affects their overall properties greatly and these include yoghurt, eggs, and jellies. The dependence of gelling on temperatures comes from the fact that in some foods, gelling is observed only when heat treatment has been applied. These types of foods (e.g. egg white) or food-gels are commonly called heat-setting gels, while those that form when the cooling process has been applied, are known as cold-setting gels. All these gel types (cold-setting and heat-setting)

may either be reversible or irreversible and since they are all temperature dependent, they may then be termed as either thermos-reversible (if they are reversible) or thermos-irreversible (for those which are irreversible). To investigate gelling behavior of foods and their relationship with changes in physical parameters such as temperature, techniques are normally employed for such purposes. Such properties may indicate cases of adulterations, authenticity, and food quality.

Generally, thermal analysis-based techniques play an important part in the field of food characterization, as they generate useful experimental data that explains how various food components behave when subjected to heat treatment. Different food types can be characterized by using thermal methods of analysis. For example, they can be used to study protein behavior upon heat treatment related to their conformation changes, thermal/freezing denaturation, and stability; and food polymers (e.g. starch, polysaccharides, fats, and oils) behavior upon treatment (gelling properties, phase transitions crystallization, stability changes, and decomposition). For frozen foods, thermal analysis techniques may be used to study their thermal stability, glass transition, and lyophilization and for food microorganisms these techniques are instrumental in establishing the heat killing food microorganisms, microbial growth, and microbial metabolism.

The thermoanalytical methods that are of interest in food forensics and food analysis include thermal gravimetric analysis (TGA), which provides a measure related to the change in weight (gain or loss) and/or rate of change in weight as a function of temperature; differential thermal analysis (DTA), which measures the amount of heat evolved or absorbed; differential scanning calorimetry (DSC), which provides a measure related to the differential temperature or heat flow either to or from the sample against the reference material (DSC and DTA are all calorimetric based methods); and thermomechanical analysis (TMA), which is useful for measuring the penetration, expansion, contraction, or extension properties of materials as a function of temperature.

Application of Thermal Gravimetric Analysis Techniques in Food Forensics

Thermogravimetric techniques are attractive and useful in food analysis due to their capabilities to continuously and simultaneously measure the mass of a food sample as it is being heated at a controlled rate of temperature. Normally water molecules are expelled first from the food materials and depending on the food molecular environment, free water molecules tends to be expelled at lower

temperatures as compared to bound water molecules The technique can thus be used to estimate the amount of water (free and/bound) that is contained in the different molecular environments of the food.

Moisture Content and Water of Crystallization of Foods Using Thermogravimetry: Measure of Food Authenticity, Quality Control and Quality Assurance

Being one of the parameters that are controlled and which may be involved in food forensic investigations because it is regulated, food moisture content of foodstuffs is always monitored, because there are guidelines and legal limits that must be adhered to and for this reason, the labeling must be verified. This stems from the fact that the water content in foods may encourage microbial growth unless foods are dried to the level that is below specific critical moisture of that particular foodstuff. Moreover, the majority of characteristics that determine the quality of foods such as taste, appearance, texture, and stability are all highly dependent on the amount of moisture contained in that particular foodstuff. In some instances, some unscrupulous business entities try to adulterate foods by adding excessive amounts of water for economic gain and this is an obvious crime that calls for forensic investigation.

By definition, the percentage of moisture content in food samples is given as the ratio of the mass of the water (mw) to that of the food sample (mfood sample) multiplied by 100 (Equation 18.1):

$$\text{Moisture percentage} = \left(\text{mw} / \text{mfood sample}\right) \times 100 \qquad (18.1)$$

NB: The mass of water in the food sample can be worked out from the data related to its molecular weight (MW), *i.e.* 18 g per mole (nW), as well as the Avogadro number (NA), *i.e.* one mole of a substance contains 6.02 × 1023 units (atoms, ions, molecules), such that the mass of water can be calculated as follows (Equation 18.2):

$$\text{mw} = \text{nwMw} / \text{NA} \qquad (18.2)$$

Moisture as water molecules in food samples exists in different forms/environments, depending on how they interact with specific matrices in

foods and they (water molecules) become imparted with different chemistries that can be categorized in different groups, including bulky water that can be contained in foods which is not interacting with the food matrix. This category of water will thus possess all the common properties of water such as density, boiling point, *etc.* Moisture or water in foods may be present as trapped capillary water that is trapped and held in tiny channels by capillary forces. There is also what is known as chemically bound water in foods, representing a water fraction that has chemical interactions with food matrices. An example is the water of crystallization, which may exist in forms of water of hydration that are characterized by relatively stronger bonds as compared to ordinary water molecules and therefore they possess different chemical and physical properties as compared to ordinary water, such as boiling point, lower melting point, *etc.* Also, they have higher density and heat of vaporization than water, but lower compressibility and different electromagnetic absorption frequencies. Apart from chemically bound water, there is also physically bound water in foods, which represents a fraction of the water in foods that is not entirely surrounded by only water molecules but also adsorbed to other biomolecules present such as sugars/carbohydrates, amino acids/proteins, or inorganic species such as salts and minerals. For this reason, this fraction of water possesses different and unique properties from that of normal water.

Practically, foods are very heterogeneous in the sense that they consist of different categories (chemically bound, physically bound, bulky, capillary water) and water of different forms (liquid water, solid water, and vapor) in different ratios and proportions. To be able to correctly determine the different categories or forms requires a proper choice of the method and technique and also the expertise and experience of the analyst.

There are thus a number of methods and techniques that are used to measure the moisture content of foods, which differ in terms of their specificity, level of accuracy, and sensitivity. Therefore the choice of analytical method will only depend on the nature of the food being analyzed and the required information. However, sample introduction to these techniques is always preceded by sample preparation steps, which will thus be discussed prior to the instrumental analytical techniques for the measurement of moisture/water in food samples.

Sampling and Sample Preparation Methods in the Analysis of Moisture/Water Content in Foods

Sampling is a crucial step that needs to be handled professionally and carefully, as it ensures a proper selection of a representative sample and also ensures that no unwanted changes in terms of the properties of the food sample will take place prior to analysis, in order to avoid unnecessary analytical errors that may compromise with the process of food analysis. There are also crucial precautions that need careful observance when analyzing food moisture content, in order to eliminate the possibility of introducing errors due to either any loss or gain of water from the surroundings. These precautions include ensuring that the food sample is protected from exposure to the atmosphere and also the food is protected from any excessive temperature fluctuations. An example of the strategies that are normally used to minimize loss or gain of water is to ensure that the storage containers for food are filled to the brim, allowing no appreciable headspace.

There are traditional wet methods for measuring moisture content and total solids in foods. These methods do not involve expensive instrumentation and do not require highly skilled personnel to handle them. They are therefore attractive, cheap, and affordable to many laboratories.

Among the traditional wet methods are the evaporation methods that are based on the fact that water in foods has a lower boiling point as compared to the other ingredients and constituents present in the food, such as biomolecules (sugars, salts, proteins, etc.). In these approaches, the amount of water/moisture is worked out by considering the difference in terms of weight before and after the evaporation process, according to Equation 18.3:

$$\text{Moisture / water percentage} = \left[\left(\text{Mbefore} - \text{Mafter}\right) / \text{Mbefore}\right] \times 100 \quad (18.3)$$

The total solid fraction in foods is related to moisture in foods, because it represents a portion of the material that remains after the evaporation of water from foodstuffs (Cai and Chen, 2008) and it can be mathematically expressed according to Equation 18.4:

$$\text{Total solids percentage} = (\text{Mafter}) / \text{Mbefore}) \times 100 = \left(100 - \%\ \text{moisture content}\right) \quad (18.4)$$

One challenge that an analyst has to overcome is the difficulty in eliminating all the moisture in the food sample without tampering with other constituents of the food, including those that are volatile. There are therefore standard techniques

that are normally used and which are known to result in reproducible and accurate data for moisture content in foods, because they employ a standardized temperature and time during the evaporation processes.

It should be noted that there are factors that control the rate and extent of water removal from food samples. These include the sample size, fineness, and shape of the sample. For samples that have been ground to a very fine size, they will possess greater surface area, which will imply that much of the sample is subjected to the drying environment and thus the sample will dry at a faster rate than those with a smaller surface area. Moreover, as discussed above, the type or category of water that is contained in the food will determine the ease of the drying process through evaporation. For example, free water can be more easily evaporated than chemically bound water and this will imply that for chemically or physically bound water, relatively more stringent temperature conditions may be required to expel all the moisture from food samples. The same consideration goes also with the water portion of food, which contains some solutes because the solutes tend to decrease the partial pressure of the water, a condition that will need an increased temperature to elevate the vapor pressure to be equal to the atmospheric pressure for the boiling to occur, making it possible for moisture to escape in the form of vapor.

However, by increasing the temperature or drying for an unusually prolonged time, care should be taken not to cause thermal decomposition, degradation, and denaturation of food constituents such as saccharides, carbohydrates, or proteins. For example, at high temperatures, disaccharides such as sucrose tend to break down to their monomeric sugar components (monosaccharides, *i.e.* glucose and fructose), a reaction which occurs when water is present. In this instance, water will be used through this reaction and it may cause serious errors as to the actual water content that was present in that particular foodstuff. In some other instances, food components such as carbohydrates at high temperatures, tend to give decomposition products that include water and this may cause errors in the estimation of the water/moisture content present in that particular foodstuff. In the case of foods that contain volatile components, such as flavoring/aroma constituents and/or odor constituents that may volatilize easily, even at normal temperature needed for evaporation, care should be taken to avoid any errors that may result in the decrease of weight/mass of food materials and where necessary an alternative drying method may be considered. For samples that may be adulterated through the addition of water (e.g. milk, juices), a multi-stage drying procedure may be considered, because heating such foods may cause spattering as well as excessive accumulation of vapor. These phenomena are undesirable as

spattering may cause loss of food constituents, especially in cases where ovens are used for drying. An example of a multiple drying stage that can be considered includes steam-bath drying before oven drying. This will mostly depend on the mode in which the thermal energy is being transferred to the food sample, whether directly or indirectly. An example of direct heat transfer may be the use of an oven and an example of indirect heat transfer may be the use a microwave, which uses electromagnetic energy to heat the food sample through the mechanism that involves the absorption of the microwave energy by water molecules present in the food.

Modern Instrumental Methods and Techniques for the Analysis of Moisture Content in Foods

TGA has been widely used in the characterization of moisture content, as well as water of crystallization of foods. Normally foods contain moisture and the characterization of this moisture/water content is important for assessing end use properties. The moisture/water levels of foods can have an impact on important characteristics including stability, shelf life times, stickiness, visual appearance, and ability to dissolve in water. Even small differences in the level of water can have a significant effect on the properties of foods.

Apart from TGA, calorimetric methods, for example differential scanning calorimetry (DSC) and differential thermal analysis (DTA), are capable of providing a measure related to changes in terms of either the amount of heat absorbed or that which is released by a food sample or other materials such as food packaging as its temperature is varied at a controlled rate. These techniques can also provide a measure of water (free or bound) contained in a particular food sample, simply by making use of a measure of the sample's melting point of water, which is largely dependent on its molecular environment. Normally free water tends to melt at a higher temperature than bound water and therefore if the enthalpy change of the food sample is measured with temperature or time, one can easily deduce from the data the amount of water that is contained in that particular food material under different molecular environments, whether free or bound.

During the measurement of moisture content, which provides the magnitude of the difference in terms of the weight of the food sample that takes place simultaneously during the heating process, the food sample is weighed before the heat treatment and also when the heating process has attained a steady state mass subsequent to drying. Since in thermogravimetry the rate of mass/weight

mass subsequent to drying. Since in thermogravimetry the rate of mass/weight loss properties is measured as a function of time or temperature, it is thus possible to use this technique to obtain valuable data on the composition, which may indicate evidence on possible adulteration, thermal stabilities, oxidative stability properties of foods, and also lifetimes, which may show the actual expiry date that may indicate any fraudulence in labeling.

Many reports have shown successful moisture measurements of different types of foods using gravimetric methods. For example, Tomassetti *et al.* (1987) employed TGA in the measurement of moisture in ten types of commercial food flours (mainly wheat meal, hard corn meal, semolina, rye flour, ground rice, corn flour, potato starch, chick pea flour, soya flour, and powdered chestnuts). In this work, the data obtained for both moisture and ash content was correlated to standard literature data for verification purposes.

Moisture content measurement is also important in authenticity tests for stored food products such as cereal grains, peas, beans, oil-seeds, copra, cocoa beans, and also many other spices used in foods, because the measure of moisture content can be used to differentiate between oily grains and non-oily grains. Tomassetti *et al.* (1986) analyzed for authentication purposes the amount of water in vegetable seeds, such as basil, cabbage, parsley, carrot, tomato, onion, and lettuce.

The authentication of hydrophilic biopolymers, such as poly(hydroxystyrene) and its derivatives, which are known to be biodegradable and thus useful as food packaging materials, can be done by measuring the bound-water content using thermogravimetry (Hatakeyama *et al.*, 1988). The analysis of the amount of bound water to biopolymers using thermogravimetry is made possible due to the fact that thermogravimetry will give a measure of heat of melting or heat of crystallization of water, and this measure can be used to work out the bound water content and in addition to these the thermogram curves will give an indication of the heat of vaporization for the biopolymers that will provide the magnitude of bound water for all the biopolymer components of the food (Hatakeyama *et al.*, 1988).

Considerations Needed to be Taken into Account During Data Interpretation from TGA Curves

- The analyst needs to understand the atmosphere (air? nitrogen? oxygen?) in

which the reaction takes place;

- The analyst must take note of the initial decomposition temperature (IDT) (the onset point) where the sample material begins to decompose. This point gives a measure of the thermal stability of the sample material;
- The point which denotes a maximum rate of decomposition (MRDT) gives an indication of the regime at which most of the sample component is undergoing decomposition;
- The D(1/2), *i.e.* d-half, denotes the temperature at which the half-weight percent of the sample material has undergone decomposition;
- The FR point (or final residue) gives a measure of the amount that remains after the end of the heating program and this point is useful in providing the final composition of the sample material, while the area under the derivative curve determines qualitatively the amount of sample component that has been decomposed;
- In case the results from the curves have either very close or similar magnitudes of the area under the main decomposition stage, it will imply that the sample component is decomposing as a function of the temperature.

Protein Identification/Authentication and Species Differentiation Using Differential Scanning Calorimetric (DSC) Methods

Differential scanning calorimetry (DSC) provides a measure of the energy required to keep a zero temperature difference between a test sample and a reference sample by either heating or cooling the sample at a controlled rate. When the sample is subjected to DSC analysis, it will undergo phase transitions and when this happens it implies that it has either absorbed or released energy (heat). The DSC can be used to keep the temperature of both the sample and that of the reference at an equivalent amount of energy (heat) by giving the magnitude of the energy that needs to be supplied to either the test sample or the reference sample. Normally, the DSC data is reported as the rate of energy absorption (Q) by the test sample relative to the reference sample with respect to the external temperature. In some cases, DSC can provide data related to the change in the heat released by the test sample as a function of time (instead of temperature) under isothermal (constant temperature) conditions.

In food forensics, differential scanning calorimetry (DSC) has proved to be very

In food forensics, differential scanning calorimetry (DSC) has proved to be very useful in the determination of energy changes in samples that are subjected to thermal treatment. Researchers and scientists can make use of DSC when investigating phase transitions that involve energy changes for the sample in question. Examples of such transitions include endothermic denaturations (endothermic: crystalline melting (heat of fusion), dehydration); exothermic denaturations/decompositions (exothermic: crystallization (ordering or freezing) polymerization, etc.); and glass transitions or melting behavior. Differential scanning calorimetry is based on measuring temperature as well as the spontaneous/compensating heat fluxes (Bershtein and Egorov, 1994).

In food forensics, physicochemical thermal methods such as DSC can be useful in species identification and authentication of foods from different species. This is because both the structural similarities and structural differences between targeted specific proteins from different plant/animal species can be revealed using thermal DSC techniques that are capable of providing the contrast in terms of the patterns and changes that are related to the protein thermal behavior of different species. DSC can provide information about the denaturation temperatures (Td) and transition enthalpies (ΔH) that can be matched/compared to the protein in various species in each of the DSC processing steps (Murray *et al*., 1985). DSC can also be used to provide information about protein thermal stability, protein overall conformation, the (apparent) thermal (endothermic/exothermic) transition midpoint (Tm) values, profile similarity values, and protein domain folding integrity that is characteristic to individual species (Ibarra-Molero *et al*., 2016 ; Wen *et al*., 2012; Xiong *et al*., 1987).

The similarities or differences in terms of protein profiles under DSC can be explained by the fact that the evolution of proteins in each species is associated with specific mutations in certain sequences of amino acids and peptides in some of the proteins to enable the proteins to adapt better to the environment, to make them more suitable for some specific functions, or make the organism successful in the population in terms of passing on its genes. In this way the same protein becomes highly conserved for all members of the same species that contain the protein. Since mutations are known to be very rare and they also occur randomly, these highly conserved proteins so specific to species may not have more than one chance for them to occur. For this reason, all organisms that possess the same protein (conserved protein), the chances are high that they belong to the same species and those organisms in which the target conserved protein is not there, belong to different species. Different proteins will have different patterns under DSC and they can thus be differentiated.

Application of DSC in the Analysis of Protein Thermal Stability

Normally biological molecules such as proteins, when they are in solutions, tend to exist in equilibrium between their native (folded) and denatured (unfolded) conformations. Thermal phase transitions can occur in such molecules, which accompany either absorption or release of heat energy. The protein stability will depend on the magnitude of the thermal transition midpoint (T_m), such that the higher the T_m, the more stable the biological molecule is. DSC is a physicochemical thermal method that provides a measure of a number of parameters, including the enthalpy (ΔH) of unfolding that is related to heat-induced denaturation, the change in heat capacity (ΔC_p) of denaturation, and also factors that govern the folding behavior and stability of native biomolecules such as proteins.

When protein samples are subjected to DSC for thermal stability analysis while the protein sample is in a dilute solution environment, the observations that may be expected include those that involve changes in the specific partial molar heat capacity of the protein at constant pressure (ΔC_p). The specific heat capacity is an essential parameter for the food industry, as it provides indication and guidance for the estimation of the measure of the amount of energy required to either be introduced or removed from the sample, so as to increase or decrease its temperature by a certain quantity. The importance of the knowledge of the magnitude of specific heat capacity of a given food material lies in the fact that it is used in the design of processes, for example chilling, freezing, warming, sterilization, and cooking. Thermal techniques such as DSC and DTA can all be useful to provide a measure of the specific heat capacities of foods. For example, when DSC is used, the measure or magnitude of the specific heat capacity (CP), can be worked out using Equation 18.5:

$$Q = m\left[C_P\right] dT / dt \tag{18.5}$$

where Q = heat flow per unit time; m = sample mass; C_P = specific heat capacity of the material; and dT/dt = the rate of change of the external temperature.

Generally, whenever there is a change in the magnitude of heat capacity of any sample, it implies that the sample possesses the tendency to absorb heat. Different substances possess specific measures of heat capacity that is characteristic to them. For example, if the specific heat capacity of water is

compared to that of many organic compounds including proteins, it will be found that water possesses a higher value of specific heat capacity than protein due to the presence of an increased measure of hydrogen bond network. Water molecules are also found forming matrices in proteins and those water molecules that are found in protein matrices have a highly ordered chemistry in terms of their molecules being tightly held near and around the hydrophobic environment found on the protein's surface, and due to the fact that water molecules cannot form hydrogen bonding with hydrophobic molecules, it makes the formation of hydrogen bonding between water molecules highly optimal (Shinoda, 1977). During the DSC heating program and as the temperature is increased, the ordered water fraction on the surface of the protein becomes distorted and starts to behave like the bulk water and this results in an increased heat capacity of protein aqueous solutions.

In order to work out the real measure of the protein's specific heat capacity calorimetrically (the protein's measure of its specific partial Cp), the subtraction of a scan of a buffer blank sample from the protein sample profile data has to be done. The magnitude of partial Cp also represents the contribution of the effects of the protein in the aqueous solution matrix, in addition to it providing a measure of the heat capacity of the protein that is being analyzed for its thermal stability properties (Bruylants *et al*., 2005 ; Freire, 1995). During the heating process, there will be a phenomenon that is associated with raising the baseline and as the heat starts to be absorbed by the protein, it will reach a certain characteristic temperature range where the protein starts to thermally unfold, which will result with the observation of an endothermic peak. The temperature range at which the protein becomes thermally unfolded is characteristic of different types of proteins and is also specific from proteins from different species (e.g. muscle proteins from different species). This means that the technique can be used for differentiation of species, authenticity tests, food adulteration cases, *etc.* The magnitude of the enthalpy (ΔH) can be worked out by integrating the data for the heat capacity of the protein and plotting this value against the corresponding temperature values used during the DSC run (Equation 18.6):

$$\Delta H = \int_{T1}^{T2} C_p dT \tag{18.6}$$

From the plot drawn using Equation 18.6, a trend will be observed, showing a shift in baseline before and after the transition and this is important as from it the

change in heat capacity (ΔC_p) of the protein (and associated water) due to the unfolding phenomena can be deduced. Another important point that can be deduced from the graph is the transition midpoint (Tm), which is also known as the melting temperature, and which represents the point where half of the protein molecules are folded and the other half are unfolded. The area under the curve plot (C_p/T vs. T), obtained using Equation 18.7, is important for providing information regarding the magnitude of entropy (ΔS) (Bruylants *et al*., 2005 ; Marky and Breslauer, 1987).

$$\Delta S = \int_{T1}^{T2} \left(C_p / T \right) dT \qquad (18.7)$$

Phase transitions such as crystallization, melting, glass transitions, and conformational changes in foods are important, because they provide useful information regarding the temperature at which these transitions take place (T_{tr}), the enthalpy change associated with such a transition (ΔH_{tr}), the type of transition involved whether it is exothermic or endothermic, and the measure in terms of quantity of sample material that undergoes this transition. However, there are certain important considerations that have to be taken into account when using DSC for the measurements of these transitions. One such consideration is that all food components are biologically and chemically complex and thus the phase transitions may occur over a wide range of temperatures. For example, the composition of vegetable edible oils may include different chemistries of different triacylglycerols and each of these may produce a different pattern in terms of the melting point profiles. Moreover, due to the different chemistries of the triacylglycerols in plant-based edible oil, there is a high possibility that they can have more than one crystalline structure due to their polymorphic nature.

Factors that Affect Thermodynamic Stability Properties of Proteins

There are several factors that govern the stability of a protein and/or its Tm value:

pH of the Environmental Matrix

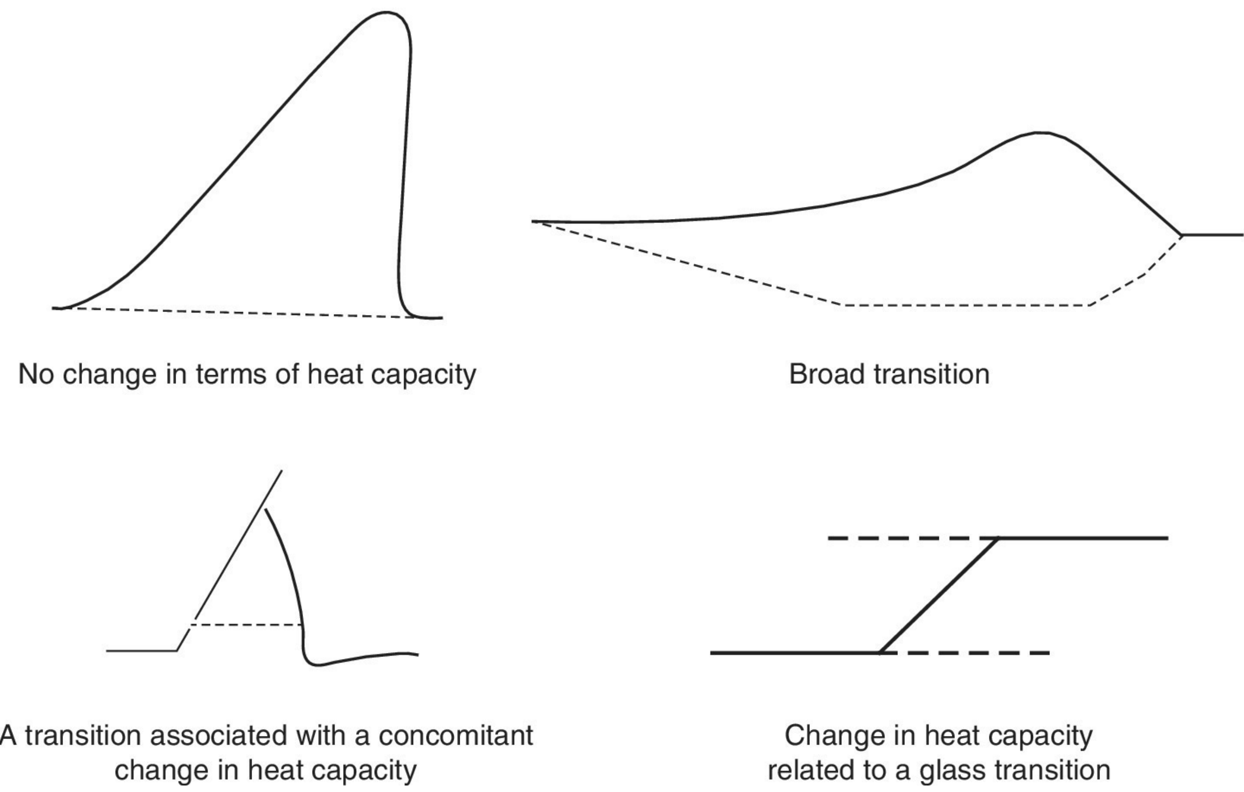
Among these factors is pH, which can cause specific proteins to be stable at a

particular characteristic pH value, and if the pH shifts or is off the range of stability, the protein loses that stability (Privalov and Khechinashvili, 1974).

Concentration of Protein

The concentration of protein can determine its conformational stability, because it is related to protein–protein interactions. It also affects the ionic strength of the solution that can be an indicative measure and role of electrostatic interactions and in addition to all these, the concentration of proteins is proportional to the scan rate (Griko *et al.*, 1995 ; Pfeil and Privalov, 1976 ; Robertson and Murphy, 1997).

One limitation of using DSC for protein analysis is mainly the amount of protein that is normally available for use as the sample is always limited, something which can seriously restrict the application of thermal biophysical methods such as the normal DSC.



Scheme 18.1 DSC interpretation illustration.

Differential Thermal Analysis (DTA) and Food Forensics

Like DSC, DTA measures the changes related to either heat absorbed or released by a test food sample while varying the sample’s temperature at a controlled rate. The changes in the magnitude of heat absorbed/released happen during the DTA processing of the sample, whereby food constituents undergo some specific phase transitions such as crystallization, melting, evaporation, glass transitions, and conformational change, or in some cases there may be chemical reactions such as oxidation, reduction, or hydrolysis that may occur instead of or in addition to phase transitions.

In DTA, the difference in the measure of temperature between the test sample and the reference sample versus time or temperature is recorded simultaneously, as the two samples (test and reference) are subjected to the same temperature treatment whereby the sample cells are either heated or cooled at a controlled rate regime.

From the DTA results, if the sample temperature is more than that of the reference, it means that heat energy was released during the phase transition and thus the process is termed as exothermic when $DT > 0$ and endothermic when $DT < 0$ (heat energy absorbed during the phase transition).

Other important information about the sample, for example the type of transitions that have taken place can be deduced from the nature of a peak, whether it is exothermic, endothermic, or it can be deduced from peak shape. In addition, signal (peak) position enables analysts to obtain information regarding the temperature range where the transition takes place. These points are characteristic to species and food types and they can be used to discriminate, classify, and provide authenticity as well as evidence in cases of adulteration.

Isothermal Titration Calorimetry (ITC) Application in Food Forensics

Isothermal titration calorimetry (ITC) is used to provide information about a measure of enthalpy changes that occur due to the interactions between different types of molecules. To perform ITC analysis, one would require having a reference sample, a test sample and an injector solution. The requirement for an ideal reference is that it must not undergo any enthalpy changes when subjected to ITC and the example is distilled water. Then a measure of the energy needed to maintain the test sample and the reference sample at the same temperature is recorded as a function of time. This will generate data that can be plotted as the amount of heat (Q) against time and from the nature of the process whether it

involved exothermic or endothermic processes or by investigating the magnitude of the area under the curve and shape, one can deduce information regarding the interactions, whether binding or conformational, which are occurring between molecules in the test sample.

Thermogravimetry and Cooking/Edible Oil Fingerprinting

Cooking or edible oils are important additions to cooked foodstuffs as they improve the taste of foods. There are parameters that are known to define the quality of cooking oil and include the fatty acid types, fatty acid composition and the oil's resistance to oxidation. In some cases, some additives (mainly antioxidants) are introduced into the oil to enhance its stability to the oxidation processes. Other approaches to control oxidation of oils and increase oil stability such as hydrogenation and modification of fatty acid composition, *etc.* are known to add to the stability and quality of oils. There are other external factors (apart from fatty acid composition, antioxidants. and water that are intrinsic factors) that contribute to the oil's lipid oxidation. The external factors that contribute to the lipid oxidation/stability include light, high temperatures, and gases, mainly oxygen (Kamal-Eldin, 2006 ; Merrill *et al*., 2008).

Despite the presence of all these factors, in most cases it is the fatty acid composition that is taken as the predictor factor for oil stability and therefore the oil's shelf life.

The stability of oils can be ascertained using thermo methods of analysis, mainly thermogravimetry (TG), differential thermogravimetry (DTG), and differential scanning calorimetry (DSC), which can also be used to verify the authenticity and differentiation of cooking/edible oils (De Souza *et al*., 2004 ; Diniz *et al*., 2008 ; Freire *et al*., 2009 ; Litwinienko and Kasprzycka-Guttman, 1998 ; Santos *et al*., 2002 ; Simon and Kolman, 2001). In food forensic cases related to edible oil products, TG can be employed for the analysis of oils, because it can reveal semi-quantitative information regarding thermal degradation processes that take place during thermochemical conversion reactions that occur under different atmospheres. This information can be coupled to that from the DTG, in which information regarding the measurement of the rate of weight loss of the oil versus temperature and/or time is obtained. The information from DTG is important as it enables the prediction of the thermal behaviors of the different types of oils.

Thermogravimetry and Food Packaging and Food Contact Substances Authenticity and Compliance

Food packaging provides a limiting barrier that separates and protects food from exposure to any potential contaminants, oxygen, moisture, etc., which may be present around it or in the immediate external environment. The ideal food packaging requires qualities that will ensure hygiene and the ability to minimize the risk of food contamination and where possible, the materials should have the advantage of extending the shelf life of foods. In addition, food packaging must be accompanied by labeling that contains all the necessary and useful information about the food product (ingredients), instructions for use, storage, processing before consumption, expiry date, and details on recycling. Normally all food packaging is required to adhere and comply with specific legal requirements.

In food industries, different materials are normally used as packaging to keep the food safe and if possible fresh so that it can be used at a later date. The types of food packaging materials that are used are in most cases single/multi-layer films, blends/composites of biodegradable biopolymers, and homopolymers, as well as copolymers.

Biodegradable Biopolymers Used in Food Packaging and Food Contact Substances

Biodegradable biopolymers are normally used for making food packaging and other food-contact substances including straws, stirrers, disposable cutlery, disposable drinking cups and their lids, disposable plates, water bottles, food overwraps, and food lamination films.

The source of these biodegradable biopolymers can be from renewable resources, which can thus be recycled and composted but are made such that they possess comparable optical, physical, and mechanical properties to their synthetic counterparts, which are normally made from oriented polystyrene (OPS) and polyethylene terephthalate (PET) materials.

There are mainly three major classes of biopolymers used in food packaging and food contact substances and they include (i) polyester biopolymers, (ii) polyhydroxyalkanoate (PHA) biopolymers and(iii) starch-based biopolymers. The classification is based on the production process that may be used and the

The classification is based on the production process that may be used and the source of the raw materials.

Polyester biopolymers can be sourced from natural biomass such as polysaccharides or they can be synthesized using starting materials such as polyetilene terephthalate (PET) containing aliphatic–aromatic copolymers and aliphatic polyesters, which are a polycondensation reaction product of glycol and aliphatic dicarboxylic acid (ADA), polylactide aliphatic copolymer (CPLA), as well as bio-based monomers, for example poly(lactic acid) and oil-based monomers such as polycaprolactones. The CPLA is a product of lactide and aliphatic polyesters such as dicarboxylic acid or glycol, with hard materials such as polyester (PS) and soft flexible materials such as polypropylene (PP), at ratios that can be determined by the content of aliphatic polyester in the blend. In addition to these, polyesters can be sourced polymeric materials, such as polyhydroxyalkanoates that can be obtained from natural sources, mainly microorganisms.

The bio-based polymers such as poly(lactic acid) (PLA), a copolymer between poly(l-lactic acid) and poly(d-lactic acid) and which can be prepared by the depolymerization procedure involving lactic acid monomers as sourced from corn and other similar products, have of late been highly promising for use in food packaging. Its attractive features include the ease in recyclability and biodegradability. An oil-based biopolymer such as polycarolactone (PCL) is used in food contact substances or in food packaging only when mixed with starch, where it is mainly used for trash bags. PCL-starch blends are thus attractive due to the fact that the polymer blend is highly biodegradable (with very short biodegradation time) and has excellent chemical resistance to solvents such as water, oil, and also chlorine. In addition, it is known to have low viscosity, and is easy to prepare and process.

Other natural materials that are used in food packaging and food contact substances include polyhydroxyalkanoates (PHA) biopolymers, which are produced in nature by microbial fermentation of sugar and lipids. PHA may exhibit properties of either thermoplastic or of elastomeric materials and may be used with or without synthetic plastic or starch in order to form the desired packaging films of the desired/specific qualities (Siracusa *et al*., 2008). An example of PHA, which does not include any blending polymer, can be the polyhydroxybutyrate (PHB) that is obtained directly from the polymerization of 3-hydroxybutyrate monomers, while an example of the blend can be the copolymer polyhydroxybutyrate-valerate (PHBV).

There are also starch-based biopolymers that are obtained from mixing of oil-based monomers and different quantities of starch, as well as other additives which play different roles such as flame-retardants, coloring, *etc.* An example of this can be low-density polyethylene film (LDPE)-starch blend.

When manufacturing food packaging, certain considerations regarding the properties of the materials are needed for specific usage or the required physicochemical and thermal properties in order to predict environmental effects as well as the impact modifications that may be incorporated. Other properties related to heat of fusion, glassy state relaxations, tan δ data, secondary transitions and storage modulus may be desirable in order to match the compatibility of various polymer composites/blends.

All these properties can be used to define and give an indication with regard to the compliance in accordance to the legal requirements for food packaging. Different materials and their blends as well as the additives incorporated into packaging give different packaging uniqueness and can also be used to determine the quality and level of compliance.

The thermal technique is one of the many techniques that can be employed to ascertain the compliance, quality, and authenticity of food packaging. For example, DSC can be employed to investigate a number of properties including the glass transition temperature (Tg), melting temperature (Tm), and crystallinity of the polymeric material, which is normally worked out using Equation 18.8:

$$X_c(\%) = 100 \times \left(\Delta H_c + \Delta H_m\right) / \Delta H_{cm} \tag{18.8}$$

where ΔH_c is the exothermic enthalpy of cold crystallization; ΔH_m is the endothermic enthalpy of fusion; and ΔH_{cm} is the endothermic heat of melting of purely crystalline sample polymer under investigation.

Another thermal based technique, thermogravimetric analysis (TGA), can be used to analyze food packaging and provide useful information regarding the decomposition temperature. The breakdown and biodegradability properties of these food packagings can be investigated using DSC and TGA.

Conclusions

Thermal methods are useful for certain types of foods and food packaging authentication. The analyst must be conversant with the principles involved in

these methods so as to correctly choose the appropriate method of analysis for a particular sample. The knowledge of data interpretation from the generated curves is very essential.

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19
Application of Electrochemical Methods and Biosensors in Food Forensics

Introduction

Electrochemical methods of analysis have attractive features which include selectivity, sensitivity, speed and also involve simple procedures and relatively simple/inexpensive instrumentation as well as simplicity of the analytical procedure, which involves less sample preparation procedures/steps (Bard and Faulkner, 1980). Electrochemical methods are capable of both qualitative and quantitative measurements of samples and there are several variants that have been applied in the analysis or detection of food components, food contaminants, *etc.*

Examples of electrochemical methods that are frequently applied in the analysis of food additives and food ingredients, include sugars (mainly glucose), vitamins, antioxidants (i.e. ascorbic acid), as well as other food and beverage components such as ethanol. Among these electrochemical techniques, applications in food analyses are those based on voltammetry, such as stripping voltammetry, an anodic stripping voltammetry technique that can be useful in the trace analysis of metal cations where the cation (analyte) is deposited onto the working electrode during a deposition step, and then oxidized during the stripping step when the current is measured.

Another voltammetric stripping technique is known as cathodic stripping voltammetry, which is useful in the trace analysis of anions whereby a positive potential is applied, oxidizing the working electrode such as a mercury electrode, thus forming insoluble precipitates of the anions. A negative potential then strips the deposited film into solution. Other voltammetric methods include linear sweep voltammetry, staircase voltammetry, square wave voltammetry and cyclic voltammetry. Cyclic voltammetry is useful in the determination of diffusion coefficients, as well as half-cell reduction potentials of the analyte. Another voltammetric method is adsorptive stripping voltammetry, which can be useful in the trace analysis of analytes. In this technique, the analyte is deposited by adsorption on the electrode surface and then electrolyzed in order to generate a measurable signal. In addition to these variants of voltammetric methods of analysis, there are other variants that make use of mercury as a working

electrode and they are commonly known as polarography. Others make use of rotated electrodes and are useful for studying the kinetics and electrochemical reaction mechanism for a half reaction. Other variants of voltammetry include normal pulse voltammetry, differential pulse voltammetry and chronoamperometry.

Of these voltammetric electroanalytical techniques, stripping voltammetric methods have been widely applied in the analysis of foods and food ingredients important for quality authentication such as vitamins, aromatic flavors, alkaloids, organic acids, *etc.* (Pisoschi, 2013). Stripping voltammetric techniques have been known for their high sensitivity over other voltammetric methods due to the fact the analytes of interest are accumulated and preconcentrated onto the working electrode by means of controlled potential electrolysis prior to the stripping step, which involves the dissolution of the deposit that will either oxidize or reduce the analyte when a linear ramp is applied to the electrode and thus generate a measurable signal at the electrode surface (Pisoschi, 2013).

From the signal generated, which is normally plotted as the current-potential plot known as the current-potential voltammogram, the shape of this plot as generated during the initial step is vital in terms of providing information about the sample. For example, the value/magnitude of the peak potential is specific and a unique characteristic of the analytes and can thus be useful qualitatively. The peak current associated with the process is very quantitative, as it is proportional to the concentration of the analyte. Moreover, since the magnitude of the stripping peaks for different analytes occur at uniquely characteristic potentials, it is then possible to have several analyte species in the same matrix/food and analyze them all simultaneously (Branina, 1974 ; Brainina and Neyman, 1993 ; Vydra *et al.*, 1976 ; Wang, 1985, 1994).

In anodic stripping voltammetry, which has been used for the trace analysis of heavy metals in foods, the working electrode that has been applied includes a hanging mercury drop electrode (HMDE) or mercury film electrode (MFE), whereby the cations/metal species deposited or preconcentrated cathodically at a controlled time and potential onto the mercury electrode are dissolved electrolytically. After preconcentration and dissolution, the potential is then scanned anodically and linearly such that during the anodic scan process, the amalgamated metallic species are stripped out of the electrode and are then reoxidized and dissolved in the sample solution. The magnitude of the anodic current associated with this process is then recorded as a function of the applied voltage. In cases where the anodic reaction generates insoluble products such as mercury salts or mercury complexes, it is then plausible to use the cathodic

mercury salts or mercury complexes, it is then plausible to use the cathodic stripping voltammetric technique (CSV) in which the oxidation of the analyte is used for its preconcentration step as an insoluble film on the electrode, and then the concentrated reduced analyte species is measured during the negative scan.

In cases where the analyte species cannot be preconcentrated by electrolysis processes at the electrode surface, then the preconcentration of the analyte can be achieved using adsorption processes (adsorptive stripping voltammetry (AdSV)), whereby the accumulation of the analyte takes place through physical adsorption instead of electrolytic deposition processes.

The use of HMDE or MFE have been discouraged in many laboratories due to the fact that they are a health concern and also not environmentally friendly. Therefore other electrode systems have been suggested to replace them. These include carbon paste electrodes (CPE) (Shams and Torabi, 2006), polymer film electrode (PFE) (Shelton and Chambers, 1991), surface-bound crown ethers electrodes (SBCEE) (Ijeri and Srivastava, 2001), carbon nanotube electrodes (CNE) (Gong *et al*., 2005), boron-doped diamond electrodes (BDDE) (Spataru *et al*., 2007), bismuth film electrodes (BFE) (Baldo *et al*., 2003) and screen printed electrodes (SPE) (Crew *et al*., 2008).

Sample preparation procedures for samples to be subjected to stripping voltammetry involve mainly acid digestion using mixtures of strong acids to decompose the sample or the use of microwave digestion approaches for elemental analysis, solvent extraction and solid phase extraction procedures.

The application of stripping voltammetry in foods has been reported in the analysis of toxic food contaminants of metallic origin, such as mercury (Capar *et al*., 1982 ; Sancho *et al*., 2001), lead (Satzger *et al*., 1983 ; Zink *et al*., 1983), cadmium (Capar *et al*., 1982), arsenic (Sancho *et al*., 1998), antimony (Locatelli and Torsi, 2004) and uranium (El-Maali and El-Hady, 1999). In another development, a simultaneous analysis of lead and cadmium in food crops, using differential pulse anodic stripping voltammetry, has also been reported (Golimowski *et al*., 1979 ; Satzger *et al*., 1982).

Differential pulse anodic stripping voltammetry has been reported in the simultaneous determination of lead and cadmium in food crops (Satzger *et al*., 1984), while stripping voltammetry was reported in the determination of lead and cadmium in vegetables (Matloob, 2003), also in wheat and rice (Ogorevc *et al*., 1987), in table salt (Ali, 1999), in liver and fish (Adeloju *et al*., 1983), in infant formulas (Esteve *et al*., 1994) and in canned soft drinks (Sabry and Wahbi, 1999).

Agrochemical contaminants residues analysis in foodstuffs including water is also one of the application areas for stripping voltammetric techniques (Ibrahim *et al*., 2001 ; Mainisankar *et al*., 2005 ; Pedrero *et al*., 1993). For example, organophosphorus pesticides such as fenthion (Diaz *et al*., 2008) and pyridafenthion (Sampedro *et al*., 1998) have been determined in olive oil and wine respectively using the adsorptive stripping voltammetric technique, while other organophosphorus insecticides such as phosalone and carbophos (malathion) have also been determined in potato and tomato with this same technique (Ulakhovich *et al*., 1998).

Certain types of carbamate insecticides have been detected in rice using anodic stripping voltammetry (Mathew *et al*., 1996). These are only a few examples of the application of stripping voltammetric techniques with application in food analysis, but there are many reports on the technique's application in the analysis of various agrochemical residues in different types of foodstuffs and drinks.

Apart from agrochemical residues, stripping voltammetric techniques have been widely applied in the determination of many pharmaceutical drug residues as well as veterinary drug residues in foods/feeds (Abu Zuhri *et al*., 1998 ; Alghamdi, 2002 ; Gratteri *et al*., 1992 ; Vire *et al*., 1989). In other developments, anodic stripping approaches have been reported in the determination of fertilizer residues in foods such as meat and other foodstuffs (Guanghan *et al*., 1997 ; Santos *et al*., 2009). Voltammetric/polarographic methods have been developed for contaminants such as heavy metals, food additives such as coloring compounds and organic compounds in foods (Alghamdi, 2010).

Biosensors for Food Analysis

Biosensors are artificial devices fabricated to sense or detect specific analytes and they are composed of three components (Figure 19.1) namely:

1. a biological recognition element (tissue, microbial cell, cell receptor, cell organelle, nucleic acids, enzymes, antibodies, protein, aptamers, synthetic oligonucleotides, etc.), commonly known as a bioreceptor. A bioreceptor is the biomimetic component that functions to bind and recognize the analyte being investigated or measured. This implies that when the target analyte interacts with the bioreceptor, it generates a signal that is measured by the detector (transducer);

2. a biotransducer, a component that plays the detection function in various ways, *e.g.* physical, optical, piezoelectric, electrochemical, *etc.* and through

these mechanisms the signal of the recognized analyte is transformed or transduced into another measurable signal, *e.g.* electrical signal (current, voltage, etc.);

3. an electronic system that may incorporate signal amplifier, processor and display platform. In some cases, the transducer component and the electronic system component are combined together.

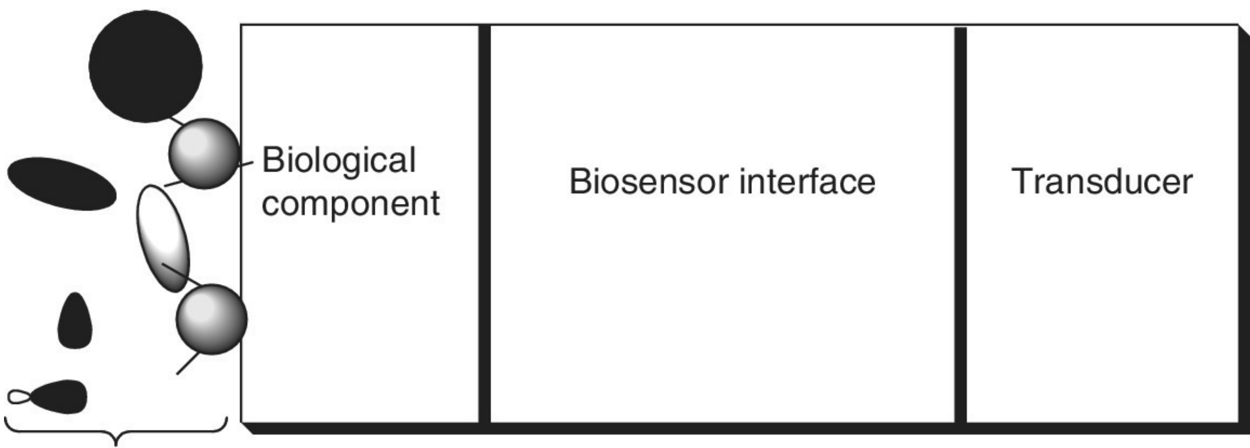

Figure 19.1 Schematic depiction of a biosensor.

The type of bioreceptor normally gives the name to the biosensor, for example if an enzyme is used as the bioreceptor to specifically convert the substrate analyte which acts like a reactant molecule into a product, then this biosensor is known as an enzyme-biosensor. In enzyme-biosensors, it is common to incorporate cofactors which are other molecules or ions that assist in the reaction in such a way that they become catalytically changed, chemically producing physico-chemical effects that can play important roles in the monitoring and detection of the enzymatic process. Antibodies also can be used as bioreceptors for some specific molecules (antibody-biosensor) and if DNA is used as a bioreceptor then we will have a DNA-biosensor and all these bioreceptors can be used to specifically recognize and bind any kind of analyte present in the food matrix for the purpose of providing the evidence required for the specific issue. The analytes may be drugs residues, toxic molecules, *etc.*

Biosensors can also be grouped based on the type of biotransducer used for that particular biosensor. There are several classes of biotransducers that have been fabricated and used in biosensor systems and they include electrochemical biosensors, piezoelectric biosensors, optical biosensors, electronic biosensors, gravimetric biosensors and pyroelectric biosensors.

There classes of biosensors, irrespective of the type of classification, have been used widely in food analysis. For example, antibody-coated optics are more often used in the food industry for the detection of pathogens or food toxins, whereby fluorescence is used as the preferred light source due to its advantage to significantly amplify the signal.

Electrochemical Biosensors in Food Analyses

Generally, electrochemical biosensors have been widely used in food analysis and are based on redox mediated enzymatic catalysis of bio-reactions that either generate or consume electrons, which are detected using a set of three electrodes (working electrode, reference electrode and a counter electrode). During the measurement process, the analyte is brought to the surface of the active working electrode where there will be a reaction with the bioreceptor that will either cause electrons to be transferred across the double layer and thus generate current or can be added to the double layer potential implying that voltage (potential) will be produced. The current or voltage produced can allow both qualitative and quantitative measurement. For example, it is possible to measure the current (the rate of flow of electrons), which will be proportional to the analyte concentration when the current measurement is done at a fixed potential (Lud *et al*., 2006). Where the potential produced is measured at zero current to give a logarithmic response with a high dynamic range, such a biosensor is generally known as the potentiometric biosensor.

One attractive feature about biosensors is that they involve very minimal sample preparation due to the fact that the biological sensing component (the bioreceptor) is fabricated to have high selective properties for the analyte being measured. The electrochemical as well as physical changes (e.g. ionic strength, pH, hydration and redox reactions) are the ones to account for the signal that is produced at the conducting polymer layer at the surface of the transducer/sensor.

For example, other electrochemical methods are based on the potential vs. pH change measurements, which occur due to changes in the ion concentration in the sample matrix, *i.e.* potentiometric techniques. Other techniques involve the use of sensors such as potentiometric biosensors, which are fabricated with a sensing bio-based transducer that monitors biochemical reactions involving variations in terms of ion concentration variation. Examples of transducers that may be incorporated into potentiometric biosensors include enzymes coupled with a glass-pH electrode such that enzymatic reactions with target analytes like sugars or food contaminants (i.e. pesticides or pharmaceutical residues) can

occur, leading to either generation of hydrogen ions or reactions that may use the generated hydrogen ions and this will be measured by the pH sensor (Blum and Coulet, 1991 ; Scheller and Schubert, 1992).

Among the various types of electrochemical biosensors there are voltammetric sensors, which provide a measure of the concentration effect of the analytes on the current potential characteristics of either the reduction or oxidation of the reaction (Bakker, 2004). Another class of electromechanical sensors are the amperometric biosensors, which are actually a subclass of the voltammetric biosensors and are based on the passing of a fixed voltage/potential to the electrochemical cell, thus generating a signal in the form of the current due to either oxidation or reduction reaction processes, and this current is proportional to the concentration of the analytes being investigated (Bakker, 2004 ; Viswanathan and Radecki, 2008 ; Wang, 2005). Amperometric biosensors make use of conventional detectors to detect and measure the metabolic substrate or product of the analyte under investigation (Patel, 2002).

Potentiometric biosensors are another type of electrochemical-based biosensor, which investigate the magnitude of the potential difference measurement between the working electrode and the reference electrode as a function of the redox reaction of the species being investigated. The potentiometric biosensors are useful in cases where the accumulation of charge at zero current as created by selective binding at the electrode surface needs to be known (Bakker, 2004). The application of potentiometric biosensors has been greatly enhanced by the development of the ion-selective electrodes including the glass electrode, which measures pH of solution, and ion-selective field effect transistors (ISFETs), which incorporate an ion-sensitive surface (Castellarnau *et al*., 2007). In ion selective electrodes, the surface electrical potential is governed by the ions interacting with the semiconductor surface, which results in a measurable potential difference.

Other classes of biosensors such as piezoelectric biosensors, employ crystals which undergo an elastic deformation when an electrical potential is applied to them such that the process leads to the generation of a standing wave in the crystal at a specific characteristic frequency that is highly dependent on the elastic properties of the crystal. For example, if a crystal is coated with a biological recognition element, the binding of a (large) target analyte to a receptor will generate a change in the resonance frequency, which results in the generation of a binding signal. The application of other classes of biosensors, such as thermometric biosensors and magnetic-based biosensors are rare in food analysis applications.

Generally, all these classes of electrochemical biosensors have been used in applications related to immobilization of biomolecules, electrode design and signal transduction for food samples as well as other applications. Currently, the advent of nanotechnology has also revolutionized the technology related to fabrication of electrochemical sensors where nanoparticles, for example gold nanoparticles and nanostructures such as nanotubes, nanofibers, nanorods, nanoparticles and thin films, have been incorporated to enhance sensitivity and selectivity of the detection of specific analytes (Bonnemann and Richards, 2001 ; Niemeyer, 2001).

For example, various nanoparticles have been found to enhance the signals of electrochemical biosensors when they are incorporated/bound to biological molecules such as antibodies, peptides, proteins, nucleic acids, *etc.* (Hernández-Santos *et al*., 2002). The ability of metal nanoparticles to enhance the amount of immobilized biomolecules in fabrication of biosensors lies in their high surface area, which is very central to even lowering detection limit of the biosensor (Cai *et al*., 2001). Apart from metallic nanoparticles, inorganic nanocrystals such as zinc sulfide, cadmium sulfide and lead sulfide have also been utilized for a multi-target electronic detection of DNA or proteins. Moreover, the application of magnetic nanoparticles, which are generally prepared in the form of either single domain or superparamagnetic (Fe_3O_4), greigite (Fe_3S_4), maghemite (g-Fe_2O_3), and various types of ferrites ($MeO\text{-}Fe_2O_3$, where Me = Ni, Co, Mg, Zn, Mn, etc.), as diagnostic tools in biosensors has also been known to separate or enrich the analyte being investigated. Other biosensors have been fabricated containing modified surfaces of various functional monolayers or thin films. Electrodes have also been fabricated with functionalized redox active components, thus enhancing the sensitivity of the biosensors, for application in food analysis (Wang, 2002a, b).

Other recent developments in the area of biosensors for food application include the incorporation of one-dimensional nanostructures, which include carbon nanotubes, polypyrrole nanotubes, conducting polymer or semiconductor nanowires that are characterized by having high surface area to volume ratios as well as excellent electron transport characteristics and strong electronic conductance (Baughma *et al*., 2001 ; Miao *et al*., 1999). The application of these nanostructures in biosensor fabrication is attractive due to the fact that they make it possible for sensing elements to be packed in high numbers within a miniaturized device (Siwy *et al*., 2005).

In other developments, liposomes, which are biological microstructures made of layers of phospholipids, have been used as the supporting substrate for immobilizing the biorecognition molecules and for the enhancement of electrochemical signals in applications related to the detection of organophosphorus agrochemical residues (Baeumner *et al*., 2003 ; Vamvakaki and Chaniotakis, 2007). Moreover, the concept of using artificial sensory mappers, such as the electronic nose and electronic tongue, has received considerable attention in the recent past as sensors for various volatile compounds. These kinds of sensors are capable of discriminating a number of volatile species based on their electronic responses such as voltage, resistance, conductivity, *etc.* that come from the different gas sensors, normally metal-oxide chemosensors. For example, Jonsson *et al*. (1997) reported the application of the electronic nose to classify cereal grains, for example to discriminate between mouldy, weakly musty and strongly musty oat samples. The same kind of sensor was able to predict ergosterol levels and fungal colony-forming units in wheat (Jonsson *et al*., 1997 ; Olsson *et al*., 2002). Olsson and colleagues also used the sensor to predict deoxynivalenol and ochratoxin A levels in barley, to indicate ochratoxin A, citrinin and ergosterol production in wheat, while other researchers used the same biosensor system to indicate mycotoxin formation by Fusarium strains (Falasconi *et al*., 2005). On the other hand, the electronic tongue has been used to discriminate between natural and artificial water, different sorts of beverages, natural and artificial mineral waters, brands of coffee, sweeteners, *etc.* (Rudnitskaya *et al*., 2002).

Conclusions

Electrochemical methods and biosensors present a cheap and rapid means of analysis. In most cases they may serve as screening methods (the absence and/or presence of adulterants, etc.). Biosensors can be fabricated as miniaturized devices that may be taken to the scene where food poisoning has taken place and they only need minute amounts of the sample to provide data that can provide a clue to the evidence needed.

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20
Application of Flow Cytometry in Food Forensics

In food analyses, it is desirable to obtain multiple properties of food microbial pathogenic cells simultaneously, because this enables the determination of the physiological status of these pathogens, i.e. knowledge of their status as to whether they are viable, dead or stressed. Moreover, in food microbiology, it may be necessary to have techniques capable of rapid enumeration of pathogenic microbes, which can distinguish between viable, metabolically active cells and dead cells as well. This is very practical because cells differ in terms of their metabolic or physiologic patterns and thus by using an appropriate technique one may obtain information regarding the cell type or even establish the presence of different populations within a given matrix based on the structural or physiological patterns of the species that are present. This knowledge is highly desirable for purposes in the food industry where food development is the key activity and also it may provide information on the mechanisms that lead to food spoilage. Techniques capable of rapid analyses and enumeration of microbial pathogens in foods are therefore highly needed and flow cytometry is one of the technologies that can be used to count and analyze cells or other particles in food, particularly in the form of fluids. Unlike other analytical techniques, which provide measurement for a single parameter for the entire population, flow cytometry enables the possibility to get data for every species/particle that has been detected. In flow cytometry the single microbial cells are stained with a specific dye, passed through a light beam and then measurement of the fluorescence pattern on a cell-by-cell basis follows (i.e. the interaction of each cell with the beam is measured and correlated with the cell characteristics or cell components).

Introduction

Flow cytometry combines attractive attributes due to fluidics and optics, as well as multiparametric data analysis (with both data discrimination and data classification possibilities) to enable quick investigation and data correlation for multiple physical and biochemical attributes of either biological systems (cells

mainly) or microspheres.

The principle of flow cytometry measurements exploits the differences that exist between the refractive indices of biological cells and their surrounding media, such that when light impinges upon the cells, it becomes scattered (Julià *et al*., 2000). Generally, the forward scattered light (FSC, light scattered at low angles) in flow cytometry measurements is useful in providing information about the cell size (Julià *et al*., 2000). In instances where light is scattered either in an orthogonal or near-orthogonal direction, there is a separate detector, for example the SSC (side scatter) detector, that collects and measures this light. Alternatively, other detectors such as the PMT (photomultiplier tube) are used with flow cytometry instruments to collect and measure fluorescence radiation from macromolecules found within the biological cells. In order to enable the detection of a number of cellular parameters that are associated with functions or structural components of the cell simultaneously, a combination of light-scattering and fluorescence measurements on either stained or unstained cells has been encouraged. In flow cytometry, fluorescent dyes, such as rhodamine 123, have been used to stain viable eukaryotic cells as well as microorganisms (Diaper *et al*., 1992; Quiró *et al*., 2007; Resnick *et al*., 1985).

Dye staining can be used to gain information on the integrity of cell membranes, depending on their dye exclusion or dye retention characteristics. Examples of exclusion dyes include propidium iodide and ethidium bromide, which normally stains nucleic acids and other macromolecules. If these dyes are used to stain cells, and happen to stain macromolecules and other biological components such as nucleic acids, then it implies that the cell membrane is permeable and therefore the cell is dead.

In order to distinguish dead and live cells, it is required that a combination of a cell-permeant dye such as those that belong to the class SYTO family in combination with cell impermeant dyes such as propidium iodide be applied in conjunction. In the case of dye retention approaches, nonfluorescent cell-permeant esterase substrates are normally used to produce fluorescent products, which become trapped inside the cell by mechanisms that can be explained by their electrical charge as well as their polarity.

In order to detect food microbial strains using flow cytometry, immunofluorescent techniques have been devised to serve this purpose, such that it is possible to detect microbial cells singly. However, the process requires the availability of antibodies which are obtained from the appropriate organisms (Álvarez-Barrientos *et al*., 2000).

In other developments, a combination of flow cytometry together with biochemical techniques has been reported in applications to investigate the heterogeneity of bacterial cultures, mainly the *Bacillus* species, where live and dead endospores have been discriminated based on either their distinct cytometric scatter (Stopa, 2000) or cytometric scatter in combination with nucleic acid stains (Comas-Riu and Vives-Rego, 2002).

Oxidative metabolism indicator tetrazolium dyes, such as cyanoditolyl tetrazolium chloride (CTC), have been reported to detect the type of respiration in aerobic microorganisms (Kaprelyants and Kell, 1993).

Generally, flow cytometry is attractive in food forensic issues due to its ability to detect microorganisms and microspheres in foods at relatively low concentrations within a short time. For example, there have been cases that involve deliberate contamination of foods and food-related products by microorganisms such as bacteria and fungi (yeasts and moulds) and the presence of these microbes in food products, even at low counts, can severely affect the quality of the food and may have the potential to tamper with the health status of the consumers. The microbial composition of food products is generally taken as an indication of the types and kinds of normal biota of the raw ingredients used to produce the food product and is reflective of how hygienic were the procedures used during processing of that particular food product.

The possibility of multiple labeling enhances the advantages of flow cytometry, as it makes it possible to detect different organisms or even microorganisms at different stages in the same food sample. Therefore, the availability of methods capable of differentiating Gram-positive and Gram-negative bacterial populations is desirable in order to provide the information needed about the source of contamination. These methods include those that are used for Gram-staining for flow cytometry. An example of such a method combines the use of two fluorescent DNA binding stains, mainly a membrane-permeable stain SYTO 13 dye and hexidium iodide (HI). The hexidium iodide is normally blocked by the lipopolysaccharide layer of Gram-negative bacteria and is therefore only permeable to Gram-positive bacteria which possess a destabilized lipopolysaccharide layer (Mason *et al*., 1998).

Apart from this, there is another technique that makes use of Oregon Green-conjugated wheat germ agglutinin (WGA) in combination with hexidium iodide (HI), such that the WGA will bind to the *N*-acetylglucosamine that is present in the peptidoglycan layer of the cell wall of Gram-positive bacteria, while the HI will bind to the DNA of all bacteria (Gram-negative and Gram-positive) after

EDTA treatment and incubation at an appropriate temperature (Holm and Jespersen, 2003).

Flow cytometry can also be used in cases where there is a need to detect specific pathogenic microbes in food products, in which case either monoclonal or polyclonal antibodies conjugated to fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin are employed (Donnelly and Baigent, 1986; McClelland and Pinder, 1994; Völsch *et al*., 1990). However, this approach suffers from insufficient sensitivity, as its detection limits for the pathogenic microbes in complex matrices such as foods as per the regulations is very low, where it cannot achieve such low levels of detection limits. To counter this shortcoming, strategies that employ the amplification of the microbial count by culture have been suggested prior to analysis using flow cytometry.

Normally food samples may be regarded as contaminated if the microbial count exceeds 100 counts/g. The whole fixed microbial cells in foods can be identified and counted directly using techniques such as fluorescent *in situ* hybridization (FISH).

Flow cytometry can be very instrumental in the dosage enumeration verification and evaluation of oral food probiotics, which are living microorganisms known to have health benefits when ingested at an optimal concentration (10^8–10^9 living microorganisms), either as a food component or as non-food preparation (Guarner and Schaafsma, 1998). For food probiotics, flow cytometry can be used to provide the evaluation regarding the viabilities of probiotics under certain treatment procedures such as storage conditions, storage time, membrane integrity, *etc.* (Lahtinen *et al*., 2006).

Conclusions

Flow cytometry is a relatively new technique and its application may depend much on the availability of the facility as well as skilled personnel to handle the instruments and correctly interpret the data generated from such techniques.

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Application of Multivariate Statistical Analysis/Chemometrics in Food Forensics

Introduction

The various measurements obtained from food samples or sources of foods (plants/animals, etc.) are desirable for analysts to acquire the target information about that food sample, for example food composition, presence of foreign substances in foods, taste properties, texture, aroma, color, origin, life-span, changes that may occur to foods during processing, storage, authenticity, *etc.* All these food properties may be revealed by different types of instruments, which provide specific signals for various food properties. In certain circumstances, food properties may undergo alterations due to changes in certain food constituents and these changes can be monitored by specific techniques that will be indicative of the occurrence of physico-chemical alterations in food composition caused by either endogenous or exogenous factors, which may include climatic conditions, agronomical practices, the ripening process, storage conditions and time, geographical origin, *etc.* When the composition of food is known, it makes it easier to establish whether there is an adulteration practice, or presence of irregularities in food processing procedures that may compromise quality or authenticity, *etc.*

However, food is a complex substance, both in terms of composition and its behavior under different biological/physico-chemical conditions and this leads to complex signals during food analysis by different types of instruments. This signal complexity from food samples, which actually produces huge amounts of data from a variety of many signals, implies that a univariate statistical data treatment of the signals, which may involve a few signals to describe the trend or behavior of certain food constituents, is misleading to a large extent. This implies that the vast majority of other signals, which are not selected or considered for statistical univariate analysis, will be lost and valuable information that would have been deduced from them will not be harnessed. However, in order to properly identify and select the most important signals, as generated from the analytical instruments (i.e. signals that represent important variables in the food samples), one would require appropriate information

concerning the properties of the food that has been analyzed. The issue will now be how to identify these crucial signals that are associated with important variables in food composition. The answer to this is to employ multivariate statistical analysis, also known as chemometric data analysis, an approach that can address adequately issues related to the identification and selection of important signals to explain the important variables in food samples, and also how to handle complex spectral patterns. Generally, multivariate methods of analysis make use of mathematical principles, statistics, informatics, and profound knowledge about the data (chemistry, electronics, and hardware) to extract the maximum useful information from a large number of data by reducing their dimensionality, with the aim of:

1. classifying and/or discriminating among groups of food samples, on the basis of certain criterion such as geographical/botanical origin;
2. mode of food processing;
3. relationship between composition and physico-chemical properties; and
4. calibration for the prediction models for identification of unknown samples and/or control food processes.

Multivariate chemometrics techniques are capable of converting signals in a value, a process parameter or an instrument diagnostic. Multivariate chemometric techniques can be tuned to work for targeted/comprehensive/metabolic profiling by identifying and quantifying constituents in food samples and classifying them based on certain criteria, in order to identify biomarkers vital for identification in cases involving adulterations, provenance, *etc.* Normally the identification of food components is aided by standards/reference/model compounds. In addition to this, multivariate techniques can use metabolic fingerprinting, where metabolites are actually not being identified or quantified, but rather the signals due to food samples obtained from the instrument are subjected to statistical analysis to identify relevant spectral features or patterns that can differentiate samples that have been analyzed. In this way, it is possible to employ multivariate techniques for samples that generate highly complex data signals, which normally make it almost impossible to accurately integrate signals.

Procedures for Sample Selection Prior to Chemometrics Data Analysis

The requirements for having data analyzed by using multivariate chemometric statistical analysis include the sample size, which must involve large numbers of samples with representative properties sought in the study, in order to guarantee or ensure that there will be variability in the data collected. If this is not done properly, then there is the high possibility of collecting inappropriate samples or samples with undesirable properties and this will result in large errors in the statistical analyses and render the use of chemometrics valueless. It is therefore imperative to devise a proper sampling regime that contains precise, accurate, and complete information about the sample (e.g. sample identity, sample size, place and date, and method of sampling/coordinates, measurement data for the rheological properties data if done onsite … *e.g.* taste, color, etc.).

Sample Preparation Prior to Analysis Intended to Generate Data for Multivariate Analysis

It may be desirable to perform some sample pre-treatment procedures such as extraction and purification. These procedures are specific to the types of food samples being analyzed, analytical procedures, the intended information required from the food samples and the type of instrumental technique being used for the analysis. After the analysis, the data signals generated (spectra, chromatograms, etc.) must be processed appropriately in terms of scales, retention times, peak areas, chemical shifts, *etc.* This step is generally known as the data pre-processing step (Alam and Alam, 2005; Araníbar *et al*., 2006; Berrueta *et al*., 2007; Izquierdo-Garcna *et al*., 2011; McKenzie *et al*., 2011; Vierec *et al*., 2008).

Targeted Metabolic Profiling Prior to Statistical Data Treatment

Targeted profiling of metabolites can be performed in steps, which include:

1. the identification of all signals that belong to the analytes of interest, as generated from the instrument. These signals can be spectra, chromatograms, etc.;
2. integrating these identified signals for analytes of interest;
3. assigning the identity of the signals to the compounds they actually represent, using either stands/reference materials or a compound library database if it is incorporated in the software; and
4. quantification of these compounds.

However, in almost all analytical data generated by analytical instruments, not all signals are associated with either of the targeted signals or important fragments that may be needed for statistical analysis and therefore such signals need to be eliminated, otherwise they will result in a weak predictive performance or poor discriminatory power. In other words, the analyst needs to do data reduction in terms of data dimensionality by performing data exploration to establish both the data quality as well as suitability, if they are to be subjected to statistical analysis (Berrueta *et al*., 2007).

The Analysis of Variance (ANOVA)

The analysis of variance **(**ANOVA) statistical technique is useful in splitting the overall total variability found in the data set. The splitting generates individual components that are important in explaining the contribution of individual/specific properties or variables to the overall total variability of the properties in the dataset. There are several statistical techniques that are known as ANOVA and these include one-way ANOVA, two-way ANOVA, and multivariate analysis of variance (MANOVA), which are useful in data analysis through experimental designs.

One-way ANOVA as a statistical technique is employed when one wants to investigate the statistical significance (as evaluated by the Fisher test or F-test) of the mean magnitudes of two or more sample groups that may be affected by one independent property/variable. Generally, the F-test is used to test the null hypothesis and determine whether the samples are in the same class or not. In cases where the F-test gives a high value for each of the variables, then the null hypothesis can be suggested to be wrong, implying that samples do not belong to the same group and therefore this particular property/variable can be suitable for use in the classification of samples.

Two-way ANOVA, on the other hand, is useful to improve and strengthen the performance of one-way ANOVA, by incorporating the effect of two differently independent variables/properties that may show on the response and also it incorporates the possible interaction factor between the two properties/variables.

In reality, far more large data is normally generated by analytical instruments, such that even two-way ANOVA will be insufficient to classify or explain the diversity. In these cases, ANOVA needs to be extended to multivariate analysis of variance (MANOVA), a statistical technique in which a linear combination of several independent variables is utilized to discriminate samples within many

classes/groups. MANOVA is far more attractive than either one-way or even two-way ANOVA, since it can provide the correlations that exist between variables and can reveal variability that cannot be shown by either one-way or two-way ANOVA.

Multivariate Statistical Analysis Techniques (Pattern-recognition Methods) in Food Forensic Samples

Multivariate statistical analysis methods, also known as pattern-recognition methods, make use of mathematical data pre-treatments in order to accomplish the intended specific purposes in terms of the reduction of variables. These methods include principal component analysis, and other multivariate techniques, which are normally used for statistical qualitative analysis, and multivariate calibration for quantitative analysis of data. Generally multivariate statistical methods are known to be the most suitable for maximum extraction of the required analytical information contained in the analyte, as provided by the signal or data generated by analytical instruments and procedures on samples. There are several multivariate techniques that are normally employed in the extraction of information from data acquired from analytical procedures and/or instruments, but all of them are capable of quantitatively relating the measured analytical variables to their respective analyte properties and thereby qualitatively group samples with similar properties or characteristics into the same classes. These methods can therefore be useful in food provenance cases, food authenticity testing, food fingerprinting, etc., for different types of foods.

Multivariate/Chemometrics Techniques in Food Classification

Analytical instruments and methods normally produce lots of data with massive information, which as such requires steps that involve variable-reduction procedures to reduce this massive information, to be able to deduce the trend that will play an important role in constructing the classification and calibration models, as well as the optimal number of correlated variables contained in the generated data.

Principal Component Analysis (PCA) as a Variable-reduction Technique

Variable-reduction Technique

Principal component analysis (PCA), a mathematically derived procedure capable of reducing variables by decomposing the data matrix composed of rows (samples) and columns (variables) into the product of a scores matrix, has been widely employed in variable-reduction in many instances. In PCA, the scores represent the positions of the samples in the space of the principal components, while the loadings represent the contributions of the original variables to the principal components (PCs). Generally, principal components successive PCs (PC1, PC_2 ….. PC_n) are mutually orthogonal, with each successive PC containing fewer total variables of the initial data set as compared to the preceding ones. However, in normal practice, just a limited number of PCs are retained and those that may arise from noise are not retained and for this reason PCA reduces the dimensionality of the data, thereby making it possible to both visualize and classify as well as perform regression of the data being analyzed (Geladi, 2003).

Multivariate Qualitative Methods

In qualitative analysis, discrete values are to be assigned to analyte characteristics/properties that represent signals generated by the instrument, such that they are the ones used to identify the product or product's state of quality, for example, the product is original, or the product is a blend, or the product is fresh, *etc*. Generally, multivariate classification methods are subdivided into two main groups, namely:

1. Supervised pattern recognition methods: which are also known as discriminant analysis methods, are used only when the group to which the sample being analyzed belongs is known. The aim of the supervised pattern recognition in which the classification group to which the sample belongs is known, is to use a set of data with known classifications to teach the software to discriminate between classes and thereafter come up with working models and comprehensive libraries that can be used predict the classes to which unknown samples belong.

2. Non-supervised pattern recognition methods: these are used when the group to which the sample belongs is not necessarily known and therefore these methods are also known as exploratory methods. The intention of using the unsupervised pattern recognition is to gain knowledge regarding the data set through exploration of natural clustering of the samples and therefore

understand the contribution of each variable to the clustering that has been worked out using unsupervised learning algorithms (Geladi, 2003).

Supervised Pattern Recognition (Discriminant Analysis) Methods

In supervised recognition (discriminant analysis) methods, the classification criteria can be based on either the data signal (as obtained from the analytical instrument) space or in a dimension-reduced factor space. Generally, certain statistical techniques for data reduction, such as the principal component (PC) or partial least squares (PLS) analyses, may be employed to reduce data, even before subjecting the data to discriminant analysis to perform both size and co-linearity reduction, and this step precedes the one that involves data-signal treatment to mark the relation between the data signal and the class/group considered to be the one to which the sample belongs. The data signal treatment can be achieved using methods such as factorial discriminate analysis (FDA) (Karoui and Dufour, 2003); linear discriminant analysis (LDA); and *k*-nearest neighbors (*k*-NN) (Sikorska *et al*., 2005). These methods are useful in creating weighted linear combinations of the data, thus helping to minimize variance within classes and to maximize variance between classes, thereby ensuring that the distances between the data means/averages of the classes surpasses those within the classes. The distance between classes is vital, as it characterizes the partition that is obtained and at the end of the day this distance interval has to validate the method.

In terms of application of supervised pattern recognition methods in food forensic issues, these methods have been used in the analyses of a number of foodstuffs for different reasons and objectives such as profiling, fingerprinting, authentication, food quality control, data interpretation, *etc.* There are several techniques that are associated with supervised pattern recognition methods and these together with multivariate calibration and prediction models such as partial least squares (PLS), partial components regression (PCR), and multiple linear regression (MLR), are widely used as statistical methods in food analysis. They include PLS regression, classification and regression trees (CART), soft independent modeling of class analogies (SIMCA), discriminant analysis (DA), artificial neural networks (ANN), and *k*-nearest neighbors (*k*-NN) (Alam and Alam, 2005; Beebe *et al*., 1998; Berrueta *et al*., 2007; Izquierdo-Garcna *et al*., 2011; McKenzie *et al*., 2011).

The attractive role these methods play is that they are capable of relating

The attractive role these methods play is that they are capable of relating multiple data sets (data signals generated from the analytical instrument) to a characteristic property or several properties of samples and thereby assist in predicting the desired trend of information being investigated. The supervised pattern recognition methods and some of the multivariate calibration and prediction methods are discussed in the subsections below.

Partial Least Squares (PLS) Regression Multivariate Calibration and Prediction Method

This technique generalizes and makes use of features in other multivariate statistical methods, mainly principal component analysis, as well as multiple regression methods to predict and/or analyze a set of dependent factors (variables) from a set of independent factors (variables) or predictors.

This technique can be of valuable importance in the analysis of food samples presented for forensic analyses, because like all samples in the research fraternity that fall under science and engineering, they use massive controllable and/or easy-to-measure variables (factors) to either explain, regulate, or predict the behavior of other variables (responses) from samples presented as evidence in food forensic investigations. In some special cases, the method (PLS) can be used, even in cases where variables/factors are numerically few (implying that they are not significantly redundant or collinear), but if these variables do present a good relationship to the responses, then one can employ multiple linear regression (MLR) to process these same data into useful information that is able to predict responses. But generally, PLS is a statistical technique used to construct predictive models to describe the variability, trend, *etc.* in the data set when variables/factors are many and highly collinear (except in cases where one needs to gain an understanding of the underlying relationship between the variables). PLS is also not suitable if one needs to employ it as a screening tool for factors that might have a negligible effect on the responses.

Food forensic PLS has been applied in many cases to different food samples, for example in the quality assessment of fruit juices (Spraul *et al*., 2009) and beers (Nord *et al*., 2004), as well as in a case where it was required to establish correlations between the composition and sensory attributes of wine (Skogerson *et al*., 2009).

Linear Discriminant Analysis (LDA)

Linear discriminant analysis (LDA) is normally used to find a linear combination of features that characterize or separate two or more classes of samples. Unlike PCA, which despite its strengths and advantages, does not include label information of the data, LDA does utilize the label information in finding informative projections. In LDA, what is being done is actually the construction of linear discriminant functions (Li), also known as canonical roots. These linear discriminant functions are actually linear combinations of the (independent) variables (xn), commonly known as the input variables or predictors. Predictors with the highest discriminatory ability that are used in the LDA equation (Equation 21.1) can be selected by ANOVA:

$$L_k = c + b_1x_1 + b_2x_2 + \cdots + b_nx_n \quad (21.1)$$

where b_1, b_2, *etc.* are the discriminant coefficients and c is a constant.

Equation 21.1 implies that there are a total of k_1 discriminant functions for k number of classes of samples that are being investigated/analyzed. Therefore, what LDA does is to select the proper discriminant functions, which leads to the maximum separation among the given classes of samples. For example, the first discriminant function, i.e., L_1 distinguishes the first group from groups 2, 3, 4, …, n groups and for the second discriminant function L_2, it will therefore distinguish the second group from 3, 4,…, n groups, etc..

Despite these advantages, LDA analysis suffers from two major shortcomings, which include:

1. cases where there are large differences in the number of samples in each class, which may result in directing the classification in the favor of the most populated class(es); and
2. for LDA to give the best predictions, it requires the presence of a larger number of samples than the number of variables, otherwise LDA as the classification model will fail to predict new data, thus resulting into what is known as over-fitting.

There are different variants of LDA that are also used in predicting patterns and they include the stepwise discriminant analysis (SDA) and the PLS-DA.

Discriminant Analysis (DA)

Discriminant analysis (DA) is a pattern recognition statistical method based on a linear function constructed by observations whose group membership is already

known and is used to predict the group membership of new samples/observations. To identify variables effectively, the stepwise discriminant analysis (SDA) model may be a better option, due to its capability to filter out variables with little contribution in the process to identify observations' group membership.

Stepwise Discriminant Analysis (SDA)

Generally, stepwise discriminant analysis (SDA) approaches are attractive for:

1. selection of useful subsets of variables; and
2. evaluation of the order of importance of variables in the dataset. SDA can be done in two main ways:
    a. forward stepwise discriminant analysis (FSDA); and
    b. backward stepwise discriminant analysis (BSDA).

The FSDA method builds a classification-prediction pattern in a stepwise manner, such that at every selection step all variables are reviewed and evaluated, in order to get the variable that mostly contributes to the discrimination between the classes of the samples being investigated and therefore this particular variable will be incorporated into the model and the same process will start again for the next discriminant function, until all discriminant functions have gone through the selection criteria.

In BSDA, all variables are included in the model and then at each selection step, the variable that least contributes to the prediction of class membership is removed and the subsequent selection step is performed in a similar fashion until the last one. This will finally create a model that is comprised of only variables with greater discriminatory power.

Partial Least Squares Discriminant Analysis (PLS-DA)

Partial least squares discriminant analysis (PLS-DA) combines PLS with DA to exploit the advantages of both statistical methods, so as to maximize or sharpen the separation amongst/between classes/groups of samples/observations under investigation. The maximum separation of groups of observations is achieved by rotating PCA components, in order to ensure that a maximum separation among classes is obtained, and to also enable a thorough understanding of the actual

classes is obtained, and to also enable a thorough understanding of the actual variables that highly influence the class separation. Generally, PLS-DA is involved in a normal PLS regression system where the response variables are given a categorical numeral one (1) in the sense that this is replaced by the set of artificial/dummy variables (zero values) that describe the categories, but which express the class membership of the statistical units. In other words, in the PLS-DA pattern recognition method, the PLS regression model can be worked out to enable the recognition of factors that relate the independent variables (the original matrix X) to an artificial (dummy) Y matrix, which is normally constructed with only zeros and ones, with as many columns as there are classes of the sample to be investigated. For example, if a vector matrix in PLS-DA is presented in the matrix form [0, 0, 1, 0], it implies that of the four possible classes, the particular sample belongs to class 3.

Therefore, PLS-DA does not allow for other response variables than the one for defining the groups of individuals. Due to this, all measured variables play the same role with respect to the class assignment. However, the PLS-DA is a more attractive approach than the LDA discussed above, due to the fact that it is applicable in all cases, even where the number of samples is lower than the number of variables (Consonni *et al*., 2011).

k-Nearest Neighbors Algorithm (*k*-NN)

The *k*-nearest neighbors algorithm (*k*-NN) can provide a platform for use as a classification tool and/or regression (Rokach and Maimon, 2008) and the kind of output is mainly dependent on whether *k*-NN is used for classification or regression. It can be useful in classifying an input by identifying the *k* data, *i.e.* the *k* “neighbors” in the known/training set that are closest to counting the number of “neighbors” that belong to each class of the target variable, classifying by the most common class to which its neighbor belongs. In other words, *k*-NN classifies unknown samples based on their closeness/similarity with samples (the training/known data set or *k*-subset) of known membership. In the process, it is required that one should look at a given unknown sample of the *k* nearest sample in the training data set and assign this sample to the class that appears most frequently in the k-subset. For this to be accomplished, *k*-NN requires:

1. an integer *k*;
2. a set of known samples (training set or *k*-subset); and

3. a metric to measure the nearness between samples, and one of the most commonly-used distances between samples is the Euclidean distance metric/measure that calculates the distance between samples x and y, which have coordinates xi and yi over the n-dimensional space (the index i ranges from 1 to n variables).

In both cases, whether used for classification or regression, *k*-NN provides a useful tool in the assignment of weight to the contributions of the neighbors (as obtained from a set of observations/samples for which the class (for *k*-NN classification) or the observation property value (for *k*-NN regression) is known), such that the nearer neighbors will be expected to make more contribution on average than the ones that are further away (Quinlan, 1986; Stone, 1984). Moreover, *k*-NN is attractive because it is analytically tractable due to its simple mathematics and implementation; it is free from statistical assumptions, for example the requirement for normal distribution of the variables; it is also nearly optimal for the large sample limit (n-N); and it lends itself easily to parallel implementations. The main limitations of the *k*-NN algorithm include the fact that it is sensitive to the local structure of the data. *k*-NN is also known to have a large storage requirement; it cannot work well if large differences are present in the number of samples in each class; and also the use of the Euclidean distance makes *k*-NN very sensitive to noisy features, which necessitates the modification of the Euclidean metric by a set of weights that stress the importance of a known sample as a neighbor to an unknown sample. This metric system ensures the nearest neighbor influences the classification more than the farthest ones.

Soft Independent Modeling of Class Analogies (SIMCA)

Soft independent modeling of class analogies (SIMCA) is a statistical software that makes use of a training (actual) set of samples with known identity. The actual (training) sample set is normally divided into separate sets, such that there is one for each class and then PCA is performed separately for each of the classes. In SIMCA, every region may contain samples either on a line, *i.e.* one principal component, or on a plane, *i.e.* two principal components, or on a 3-D space, *i.e.* three principal components. It can also be extended to higher-dimensional regions, *i.e.* “n” principal components. When SIMCA is performed on each class in the data set, it thus generates sufficient numbers of principal components that are retained and which play an important role in accounting for

most of the variation within each class. This presence of a principal component model will be used to represent each class in the data set.

In each of the classes in a data set, the number of principal components retained are unique to that class and therefore care has to be taken to decide on the appropriate number of principal components that may need to be retained for each class, such that they are not too few to distort the signal or information content contained in the model about the class or too many to diminish the signal-to-noise. Generally a method known as cross-validation, which also ensures that the model size can be determined directly from the data, can be employed to determine the number of principal components in the training set. Cross-validation procedures for the determination of the number of the appropriate principal components number begins by omitting a certain portion of the data during the PCA by repeating the process, making use of PC1, PC2, PC3, *etc.* until every data element has been kept out (omitted) once and the omitted portion/segment of the data as well as the unknown samples are then predicted, matched, and compared to the training samples or actual values in the class models and assigned to classes according to their analogy with the training samples. From this process, the principal component model that yields the minimum prediction error for the omitted data is the one that will be retained and therefore cross-validation will have served its purpose to find the number of principal components necessary to describe the signal in the data, while ensuring high signal-to-noise by excluding secondary or noise-laden principal components in the class model.

Classification of the unknown sample (e.g. in samples presented as evidence in food provenance issues where the origin, authenticity, identity, *etc.* is being sought) can be achieved by comparing the residual variance of an unknown food sample to the average residual variance of those samples that make up the class. Having done that, it is possible to obtain a direct measure of the similarity of the unknown to the class. In other words, the sample being investigated can only be grouped as a member of a particular class if it is sufficiently similar to the other members of this class, otherwise it will be rejected and considered as an outlier.

Decision Trees: Classification and Regression Trees (CART)

Classification and regression trees (CART) are statistical tools that are useful for constructing prediction models from data sets. CART are actually tree models in which the target variable can take a finite set of values They make use of a

which the target variable can take a finite set of values They make use of a decision tree as a predictive model, which maps out observations about the samples being analyzed to conclusions about the sample's target value. In the CART tree model, leaves denote class labels, while branches denote conjunctions of features that lead to these class labels.

Decision trees as a statistical technique, useful in data mining, can be subdivided into two main types:

1. Classification tree analysis: this is only applicable in cases where the predicted outcome is actually the class to which the data belongs.
2. Regression tree analysis: this is applicable when the predicted outcome can be considered a real number (value), *i.e.* it is a class of decision trees in which the target variable takes continuous real numbers (values).

Generally, decision trees are meant for dependent variables that take a finite number of unordered values, with the prediction error measured in terms of misclassification cost and also for dependent variables that take continuous or ordered discrete values, with prediction error normally measured by the squared difference between the observed and predicted values. The classification and regression trees (CART) are used to generate a set of simple rules, which are suitable for predicting the origin of any new (unknown) sample being investigated. If the predicted outcome happens to be the class membership, then a classification tree is the one that will be constructed, but if a regression tree is created, then it implies that the predicted outcome is a real number, for example the value of a variable (Berrueta *et al.*, 2007; Breiman *et al.*, 1984).

Artificial Neural Network (ANN)

According to Marini (2009), the application of artificial neural network **(**ANN) in food analysis is scarce and less preferred as compared to other chemometric techniques, for example PCA, LDA, and PLS-DA (Marini, 2009). ANN mimics the functioning of the neural network in the brain, such that just as the biological neurons systems in the central nervous system receive signals through synapses, so do the artificial networks receive input data that are then multiplied by weights that mimic the strength of the signals, and are then computed by a mathematical function, which determines the activation of the neurons (Berrueta *et al.*, 2007; Marini, 2009). The operation of ANNs is as follows: as natural neurons receive signals through synapses, so the artificial networks receive input data that subsequently are multiplied by weights that mimic the strength of the

signals, and then are computed by a mathematical function, which determines the activation of the neurons. Another mathematical function computes the output of the artificial neurons. In ANN, the higher the weight of an artificial neuron, the more influential is the input variable multiplied by it. By adjusting the weights and the variables that are used to feed the artificial neural network, specific outputs that explain the particular complex problem are obtained.

There are several variants of ANN that differ in terms of the input and/or output functions, the accepted values, and the learning algorithms, *etc.*

ANN is attractive in the statistical analysis of food samples presented as evidence in forensic cases, because it completes tasks that a linear program cannot and also its parallel nature guarantees continuous function, even when an element of the neural network fails and moreover learns and does not need reprogramming, and it can be implemented in applications without serious problems. The shortcomings associated with ANN include the fact that there is a need for training of the model, high processing time, and difficulty to interpret the original variables.

Non-supervised Pattern Recognition Methods (Exploratory Methods)

Non-supervised methods examine the natural classification or clustering of either samples or variables, or both, and are applicable in the first stages of the investigation in order to reveal the different property/characteristic levels, such as ripening levels of fruits within the subgroups in a data set and thereby the method served to cluster the products. Examples of cluster analysis techniques that can be employed include (i) hierarchical cluster analysis (HCA); and (ii) principal component analysis (PCA).

The Hierarchical Cluster Analysis (HCA)

The hierarchical cluster analysis (HCA) is normally used to assess the similarities between product samples according to their measured characteristic properties or variables whereby product samples are grouped into clusters based on their nearness in multi-dimensional space. Normally, HCA results are depicted in the form of dendograms, such that either samples or variables that are similar or that show strong correlation with one another, fall in the same cluster of dendograms and also samples that are not similar or that show weak

correlation are clustered in different groups in the dendograms (Poulli *et al*., 2005).

The HCA is further classified into two main subtypes, namely:

1. agglomerating HCA: in which the process of clustering begins with each sample product forming a unique and specific cluster, then iteratively merges pairs of individually formed clusters that are closest to one another until one larger cluster is formed. HCA involves the calculation of the magnitude of distances among all samples that constitute different clusters that are joined to form one larger cluster, by using amalgamation rules such as Euclidian distance and also the single linkage.
2. divisive HCA: which is the opposite of agglomerating HCA. Of these two HCA types, the agglomerating clustering is the one that is most widely used due to its simplicity and clustering power.

The Principal Component (PCA) Cluster Analysis

The principal component (PCA) cluster analysis is very useful in cases when one needs to find relationships between different parameters in either samples and variables and/or the detection of possible clusters within the samples and/or variables. This method involves the reduction of the dimensionality in terms of the number of variables of the data set, using as few axes or dimensions as possible. The new axes, which are known as the principal components (define the linear combinations of the original variables), are orthogonal to one another and serve to describe the variation within the data set.

PCA provides the analyst with a tool for initial investigations of large data sets to explore trends in terms of similarities and classifications, as well as the detection of outliers. Generally, in PCA, the first principal component presents the maximum magnitude of the variability in the data set, and each subsequent principal component accounts for the remaining variability. In the form of matrix notation, the data matrix X can be described as the product of a score matrix S and a loading matrix L, *i.e.* $X = SL^T$, where L^T = the transpose of the loading matrix. The S matrix contains the information about the magnitude of variance each principal component describes, while the loading matrix denotes the contribution of each variable to the construction of the principal components.

Multivariate Calibration for Quantitative Analysis

For quantitative analysis, multivariate regression methods are the ones widely used. Among the multivariate regression methods are:

1. partial least-squares regression (PLSR): which is capable of searching the directions of highest variability by comparing both signal of interest as generated by the analytical system and target characteristic property information with the new axes known as PLSR components or PLSR factors. In other words, PLSR represents the most relevant variations showing the best correlation with the target property values.
2. principal component regression (PCR): which makes use of the principal components generated by the PCA to perform regression on the sample characteristic property that is to be predicted. Therefore, in this approach, the first principal component or factor in PCR denotes the widest variations in the sample signal generated by the analytical system (Geladi, 2003).

Validation Approaches for Chemometric Pattern Recognition Models

In chemometrics, there is a possibility of obtaining a close fit that may look like it is the convincingly desired one using more and more principal components in PCA experiments, but which may not present meaningful information. Under these conditions, a validation procedure becomes necessary in order to define and limit the number of principal components that will yield a sufficient number of components that are necessary to describe the trend and the data in general. Validation of the statistical pattern recognition chemometric models is also of significant importance for the purpose of the evaluation of the significant variables required to construct the model, as well as for the recognition and prediction ability of the model. Validation of chemometric pattern recognition models can be done by either employing:

1. external validation: which is more applicable in cases where there are large number of samples;
2. internal validation: also known as cross-validation, k-fold cross-validation or the jack-knife method, which is mostly applicable in cases where the number of samples is limited.

The external validation approach does not depend on the model building procedures that incorporate the actual data (training) set in cross-validation as

procedures that incorporate the actual data (training) set in cross-validation as internal validation does.

Factors that Govern a Proper Choice of Statistical Technique in Food Forensic Samples

A number of factors may need to be considered in order to make an appropriate choice for the chemometric technique for food forensic samples and they may include the number of purposes of the analysis and chosen model, data structure, the variables required, the number of samples under investigation, as well as the metabolomic protocol that has been chosen for the samples (Berrueta *et al*., 2007).

For example, unsupervised pattern recognition methods, such as HCA and PCA, may be mostly suitable in cases where there is a need to investigate similarities and/or differences amongst food samples. The supervised pattern recognition models are mostly suitable in cases where there is a need to predict the class membership of future samples.

Conclusions

Multivariate techniques are highly useful as validation methods that are required to validate and prove the reliability of the methods and data generated with regard to the authenticity of the procedures and results. Every technique and method applied in food forensics needs to incorporate multivariate statistical methods, as well as data mining using these chemometric methods.

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Conclusions and Future Trends

This book has dealt with a number of cases that have been reported frequently, of deliberate food poisoning in various places for various reasons, ranging from general criminality, tendencies to violence, economic gains and political interests. The first ten chapters have highlighted food forensic issues related to food provenance (authentication of originality of foods, fingerprinting and adulterations), deliberate incorporation of ingredients that may cause allergenic reactions in certain individuals, food forensics cases related to food bioterrorism/food bio-weapons and food poisoning agents, such as agrochemical food poisoning agents and bioterrorism agents (chemical and bio-agents) that be used to inflict terror in groups of people.

Other issues in food forensics are related to genetically modified organisms (GMOs), whereby a huge debate is still on-going with regard to their regulation. There are ethical considerations that have divided people and governments, due to varying opinions from every side. Radionuclide food poisoning at both levels (individuals, as well as groups of people) has been discussed in detail. Drinking water is one of the targets where these agents may be applied deliberately to poison whole communities. Forensic cases involved in the application of nano- and novel/intelligent foods, feeds and agroproducts has been well covered. In addition, the application of various food additives and food improvement agents, such as food coloring, flavorings, *etc.* and their regulations, has been discussed.

In all of these, the fate of the molecules incorporated in foods unlawfully and their degradation products has been narrated. The choice of representative sample specimens as evidence and how to process and analyze these have been described in detail.

The following chapters (11–21) have been dedicated to the methods and techniques that are normally used to verify the transgressing of food regulations by distributors, processing industries, vendors and criminals, who may deliberately intoxicate foods. The methods discussed include those that make use of the principles of molecular biology, which in turn make use of specificity of the macromolecular signatures such as nucleic acids and proteins, which are specific to each organism and thus provide concrete evidence.

Spectroscopic methods rely on the pattern displayed by various molecules when

subjected to various regions of the electromagnetic radiation (EMR). These methods include infrared, UV-Vis, Raman, microwave, and nuclear magnetic resonance (which utilizes the radiowaves region in the EMR), *etc.* Different techniques that fall under the microscopy class of techniques and which find application in food forensics have been presented with examples. The application of ionizing radiations, such as X-rays and γ-rays in providing the necessary evidence in cases which are related to food forensics and their application in ensuring foods free of microbial pathogens and consequences, has been detailed in this book.

Chromatographic methods, electromigration-based methods (electrophoresis) and hyphenations involving chromatographic and spectroscopic/spectrometric methods, are other sets of methods that are widely used for food adulteration confirmation. Moreover, thermal methods of analysis, electrochemical methods and bio/chemical sensors, as well as the application of cytometry in food forensics, have all been presented. Multivariate statistical methods of analysis (chemometrics) are very central as validation methods that need to be applied to all of the techniques discussed in this book. An in-depth narration of multivariate methods and techniques has been well covered.

Generally, the trend of deliberate food poisoning has been on the increase in the recent past and as conflicts within societies or nations increase, the possibility of using food as one of the possible means to harm others may increase as well. This requires programs to prepare people as a precautionary measure in terms of education on the possible food targets, poisoning agents, and biomarkers of poisoning in the body. Moreover, knowledge is required regarding the detection of poisoning agents in foods, and their metabolites in body specimens such as fluids (blood, serum, urine, etc.). It is therefore required that training of eminent scientists and technicians be taken seriously to provide effective diagnosis that may enable physicians and clinical officers to provide the correct solution (treatment) in as timely a manner as possible.

It may be expected that future poisoning agents may have different mechanisms of action that may produce different metabolites in the body. Therefore the development of analytical techniques needs to be kept up to date.

On the other hand, market competition may be so intense in the future that this may well lead to even more unscrupulous behavior relating to the adulteration of food products. More accredited food forensic laboratories need to be in place to ensure that the quality of food is as indicated on the labeling and meets the quality standards as stipulated by the guidelines and legislation. Inter-

laboratories testing of food products may need to be emphasized to provide reliable authenticity of the food products sold to other communities/countries.

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CSIA-GC-C-IRMS
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