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Food Quality and Safety.
A Mass Spectrometric Approach for Traceability and Control of Chemical Contaminants in Foodstuffs

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Chapter 1

Food quality and safety

1.1 Food traceability

The Codex Alimentarius Commission (1999)[1] defined traceability as the ability to trace the history, application or location of an entity by means of recorded identifications. Traceability is closely linked to product identity, but it can also relate to the origin of materials and parts, product processing history, and the distribution and location of the product after delivery. The European Union has attached to traceability and labeling the solution to consumer concern about mad cow disease, dioxin in chicken feed, and GM food products.

Traceability became a political matter when the European Commission approved proposals requiring the traceability and labeling of GM foods, including highly-refined starches and oils in which unaltered DNA or proteins cannot be detected.

Food manufacturers develop and adopt internal traceability systems and traceability chains mainly to improve food safety, since traceability can be seen as a subsystem whose presence is essential to the management of food quality.[2] Traceability is an essential tool for ensuring both production and product quality.[3,4] The opportunity to connect traceability with the whole documentation and control system represents an effective means for boosting the consumer's perception of a food safety and quality.[5]

Meanwhile, the Codex Committee on General Principles has been asked to examine the role of traceability as a potential risk management tool for public health purposes. Other Codex committees will seek to determine how traceability might be used in their standards and guidelines.

Regulation EC No 178/2002, 2002,[6] which provides the legal basis for the establishment of the European Food Safety Authority (EFSA), defines traceability as the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution.

Through the documented identification of material flows and operator chains, the UNI 10939, 2001 considers traceability in food chains as a system which enable a product's history to be reconstructed and its use followed up.[7] This standard provides the overall principles for the design and management of a traceability system. In addition, the UNI

11020, 2002 establishes the principles and requirements for the development of internal traceability systems in the farming and food industries. [8]

At the same time, these standards provide that industry identifies Critical Control Points with a view to monitoring traceability systems and defining methods for their monitoring, assessment and documentation.

Moreover, traceability systems represent the most suitable tool for circulating information on product quality to end consumers, and for making the manufacturing system and the whole supply chain more transparent.

They also enable the identification of the parties responsible for the production of a given food and the transfer of this information to the consumers, who, as a consequence, may become more loyal customers.[2,3,9]

In order to develop a traceability system, the whole system must ensure that all the information concerned is quickly and easily available to the person involved in the activities and flows of the production process.[10,11]

Many papers emphasize that the main difficulties lie in the design of an internal traceability system for a given, complex production process.[4,5] They showed that a good system for internal quality control and traceability of a production process can yield several competitive advantages such as:

- improving process control, through cause-and-effect indications for products that do not conform to the company standards;
- directly linking end product and raw material data, which can help to improve specific production processes and ensure a better use of raw materials in the final product;
- avoiding the uneconomical and unrewarding mixing of high-quality and low-quality raw materials;
- carrying out an easier quality auditing process.

The desire for the integration of more and more information in food production management and the increasing demands for information along the food processing chain will set higher requirements for well structured traceability systems in the future.

There is a need for more work on analysis of the fundamental and practical aspects of traceability in food manufacture. From this, guidelines that can help companies to

assess their particular need for the degree of detail in their internal and chain traceability systems can be made.

In the future, the information owing in the food manufacturing chain might provide a competitive advantage since it can be sold along with the product.

1.1.1 The importance of food traceability and safety

The market for imported, premium priced foods has increased dramatically over recent years, a consumers become ever more aware of products originating from around the world. There are many foods products that are of superior quality (taste, texture, fragrance etc.) because of the local in which they are cultivated. Environmental conditions, such as local climate and soil characteristics, combine to yield crops that exhibit specific traits. Clearly, higher quality products demands higher market prices, therefore unscrupulous traders may attempt to increase profits by deliberately mislabeling foods, or by increasing the volume of a good quality batch through adulteration with sub-standard produce.

There may also be safety issues attached to food adulteration, for example powdered or smashed hot chili pepper imported from India and Pakistan was heavily contaminated with a carcinogenic azo dye known as Sudan[12-14].

Many people are allergic to cow's milk and therefore choose milk, cheese and yoghurt from other animals, such as ships and goats. But in many cases, if a significant quantity of cow's milk is added to these products, without proper labeling, health problems could result. Similarly, in order to ensure consumer confidence, it is important to know that food commodities do not originated in a country where food-borne disease is an issue, *e.g.* British beef/BSE.

In recent years, there has been increasing legislation to protect the rights of both the consumer and honest producers.[15,16] To enforce there laws, a measure of the authenticity of samples must be made, most often in the form of providing the presence/absence of adulterants, or verifying geographical or cultivar origin by comparison with known and reliable samples. The latter method often includes the use of multivariate statistical techniques to investigate sample data.[17]

1.1.2 Analytical methods used in food traceability and safety

A large number of techniques have been used for food study from safety ad authenticity point of view.

DNA analysis has been applied to prove the species origin of several meat,[18,19] fish,[20,21] and plants products,[22] as a matter of concern for reasons of lifestyle, religion and health. IR spectroscopy has been widely applied to the detection of adulterants and the assignment of geographical or cultivar origin.[23] Products analyzed by this method include fruit juice,[24] olive oil,[25] fish and meat, beverages, maple syrup, starch, and honey.[26]

Recently, gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) have been widely used in the authentication analysis of food products. The fat,[27] protein,[28] carbohydrate or other natural compound profiles[29,30] have been used to provide species an geographical differentiation. In several cases, enantiomeric composition has been used.[28,30] These method of discrimination rely on samples of different species and/or different geographical origin having different chemical compositions. This is not always the case, samples from the same location have been found to contain different components and conversely samples from different regions may be display identical chemical composition.[31] Detectors usually used, in combination with chromatographic techniques (GC and HPLC), may be more or less selective and sensitive, but lack information about the identity of compounds. Therefore, the coupling of chromatographic techniques and mass spectrometry (MS) overcomes this drawback. MS is a sensitive and selective detector, sometimes allowing preparation steps to be avoided. GC-MS is a robust techniques, used routinely in many laboratories for food analysis, for example for the determination of aroma compounds, contaminants and pesticides analysis. More recently, LC coupled to quadrupoles, magnetic sectors or time-of-flight (TOF) detectors, has also had a great expansion into the field of food analysis. The recently introduced spray methods (ESI) have fostered qualitative and quantitative analysis of medium to high polar analytes by mass spectrometry.

The designing of ion source houses for ESI has also fostered the rediscovery of Atmospheric Pressure Ionization (API) methods such APCI where the chemical

ionization (CI) is achieved at atmospheric pressure. Both techniques produce soft ionization, but additional fragmentation can be achieved by performing in-source collision induced dissociation (CID) in tandem or trap instruments. MS/MS in space and in time provide additional and unique information on the structure of analytes. ESI is useful for polar and ionic solutes ranging in molecular weight from 100 to $150x10^3$ dalton. APCI is applicable to non-polar and medium polarity molecules with a molecular weight from 100 to 2000 dalton. Although the choice of the right interface, as well as the detection polarity re based mostly on the compounds polarity and thermal stability and the HPLC operating conditions, many classes of compounds can give good response with both ionization techniques. In some cases both positive and negative ionization modes are needed, while in most of the cases the choice of only one operation mode is enough.[32]

The number of applications of HPLC-API-MS to food analysis has rapidly increased on recent years. ESI is much more widespread than APCI, but for both techniques the trend is towards an increase in the number and the variety of applications.[33-34]

Other techniques applied for food traceability studies include capillary electrophoresis, sensory arrays[35] and examination of physical characteristics.[36] This latter approach has been shown to be subject to human inconsistencies in the case of wine tasting.[37]

Stable isotope techniques enable differentiation of chemically identical substances through alterations in their isotopic fingerprint and have been used in authenticity studies for many food products.[38] The isotopic composition of light elements in plant material can vary depending on location[31] but, the dominant factor is the influence of latitude on the fractionation of the elements in groundwater.[39]

Fractionation occurs during physical processes such as evaporation. Lighter isotopes evaporate very slightly faster than heavier counterparts, therefore in warmer regions where the amount of evaporation is higher , the isotopes are fractionated to a greater degree. The discrimination between isotopes in such physical processes is only significant for light elements, with an high relative mass difference between the isotopes.

Thus hydrogen ratios, measured by site-specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR) and carbon, nitrogen, oxygen and sulphur isotope ratios measured by isotope ratio mass spectrometry IRMS) have been applied to the

foods authentication. Fats and oils,[30,40,41] flavours,[42] wines,[43-44] fruit juice[45] and several other products have been classified using stable isotope analysis.

The elemental composition of vegetation reflects the soil in which it has grown,[46] which in turn will depend on the topography, geology and soil characteristics, therefore, no two countries will have identical soil maps and the concentration of elements in food products can then be used to assign their geographical origin. As with other methods of traceability, a database of samples of known origin must be available, against which unknown samples can be compared.

The most useful elements for the assignment of origin are those that are not homeostatically controlled. Elements such as K, Ca and Zn are actively absorbed by organisms and will therefore be present in samples at similar levels, regardless of the environmental conditions experienced. Elements that have no role in normal physiological processes, such as the rare earth elements (RREs) and the heavy metals are passively absorbed and the concentration of these elements in an organism will strongly reflect the environmental levels to which the organism has been exposed.

Several studies have used multi-element concentration profiles in the determination of food authenticity, either alone or in combination with chromatographic or stable isotope ratio data.[47-48] Elemental concentrations were determined by AAS,[49] ICP-AES[50] or ICP-MS.[51,52]

Several trace elements have variable natural isotopic abundance due to the decay of radioactive isotopes. These include Li, B, Sr, Nd, Hf, Pb and U.[53] The composition and age of the local rocks dictates the abundance of the radioactive precursors and their daughter species.[54] This fact has been widely exploited for environmental applications, archeology and geochronology and geochemistry. Elements are taken up into plants in the same isotopic proportions as they occur in the soil and in precipitation.[55-57] Therefore isotope ratios in plant-derived food products depend on the geology of the region in which the source crop was grown and are different in produce of different geographical origin. There were, however, relatively few reports of the use of heavier stable isotope ratio measurements of the authentication of foodstuffs. For many years Thermal Ionization Mass Spectrometry (TIMS) was the only technique capable of performing isotope ratio measurements with sufficient precision to allow geographical assignment of food products based on trace element isotopic composition. Sample for

TIMS analysis must be loaded in the form of the pure element, meaning that extensive sample preparation is required if this technique is to be applied. Nonetheless, TIMS has been used for Sr isotopic measurements for the regional assignment of wines[58] and butter.[31] In the latter case Sr, C, N, O and S isotopic profiles were subjected to discriminant analysis, allowing assignment of the regional origin of unknown samples by comparison with samples of certified origin.

Inductively coupled plasma mass spectrometry (ICP-MS) is now a well established technique for isotopic trace element determinations.[59] ICP-MS allows rapid analysis of a large range of sample types, requires minimal sample preparation and due to the ionizing power of the ICP, can be applied to a wider range of elements than TIMS. The precision of isotope ratio analyses by ICP-MS has only recently matched that achievable by TIMS, through the application of double focusing mass analyzers coupled to multi-collector detection arrays. The increasing availability of multi-collector ICP-MS is likely to lead to wider application of heavier stable isotope ratio measurements to the authentication of food products. Two recent reports used Sr isotope ratios measured by ICP-MS for the regional assignment of wines and the Pb isotopic profiles of port wines have also been measured by ICP-MS and found to a certain extent to depend on the age of the wine.[60,61] Other studies of trace element isotope ratios for food authentication have been carried out.[62-63]

1.2 Metabolomics applications in food traceability

The term "metabolome" describes the complement of all metabolites expressed in a cell, tissue or organism during its lifetime. Already at the 19th century it was suggested that two classes of metabolites exist; primary (or basic) and secondary. Secondary metabolism refers to compounds that are not needed for the cell survival and propagation but are believed to be of importance to continued existence in particular environmental conditions. Secondary metabolites are derived from primary metabolites (e.g. amino acids and carbohydrates), through modifications, such as methylation, hydroxylation, and glycosylation. Evolutionary processes directed towards enhancing plant fitness most probably stimulated formation of new structures. Secondary

metabolites are formed both as part of normal plant developmental pathways and upon diverse endogenous and environmental stimuli. Examples of secondary metabolites are fruit flavor and aroma compounds, flower and fruit pigments and "sun screen" metabolites such as the flavonols. Up to date, a few hundred thousand different secondary metabolites structures have been identified in plants, the largest of them are the phenylpropanoids, isoprenoids and alkaloids.

An estimated 20% of all carbon fixed in the leaves passes through the Shikimate pathway (Figure 1.2.1) and 30% of the plants dry weight comes from this pathway products, the aromatic amino acids Phenylalanine, Tryptophan and Tyrosine. These amino acids are the primary source of carbon for the formation of a myriad of secondary metabolites in both plants and microorganisms. For example, the insects deterring glucosinolates are products of Phenylalanine and Tryptophan metabolism and the phytohormone salicylic acid is produced from Chorismate, the final product of the Shikimate pathway.

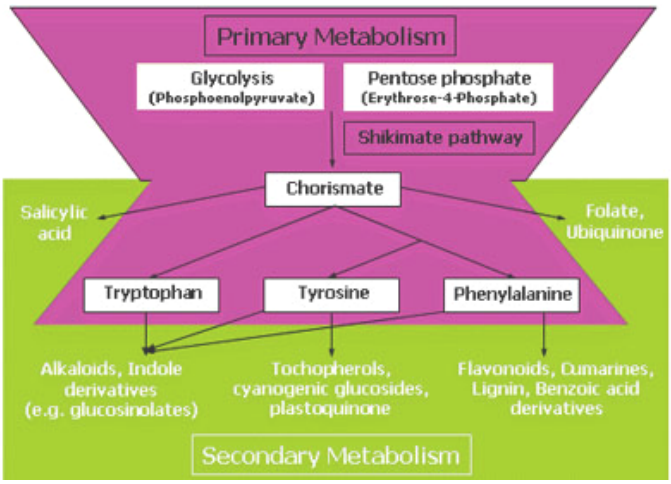

Figure 1.2.1 The primary-secondary metabolism interface scheme.

1.2.1 Flavor formation in fruits and vegetables

Knowledge of precursors and pathways leading to the formation of flavor in fruits and vegetables has progressed slowly over the years. Historically, emphasis has been placed on determining what constitutes flavor rather than on the mechanisms of flavor formation.

Also, the task of elucidating metabolic pathways in very complex biological systems is difficult at best. The continued demand for natural flavorings has renewed interest in this research area since a knowledge of biological pathways facilitates their production under conditions that permit their labeling as natural.

Considering that flavor components are reasonably low molecular weight materials, we might expect that they are a part of the normal biosynthesis process. While this is often true for the nonvolatile components of flavor, such as sugars or acids, this is generally not true for aroma components. It is interesting that few if any aroma compounds are known to serve a biological function in the plant. In fact, most aroma compounds arise as a result of degradation reactions. In fruits, the plant cell walls soften and internal organization is lost during ripening. This loss of organization permits enzymes normally associated with growth to attack various substrates normally not available to the enzymes. Enzymes typically involved in synthetic processes are involved in degradation reactions. This attack results in the formation of a host of low molecular weight products many of which have significant sensory properties.

In vegetables, there is no ripening period in which aroma compounds are formed via enzymatic reactions upon cellular damage. If flavor is the result of an evolutionary process, it may be in how it influences animal behavior or microbial degradation. Animals may be attracted to a pleasant flavor, promoting the consumption of the plant and the spread of its seeds. An unpleasant flavor may deter an animal from eating the plant until it has had time to come to reproductive maturity. Some flavor components have antimicrobial properties that may protect the plant's reproductive elements to enhance survival.

1.2.2 Biogenesis of flavor compounds in fruits

There are some important differences in the biogenesis of flavor constituents in fruits and vegetables. In general, in fruit the flavor constituents are not present during early fruit formation, but are biosynthesized in the intact tissue during a short ripening period which is strongly related to the climacteric rise in respiration.

During this period, metabolism of the fruit changes to catabolism, and flavor formation begins. Minute quantities of lipids, carbohydrates, proteins and amino acids are enzimatically converted to simple sugars or acids and volatile compounds. The rate of flavor formation reaches a maximum during the postclimacteric ripening phase.

An overall view of flavor formation during ripening of fruit is shown in figure 1.2.

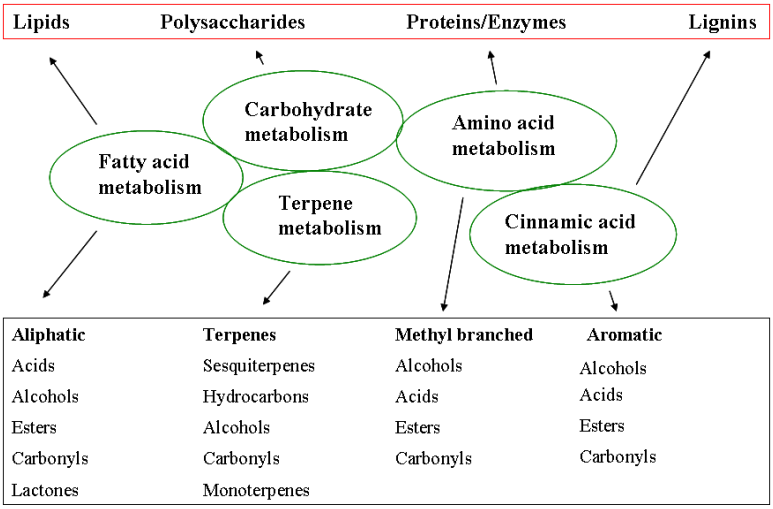

Figure 1.2.2 Formation of aroma fruits from major food components.

Flavors are formed from major plant constituents under genetic control. Each metabolic pathway is connected to other metabolic pathway. As direct products of a metabolic pathway, or as a result of interactions between pathways or end products, a host of volatile compounds are produced which contribute to the flavor of a ripe fruit. The precursors of some aroma compounds in table 1.2.1.

In most vegetables the intact tissues contain non-volatile precursors which are split by enzymatic action when the tissue is disrupted by crushing, cutting or slicing. Typical fruit volatiles include esters, alcohols, acids, lactones, carbonyls and terpenes while typical vegetable volatiles are nitrogen and sulfur compounds and carbonyls.

Nutrient	Aroma Component
Carbohydrates	**Organic acids:**
Glucose	Pyruvic acid, acetic acid, propionic acid, acetoacetic acid, butyric acid, hexanoic acid, octanoic acid
Fructose	**Esters:**
Sucrose	Pyruvates, acetates, propionates, butyrates, acetonacetates, hexanoates, octanoates
	Alcohols :
	Ethanol, propanol, butanol, hexanol, octanol
	Aldehydes :
	Acetaldehyde, propanal, butanal, hexanal, octanal
	Terpenes:
	Monoterpene, linalool, limonene, α-pinene, citronellal, citral, geranial
Amino acids	Pyruvic acid, acetaldehyde, ethanol
Alanine	isopropanal, isopropanol, α-keto-isobutyric acid
Valine	3-methylbutanal, 3-methylbutanol, α-keto-isocaproic acid
Leucine	2-methylbutanal, 2-methylbutanol
Isoleucine	Benzaldehyde, phenylacetaldehyde, cinnamaldehyde, hydroxycinnamaldehyde, p-hydroxybenzaldehyde, p-hydroxyphenylacetaldehyde, p-hydroxycinnamaldehyde
Phenylalanine	
Serine	
Threonine	Pyruvic acid
Glycine	Thiazoles
Cystine/cysteine	Glyoxal
Fatty acids	Trans-2-trans-4-decadienale, trans-2-octenal, Trans-2-pentanal, trans-2-hexenol, hexanal, cis-3-hexenal, cis-3-hexenol, trans-2-trans-4-heptadienal, propanal
Linoleic acid/Linolenic acid	
Vitamins	
Carotene	β-ionone
β-carotene	

Table 1.2.1 Precursors of aroma compounds in food.

1.2.2.1 Aroma compounds from fatty acid metabolism

Volatile flavor compounds may be formed from lipids via several different pathways. The primary pathways include α- and β-oxidation via lipoxygenase enzymes. The widest variety of flavor compounds formed from lipids arises via lipoxygenase activity. Many of the aliphatic esters, alcohols, acids and carbonyls found in fruit and vegetables are derivative from the oxidative degradation of linoleic and linolenic acids. Different studies on the generation of flavor in banana,[64] tomato,[65] apple, tea[66] and olive oil[67] elucidate and point out the importance of lipoxygenase activity to flavor development in fruit (Figure 1.2.3).

Acids and ketones as well as other intermediates in the oxidation process are readily converted to alcohols, aldehydes and esters by other enzyme systems in the plant. Both α- and β-oxidation pathways have been demonstrates to exist thereby providing a wide range of volatiles for further conversion to flavor compounds. The discussion above outlines how many volatile compounds are formed from lipid degradation. Yet, one may be inclined to question the importance of these pathways since there is very little lipid in fruits. First, one must recognize that many aroma compounds have low sensory thresholds. It does not take much precursor to yield ppm quantities of aroma compounds. Second, there is a significant quantity of linoleic and linolenic acid in plant chloroplasts. As fruits ripen, they loose their green color due to chloroplast degradation, which then releases membrane lipids which are rich in these key aroma precursors. These precursors then enter the pathways described above to form the host of esters and carbonyls that characterize the aroma of many fruits.

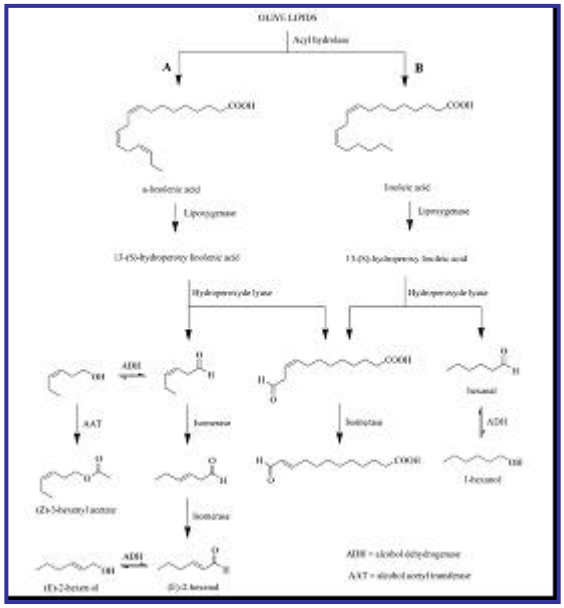

Figure 1.2.3 Metabolic pathway for the origin aliphatic aroma compounds via lipoxygenase enzymes.

1.2.2.2 Aroma compounds from amino acid metabolism

Amino acid metabolism generates aromatic, aliphatic and branched chin alcohols, acids, carbonyls and esters that are important to the flavor of fruit. Yu et al.[68] were amongst the earliest researchers to demonstrate that valine, leucine, alanine and aspartic acid can be converted to short chain carbonyls by tomato extracts. The enzymes involved in these transformations were found to be located in different sites of fruit. In tomato, in fact, the soluble fraction isolated via centrifugation acted on leucine while the mitochondrial fraction metabolized both alanine and aspartic acid.

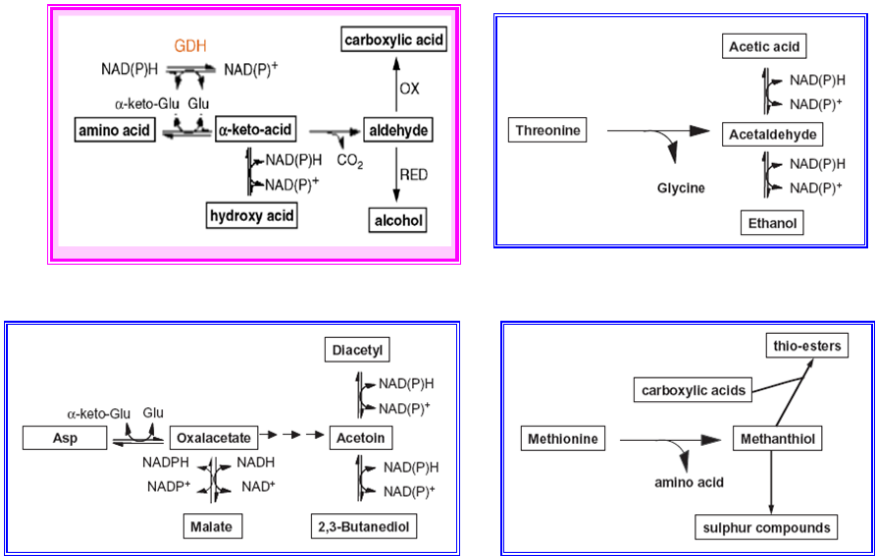

Figure 1.2.4 Principal reactions involved in the formation of aroma compounds from amino acids.

Although aldehydes and alcohols derived from the degradation of branched-chain and aromatic amino acids or methionine constitute a class of highly abundant plant volatiles, their pathways have been barely analyzed in plants (Figure 1.2.4). Especially important are branched-chain volatiles derived from branched-chain amino acids. Isoamyl acetate, an ester with a strong fruity odor described as similar to banana or pear, is one of the key constituents of banana flavor.[69] 2-methyl-butyl acetate has a strong apple scent and is associated with apple varieties that are rich in aroma such as 'Fuji', 'Gala' and 'Golden Delicious'.[70,71] Methyl 2-methyl butanoate determines the characteristic aroma of prickly pear,[72] while a combination of several volatile esters imparts the unique aroma of melons, with isoamylacetate and 2-methyl-butyl acetate being prominent in many varieties.[73]

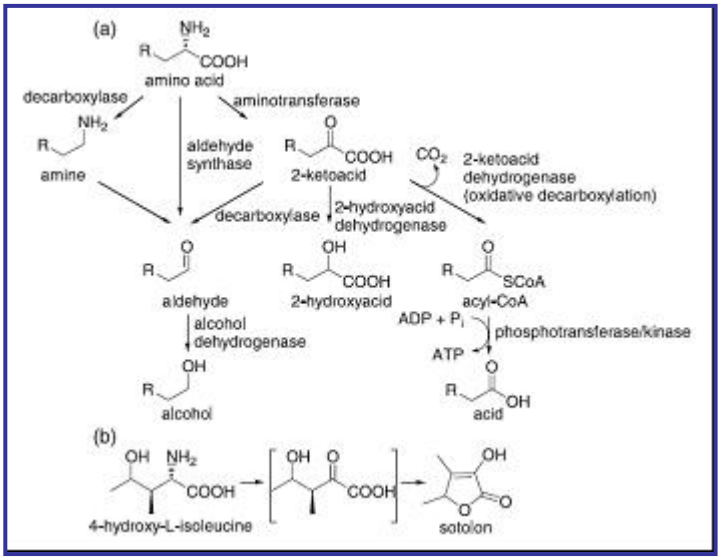

Figure 1.2.5 Biosynthesis of amino acid-derived flavor compounds. (a) Catabolism of branched-chain amino acids leading to methyl branched flavor compounds, and (b) postulated biosynthesis of sotolon. Formation of aldehyde (a) from amino acids requires the removal of both carboxyl and amino groups. The sequence of these removals is not fully known and could be the opposite to that shown or aldehyde could be formed in one step by aldehyde synthase.[74]

1.2.2.3 Aroma compounds formed from carbohydrate metabolism

Observing the table 1.2.1, one can see that a large variety of volatile flavors can be traced to carbohydrate metabolism. Considering plant metabolism, it is clearly established that plants obtain all of their energy directly from photosynthesis. The photosynthetic pathways involve turning CO_2 into sugars that are metabolized into other plant needs, lipids and amino acids. Therefore, nearly all plant flavors come indirectly from carbohydrate metabolism since all of the other flavor precursors come from carbohydrate metabolism. However, there are few flavor constituents that come directly from this metabolism.

Terpenes, for example, arise both from carbohydrate and lipid metabolism.

Terpenes are classified by the number of isoprene units they contain. Monoterpenes contain two isoprene units (10 carbons), sesquiterpenes contain three isoprene units (15 carbons) and diterpenes contain four isoprene units (20 carbons). Of these groups, the

monoterpenes, and more specifically, the oxygenated monoterpenes, are considered most important to the aroma of certain fruits, such as citrus products.

Limonene, a monoterpene hydrocarbon possessing little odor, is the major terpene in most citrus oils accounting for up to 95% of some oils. The oxygenated terpenes, often comprising less than 5% of the oil, generally provide the characteristic flavor of different citrus species.

The biosynthetic pathway proposed for the synthesis of isopentenyl diphosphate (IPP, isoprene building block) is shown in figure 1.2.6.

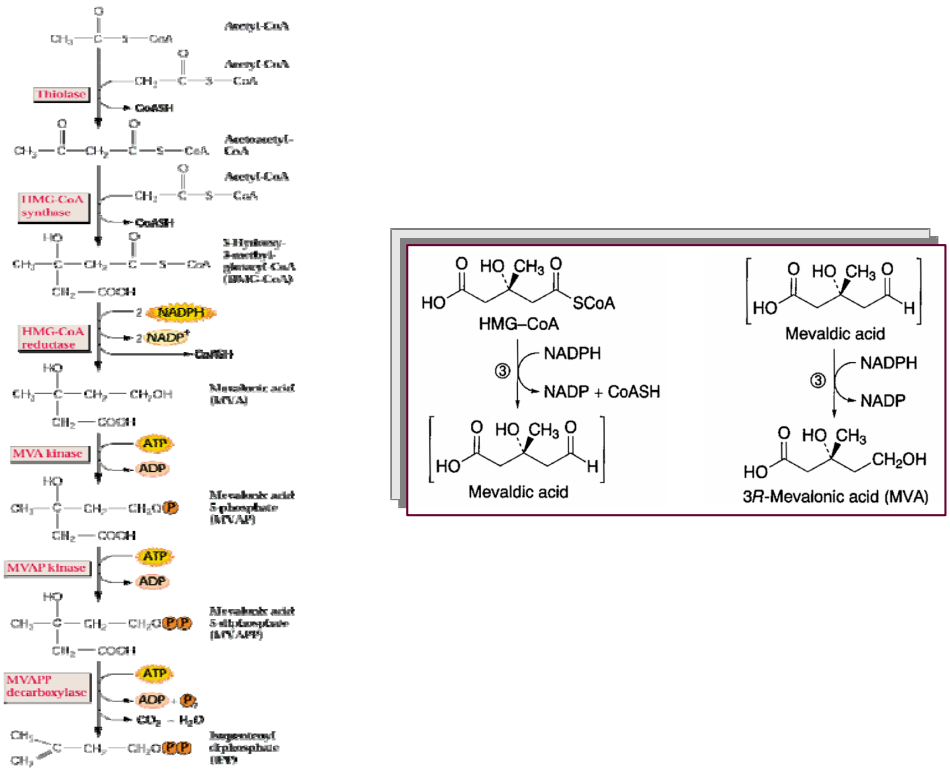

Figure 1.2.6 The biosynthetic pathway proposed for the synthesis of isopentenyl diphosphate, IPP. This step is catalyzed by hydroxymethylglutaryl-CoA (HMG-CoA) reductase.

This pathway combines three acetyl-Co-A molecules to form 3-hydroxy-3-methylglutaryl-Co-A (HMG-CoA). The HMG-CoA is reduced to mevalonic acid which is then phosphorylated and decarboxylated to form IPP, the key building block in

terpene biosynthesis. Some researchers has found that IPP can also be formed via a pathway that does not include mevalonic acid.[75] While there is evidence to support the existence of this alternative pathway, it has not been adequately determined.

The next series of steps involves the combination of IPP units to form geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate, which serve as the precursors of the mono-, sesqui- and diterpene families, respectively, figure1….. In effect, all of the other members of these families arise from the cyclization or secondary modification of these three precursors.

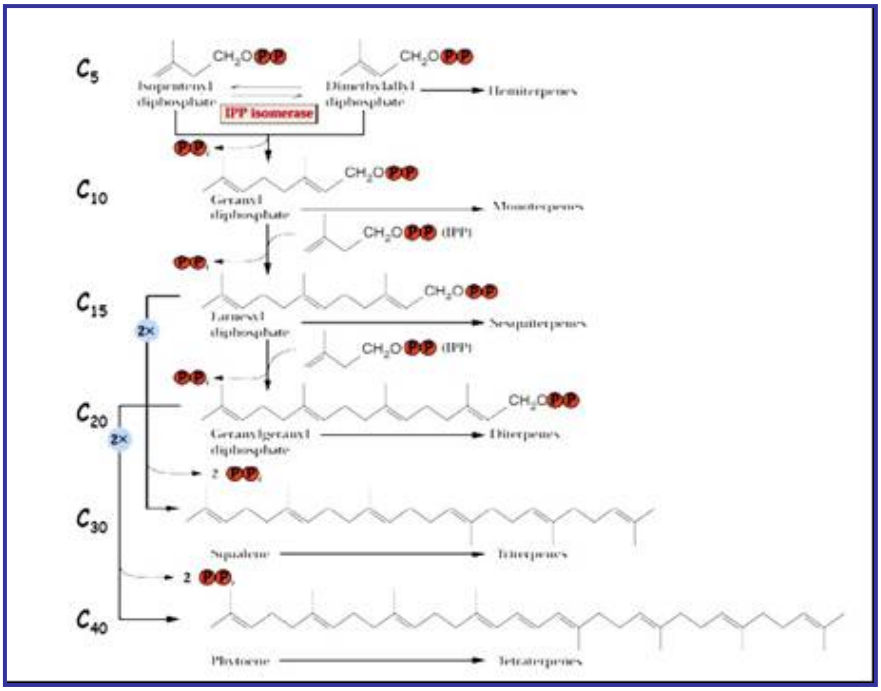

Figure 1.2.7 Formation of different families of terpenes from the universal precursor isopentylphosphate (IPP).

In addition to terpenes, carbohydrates serve as a precursor of furanones in plants. The formation of 2,5-dimethyl-4-hydroxy-2H-furan-3-one (DMHF) is an example (Figure 1.2.8). Incorporation experiments using labeled precursors revealed that D-fructose-1,6 diphosphate is an efficient biogenetic precursor of furaneol. In strawberry (Fragaria ananassa) and tomato (Solanum lycopersicum), the hexose diphosphate is converted by

an as yet unknown enzyme to 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone, which serves as the substrate for an enone oxidoreductase recently isolated from ripe fruit.[76,77]

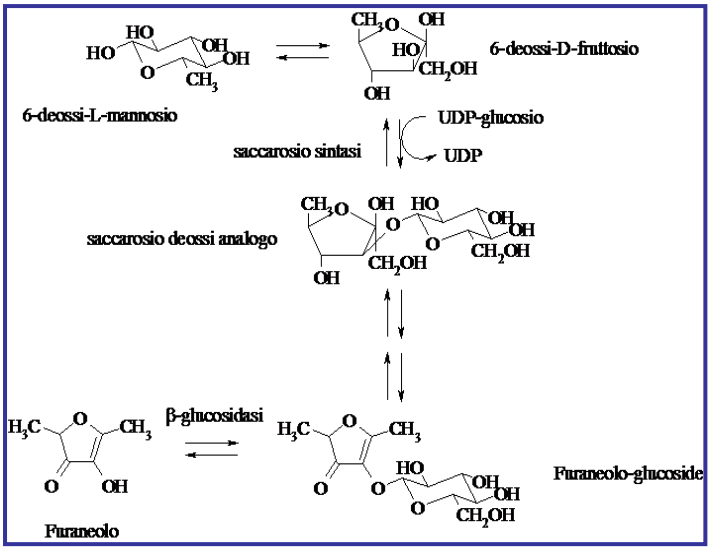

Figure 1.2.8 Formation of 2,5-dimethyl-4-hydroxy-2H-furan-3-one (DMHF) in strawberry fruit.

Furanones and pyrones are important fruit constituents or have been isolated from the bark and leaves of several tree species.[78] Although hexoses and pentoses are the primary photosynthetic products and serve as excellent flavor precursors in the Maillard reaction, only a limited number of natural volatiles originate directly from carbohydrates without prior degradation of the carbon skeleton. Such compounds include the furanones and pyrones.[79]

The furanones are mutagenic to bacteria and cause DNA damage in laboratory tests. However, they are also very effective anti-carcinogenic agents in the diets of animals, and their antioxidant activity is comparable to that of ascorbic acid.[80]

1.2.3 Biogenesis of flavor compounds in vegetables

As mentioned earlier, the process leading to aroma formation in vegetables is quite different than that of fruits. Vegetables do not have a ripening period as fruits do. While some vegetables will develop flavor during growth, much of taste and a limited number of aroma compounds, the remaining flavor, particularly aroma, develops during cellular disruption.

Cellular disruption permits the mixing of enzymes and substrates, which had been separated within the cell, thereby resulting in the generation of volatile substances. A few examples of vegetables that contain a typical aroma prior to cellular disruption are celery, containing phthalides and selines, asparagus, with 1,2-dithiolane-4-carboxylic acid, and bell pepper, containing 2-methyl-3-isobutyl pyrazine.

Similar to aroma development in fruit, fatty acid, carbohydrate and amino acid metabolism serve to provide the precursors of vegetable aroma.

However, while sulfur-containing volatiles are important to some fruits, for example passion fruit, grapefruit or pineapple sulfur-containing are generally much more important to vegetable flavor. This is due to the type of sulfur-containing flavor precursors present in vegetables respect to fruits. In fresh vegetables, thioglucosinolates and cysteine sulfoxides serve as primary precursors.

Most of the vegetables belonging to the Cruciferae family depend to varying degrees on glucosinolate precursors for aroma. More than 50 different glucosinolates have been identified. Glucosinolates are nonvolatile flavor precursors, which are ezimatically hydrolyzed to volatile flavors when cellular structure is disrupted.

The initial products are isothiocyanates and nitriles. Secondary reactions lead to the formation of several other classes of flavor compounds. Figure 1.2.9 outlines the pathway proposed to explain the formation of flavor compounds in different vegetables belonging to *Cruciferae* species.

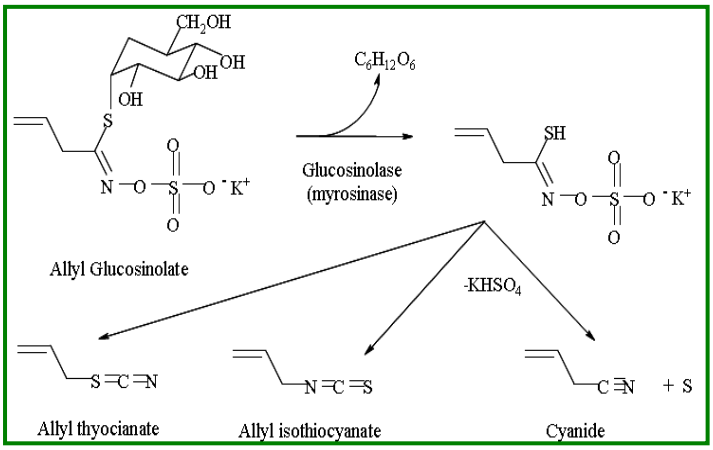

Figure 1.2.9 Conversion of glucosinolate precursors to aroma compounds by myrosinase enzyme.

The glucose moiety is initially hydrolyzed from the glucosinolate. This results in an unstable molecule that readily splits out HSO_4^-. Depending upon molecule rearrangements, either an isothiocyanate or a nitrile result. Further reactions result in the formation of thiols, sulfides, disulfides and trisulfides.

Terpenes also are rather common to vegetables. They arise from mechanisms similar to those active in fruit biosyntheis.

1.2.4 Glycosidically bound aroma compounds

Some aroma compounds are bound as glycosides in fruits and vegetables, and thus make no contribution to food aroma unless released. They may be released from the carbohydrate moiety during maturation, storage, processing, or aging by enzymes, acid or heat. Since their discovery, this has been a very active area of research. At this time, more than 50 plant families have been found to contain glycosidically bound aroma precursors.

Research has been driven by two factors, the first being the desire to enhance flavor strength, or yield, in an application. The pool of glycosidically bound aroma compounds in some plants exceeds the free aroma pool as much as 10:1, thus the ability to free this aroma pool would appear to be desirable. A second factor driving research is that the aging of some products, most notably wine, was postulated to be related to the freeing

of some of the bound aroma constituents over time, thereby maturing or balancing the aroma. If one could determine how to free the bound aroma components, it may be possible to obtain aged wines more quickly. There was not only academic interest behind this research but also considerable financial implications.

Glycosidically bound flavor compounds typically exist as glucosides, diglycosides or triglycosides. The aglycone, flavor compound, is normally bound to glucose and the remainder of the glycoside may be composed of a number of other simple sugars such as rhamnose, fructose, galactose or xylose. Over 200 aglycones have been found including aliphatic, terpene and sesquiterpene alcohols, norisoprenoids, acids, hydroxyl acids and phenylpropane derivatives as well as related compounds (Figure 1.2.10).

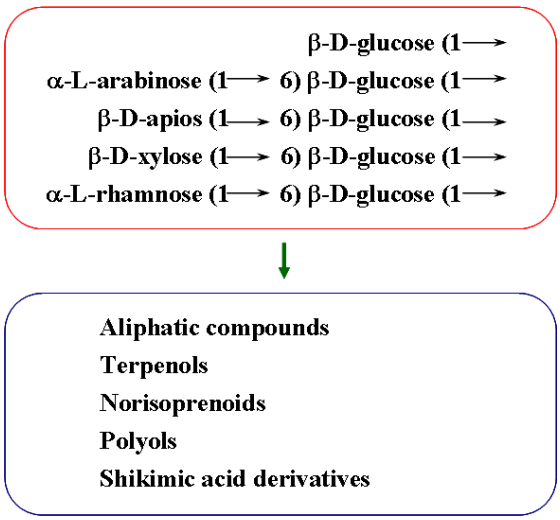

Figure 1.2.10 Main glycosidically bound aroma compounds found in fruits.

As mentioned earlier, the aglycones may be freed from the glycosides by acid, heat or enzyme catalysis. In many fruit juices, the pH is low enough to cause slow hydrolysis during storage. This accounts for some flavor changes during aging/storage of fresh plant foods.

The aglycones may also be freed by enzymatic action. An example where this is a major pathway to release is in vanilla. There is very little free vanillin in the green vanilla bean, because it is bound as a glucoside. The making of vanilla requires a curing step where glucovanillin is converted to free vanillin by an endogenous •-glucosidase

freeing it for extraction.[81] most often this enzymatic freeing of aglycones is a slow process since the enzymes responsible for this hydrolysis are inhibited by low pH, typical of fruit juices.

The most part of all metabolic pathways described in this chapter are strictly influenced by many factors, such as temperature, this is an important factors that can produce stress in plants, in fact plants subjected to low temperature are known to accumulate low molecular weight metabolites. This presumably is an evolutionary response directed toward lowering the freezing point of the plant tissue to enhance survival of frost damage.

Additional temperature effects on flavor development may be due to temperature and pH dependency of enzymatic reactions.

Another factor known to influence flavor is the water content of the soil during plant growth. Plentiful rainfall often results in large, lush vegetables that unfortunately lack flavor. While years with inadequate rainfall often result in fruits and vegetables of smaller size and less attractive appearance but yet with more intense flavor.

Probably this effect can be due to increased amounts of flavor precursors caused by stress put on the plant by restricted water availability.

In this variability of flavor also soil conditions and compositions, maturity stage of fruits and postharvest storage conditions have an important role in the biogenesis of volatile compounds. But also genetics determine the enzyme systems and precursors involved in flavor formation.

In this complex contest the study of aroma compounds are widely used to trace the origin of food and to front the traceability and authenticity problems of a product.

1.3 Olive oil

The extra virgin olive oil is the principal source of fat in the Mediterranean diet with important nutraceutic effects due to its abundance of oleic acid, a monounsaturated fatty acid controlling the cholesterol level, and an adequate content of linoleic and linolenic acids, the major essential fatty acids that lower the risk of coronary heart diseases and cancers.[82] Virgin olive oil is the only vegetal fat that can be eaten crude (also called ‘‘olive juice’’) with no refining operations. This allows to preserve its natural

composition, including the minor, non-saponificable compounds, making up to 1–2% of total content, e.g. hydrocarbons, phenols, alcohols, sterols, pigments, tocopherols and vitamins.

These compounds are crucial both for the oil oxidative stability (improving the shelf life) and for its unique flavor. Aroma and taste are the only parameters that consumers can appraise directly, while other quality features (e.g. chemical composition) are not always labelled on the bottle.

One of the parameters determining the success of a product in the food industry is aroma. Aroma is a very complex mixture of components of most of the various molecular classes and volatile compounds and they are generally responsible for the fragrant 'note' of which fragrance is composed. Olive oil aroma in particular, is unique among the alimentary oils because the wealth of components making its fragrance pleasant to the taste.

The various organoleptic 'notes' are evidenced by means of sensorial analysis from a panel of tasters and the most diffused and credible method of characterization. That is so because the components concentrations range of the mixture is very ample and using always the same analytical technique could carry to a loss of sensibility toward the volatile material present in traces.[83-85] These ones could, however, be essential in the in most of fragrance.

1.3.1 Olive oil flavor and his biogenesis

The unique and delicate flavor of olive oil is attributed to a number of volatile components. Aldehydes, alcohols, esters, hydrocarbons, ketones, furans, and other compounds have been quantified and identified by gas chromatography–mass spectrometry in good-quality olive oil. The presence of flavor compounds in olive oil is closely related to its sensory quality. Hexanal, *trans*-2-hexenal, 1-hexanol, and 3-methylbutan-1-ol are the major volatile compounds of olive oil.

Volatile flavor compounds are formed in the olive fruit through an enzymatic process. The C6 and C5 compounds,[86-88] especially C6 linear unsaturated and saturated aldheydes represent the most important fraction of volatile compounds of high quality virgin olive oils from a quantitative point of view. But other neo-formation volatile compounds,

namely C7–C11 monounsaturated aldehydes,[89,90] or C6–C10 dienals,[91] or C5 branched aldehydesand alcohols[92] or some C8 ketones,[93] reach high concentrations in the aroma of virgin olive oils affected by organoleptic defects.

C6 and C5 compounds are enzymatically produced from polyunsaturated fatty acids through the so-called lipoxygenase (LOX) pathway and their concentrations depend on the level and the activity of each enzyme involved in this LOX pathway.[98-102]

The pathway starts with the production of 9- and 13-hydroperoxides of linoleic (LA) and linolenic (LnA) acids mediated by lipoxygenase (LOX) (Figure 1.2.3). The subsequent cleavage of 13-hydroperoxides is catalysed by very specific hydroperoxide lyases (HPL) and leads to C6 aldehydes, whose unsaturated ones can isomerize from *cis*-3 to the more stable *trans*-2 form. The mediation of alcohol dehydrogenase (ADH) reduces C6 aldehydes to corresponding alcohols, which can produce esters because of the catalytic activity of alcohol acetyl transferases (AAT).[103-104] But an additional branch of the LOX pathway is active when the substrate is LnA. LOX would catalyse, besides the hydroperoxide formation, also its cleavage via an alkoxy radical giving rise to the formation of stabilized 1,3-pentene radicals. These last can dimerize leading to C10 hydrocarbons (known as pentene dimers) or couple with a hydroxy radical present in the medium producing C5 alcohols, which can be enzymatically oxidated to corresponding C5 carbonyl compounds.[86]

A recent investigation pointed out that olive seeds should contain enzymatic activities metabolising 13-hydroperoxides other than hydroperoxide lyase that are responsible for a decrease in the content of C6 unsaturated aldehydes during the olive oil extraction. Moreover they would contribute with an alcohol dehydrogenase activity more specific for saturated C6 aldehydes and especially with an AAT activity scarcely specific in terms of substrate that would be responsible for all kind of esters.[105]

The other accumulation products come from possible fermentations or conversion of some amino acids or from enzymatic activities of moulds or finally from oxidative processes but are generally related to the off-flavour of virgin olive oil (Table 1.3.1).

Compound	Odour quality
Aldehydes	
Acetaldheyde	Pungent, sweet, floral
Propanal	Sweet, pungent, floral
2-Methyl-propanal	Cooked, caramel
Hexanal	Green, apple, cut grass
Heptanal	Fatty
Octanal	Citrus-like, soapy
Nonanal	Soapy, citrus-like
Decanal	Soapy, citrus-like
2-Methyl butanal	Malty
3-Methyl butanal	Sweet, fruity, malty
2-Methyl-2-butenal	Apple
trans-2-Pentenal	Green, apple, floral
cis-2-Pentenal	Green, pleasant
trans-2-Hexenal	Bitter, almonds, green, green apple-like, fatty, bitter almond like, cut grass
cis-2-Hexenal	Green, fruity, sweet
trans-3-Hexenal	Artichoke, green, floral
cis-3-Hexenal	Green leaves, grassy, green, apple-like, leaf-like, cut grass
2-Octenal	Fruity, soap, fatty
cis-2-Nonenal	Green, fatty
trans-2-Nonenal	Paperlike, fatty, sharp, cut grass
2-Decenal	Fatty
2,4-Hexadienal	Cut grass
2,4-Heptadienal	Fatty, nutty
2,4-Nonadienal	Deep-fried
2,6-Nonadienal	Cucumber-like
2,4-Decadienal	Deep-fried
Benzaldehyde	Almond
Phenylacetaldheyde	Pungent, phenolic
Ketones	
Pentan-3-one	Sweet
1-Penten-3-one	Sweet, strawberry, sharp, Pungent, green, metallic
1-Octen-3-one	Mushroom-like
Alcohols	
Ethanol	Alcoholic, ripe apple, floral
Pentan-1-ol	Pungent
Hexan-1-ol	Fruity, aromatic, soft, cut grass
2-Methyl-propan-1-ol	Ethyl acetate-like
2-Methylbutan-1-ol	Fish oil
cis-2-Penten-1-ol	Banana
trans-3-Hexen-1-ol	Fruity, fatty, pungent, cut grass
cis-3-Hexen-1-ol	Banana, leaf-like, green-fruity, pungent
trans-2-Hexen-1-ol	Green, grassy, fruity, fatty, pungent
cis-2-Hexen-1-ol	Green fruit, green-fruity
1-Penten-3-ol	Wet earth

Compound	Odour quality
Esters	
Methyl acetate	Ester
Butyl acetate	Green, pungent, sweet
Ethyl acetate	Sweet, aromatic
Ethyl propanoate	Sweet, strawberry, apple
Ethyl butyrate	Cheesy, fruity
Ethyl isobutyrate	Fruity
Ethyl 2-methylbutyrate	Fruity
Ethyl 3-methylbutyrate	Fruity
cis-3-Hexenyl acetate	Green-banana, fruity, Green, green leaves, floral, ester
Hexyl acetate	Sweet, fruity, floral
3-Methylbutyl acetate	Banana
Methyl 2-methylbutyrate	Fruity
Methyl decanoate	Fresh
Methyl nonanoate	
	Fruity, sweet, floral
Acids	
Acetic acid	Pungent, like acetic acid
Propanoic acid	Aromatic, pungent
Butanoic acid	Buttery, rancid
Pentanoic acid	Sweaty, pungent, putrid
Hexanoic acid	Sweaty, pungent
3-Methylbutyric	Sweaty
2-Methylbutyric	
	Sweaty
Others	
Methylbenzene	Glue, solvent-like
Ethylbenzene	Strong
Ethylfuran	Sweet, rancid
Dimethyl sulfide	Organic, wet earth
Dipropyl disulfide	Cooked meat
Cyclopropane	Musk

Table 1.3.1 Odour qualities of some volatile compounds found by different authors by means of sniffing technique.

1.3.2 Effect of agronomic and technological aspects on the volatile fraction of olive oil

A number of investigations were aimed to find some correlations that can explain the presence of positive or negative sensory notes that are perceived by tasters during the virgin olive oil tasting.

The most important positive attribute is represented by fruity sensory note, the sensation reminiscent of healthy fruits harvested at the right ripening degree. To the aroma of high quality oil, in addition to fruity, generally contribute the “green” sensation reminiscent of just cut grass, leaf, tomato, artichoke, walnut husk, apple or other fruits. The flavour

of these oils is accompanied by more or less intense notes of bitterness and pungency, ascribable to secoiridoid compounds.

The determination of volatile compounds highlights that C6 and C5 compounds mainly form the volatile fraction. The concentration of each of them, responsible for the different nuances of the positive attributes,[106] is dependent on the level and the activity of enzymes involved in the LOX pathway. The enzymatic levels are genetically determined, whereas a number of factors affect their activities.

Two recent works reviewed the several factors influencing aromatic quality of virgin olive oil, i.e. biogenesis and composition of volatiles, relationships with sensory notes, possible influence of agronomic and processing factors, and oil oxidation.[107,108] All these findings show that volatiles content, mainly C6- and C5-skeleton compounds from the lipoxygenase pathway, are strongly influenced by the genetic origin (cultivar) for the enzymatic expression and by horticultural and processing parameters for the enzyme activity.

This feature, together with the different concentration of *trans*-2-hexenal, represents an effective tool to differentiate monovarietal oils from different cultivars.[95]

The unique flavor of virgin olive oil is mainly attributed to the volatiles that develop during and after oil extraction from the fruit. These compounds become less important during oil storage due to oxidation. These changes on volatile composition, together with genetic, horticultural and processing influences, explain quality differences in olive oils.[109]

The concentration of compounds in the volatile fraction depends on the level and activity of enzymes which are genetically determinant, climate and soil type as well as the fruit ripening cycle.[108,110] Removal of the stone in order to obtain oil from just the pulp is another factor that influences the volatile compounds. A number of authors have studied the volatile composition of oils obtained from whole olives and from olive pulp only.[111-112]

An essential point to obtain high quality olive oil is the processing of healthy fruits. Unfortunately when *Bactrocera oleae* attacks fruits damages cause a considerable increase of carbonyl compounds and alcohols is related to the stage of the development of the olive fly and the intensity of attack.[113,114]

Several researches[115-117] proved that during the olive ripeness the amount of volatile compounds, especially of *trans*-2-hexenal, increases until a maximum concentration occurring when fruits turn their skin colour from yellow-green to purple.

Beyond this time the concentration of volatile compounds decreases because of a lower activity of enzymes involved in their production with a weakening of the intensity of some "green" sensory notes. This trend was not found by Aparicio and Morales[97] who described a steady decrease of the concentration of the volatile compounds, including *trans*-2-hexenal, from the unripe to the over-ripe stages, except for oil from Coratina fruits that, on the contrary, confirmed the behaviour observed by the other researchers.[115-117]

The genetic effect related to the cultivar is one of the most important aspects of volatile composition of olive oil. However, climatic and agronomic conditions of olive growing can affect volatile composition of olive oils obtained by the same cultivar. In this ambit the relationships between the water availability during fruit ripening and the volatile composition has been studied.[118-119] Results showed that, in the climatic conditions of central Italy, the rainfall effect is pre-eminent with respect to temperature and that some compounds, such as hexanal and isobutyl acetate, were negatively correlated to rainfall.

Also the geographic origin of oils plays a fundamental role in defining the volatile compounds profile of virgin olive oils. Early studies conducted by Montedoro et al.,[120] have shown the ability of volatiles, sampled with the static headspace, in discriminating different oils coming from different Italian regions.

In conclusion we can consider that several agronomic and climatic parameters can affect the volatile composition of olive oils. For this reason volatile compounds can be considered as markers, with sensory impact, that can be used to differentiate them.

The different technological operations also have repercussion on the volatile composition. This factors are: harvesting methods, the olive fruit storage, the olive crushing and malaxation, the extraction system of oil and its storage methods.

A considerable increase of volatile alcohols and carbonyl compounds having unpleasant aroma and the appearance of a typical defect, reminiscent of "mouldy" and "earthy" tastes at the same time,[121] can be considered as a consequence of the prolonging of contact time of fruits with the ground.

The storage of olives in unsuitable conditions, into sacks or in piles, has heavy negative repercussions on the sensory quality of resulting oils. The production of different metabolites, according to the type of microorganisms from environment whose development is promoted by the temperature reached in the pile and the humidity degree, gives rise to different sensory defects, better evidenced by the weakening of positive perceptions related to the decrease in concentrations of compounds from LOX cascade.

Almost all volatile compounds of a good quality olive oil give rise at the moment of tissue disruption of the olive pulp, therefore the effectiveness of crushing plays an important role in their production. The use of an hammer mill crushers, which determining a more violent grinding of pulp tissues causes an increase of the olive paste temperature and the reduction of hydroperoxide lyase activity,[122,123] has as a consequence the production of oils characterized by a lower amount of volatile compounds, especially of *trans*-2-hexenal, hexanal and *cis*-3-hexen-1-ol, compared with the concentration of the same compounds in oils obtained with the same processing diagram except for the crushing performed by means of a stone mill.[99]

Time and temperature of malaxation affect the volatile profile and therefore the sensory characteristics of the resulting oils. In fact high temperatures in the malaxation step make active the amino acid conversion pathway with production of considerable amounts of 2-methyl-butanal and 3-methyl-butanal, but without accumulation of corresponding alcohols,[102] correlated with "fusty" defect.[92]

The LOX is not the only oxidoreductase active in the olive pastes during malaxation. In fact, peroxidase and polyphenoloxidase are activated during crushing and oxidize phenolic compounds during malaxation reducing their concentrations in the pastes and in the oils. For this reason during the last 10 years several works were performed to control selectively endogenous oxidoreductases in the pastes during this technological process. In this ambit the use of inert gas to remove oxygen in the headspace of malaxer was studied.[124-127] The results show that the use of N_2 during malaxation not only reduces the oxidative degradation of phenolic antioxidants but, at the same time, hardly modifies the volatile composition of oil.

The final volatile profile is also influenced by the system used for extracting oil. The most widespread systems for the separation of the oil from the olive pastes are the

centrifugation and pressing methods. The losses in volatile compounds depend on the importance of the interactions between on one hand oil and solids and on the other oil and vegetation waters that are reduced to a minimum when the traditional pressing plant is adopted. But it must be underlined that for obtaining high quality olive oils pressing plant needs to work fruits of the same good quality and in a continuous way to prevent possible fermentations and/or degradation phenomena of residues of pulp and of vegetation waters on the filtering diaphragms, which could give rise to the defect named "pressing mats".[128]

The olive oil profile changes during its storage because of the simultaneous drastic reduction of compounds from LOX pathway and the neo-formation of some volatile compounds[90,129,130] responsible for some common defects known as "rancid", "cucumber" and "muddy sediment" attributes. The newly formed volatile compounds arise from the fragmentation of odourless and tasteless hydroperoxides.[129] They are radically produced from the oxidation process of lipids, promoted by several factors like as light, temperature, metals, pigments, unsaturated fatty acid composition, quantity and kind of natural antioxidants and amount of sterols.[130] The most important contributors, because of their low odour thresholds,[131] are unsaturated aldehydes whose concentration increases with prolonging the storage time, but other chemical species belonging to saturated aldehydes, ketones, acids, alcohols, hydrocarbons, lactones, furans and esters contribute to the complete definition of the typical undesirable oil aroma.

Morales, Rios and Aparicio suggested that the ratio hexanal/nonanal can describe the evolution of oxidation,[132] whereas Solinas and co-workers found *trans*-2-heptenal to be correlated with the rancidity perception.[89,90]

1.4 Tomato

The tomato (*Lycopersicon esculentum*, Mill) originated in Tropical America, probably in Mexico or in Peru. The name is of South American origin and is derived from the Aztec word "xitomate" or "zitotomate". It was taken to Europe in the early sixteenth century, and later in the eighteenth century to North America, and from these areas spread out to the rest of the world.

The tomato is a member of the *Solanaceae* family. Botanically, it is a berry fruit, but it is cultivated and used as a vegetable.[133] It is a tender, warm season perennial. Nutritionally, the tomato is a good source of vitamin A and C.[134] Composition data varies due to the wide range of species, stage of ripeness, year of growth, climatic conditions, light, temperature, soil, fertilization, irrigation, and other conditions of cultivation, and handling and storage.

Analysis of volatile compounds appears to be difficult with fresh fruits and vegetables due to unstable nature of some of the most important flavor compounds that origin during the ripening of fruits and to the presence of other important flavor compounds that are formed enzymatically in appreciable amounts only when the tissue is disrupted by slicing, chewing, or blending and may also be degraded due to enzyme action.

The nutritional importance of fresh tomatoes and tomato products is now well-known. Flavor and aroma are essential parameters of quality in tomatoes. The main precursors of volatile compounds in tomato are the free amino acids, fatty acids and carotenoids. Over 400 volatile components have been identified in fresh tomatoes, while during processing the endogenous enzymes catalyze the formation of important volatile compounds of tomato. In fact saturated and unsaturated C6 and C9 alcohols and aldehydes, that are impact compounds of fresh tomato, are originated by lipoxygenase activity, while terpene and carotenoid derivatives can be released from odourless glycosidic compounds by glycosidase activities.[135,136]

It's known that the flavor of fresh fruits and vegetables, and also the flavor of fresh tomato, is strictly influenced from the pedoclimatic characteristics of the growing areas.[137]

Some official method, using various markers, were developed to selfguard the product and consumers from fraudulent attributions about its origin.

Research with many other foods indicates that only a very small fraction of the volatile compounds occur in concentrations above their odour thresholds and, thus, are actually contributors to the aroma of food. The pleasant sweet-sour taste of tomatoes is mainly due to their sugars (primarily the reducing sugars, glucose and fructose) and organic acids (citric and malic acids are the major organic acids) content.

It seems generally accepted that ripe tomatoes, purchased in supermarkets in the United States, lack the desirable aroma and flavor associated with ripe tomatoes picked directly

from the plant in the field. There have been a large number of studies carried out on the identification of the volatile flavor component of fresh tomatoes, which have been reviewed.[138] Despite these (largely qualitative) studies there are some questions unanswered particularly regarding the actual quantitative concentrations of the identified volatile components. Such quantitative data are necessary for the full understanding of the role of the individual components in fresh tomato flavor. One major problem with quantitative analysis has been that some of the enzyme produced volatile flavor components are themselves degraded by other tomato enzymes before or during the volatile isolation by conventional methods.

Volatile compounds contribute to the tomato overall aroma intensity and numerous studies have been devoted to identifying the major constituents responsible for tomato aroma. Some fruits or vegetables have one or two odour impact compounds that dominate the flavor of that particular commodity. This is not the case for tomato, however, since no single compound has been found in this fruit that is reminiscent of a ripe tomato.

However, the effect of genetic variation and growing conditions of tomato on aroma compounds is not well understood. Reasons for this lack of information are the complexity of analysis of volatiles, the difficulty in developing a consistent methodology for sensory evaluation, and the challenge to link these analytical tools to well-defined raw materials. In addition, little quantitative data is available on tomato flavor volatiles.

1.4.1 Tomato aroma biogenesis

Flavor volatiles in tomato are formed in the intact tomato fruit during ripening as well as upon tissue disruption, which occurs when tomatoes are macerated, blended, or homogenized.[139] These two main routes of volatile production point out the involvement of certain enzymes in the biogeneration of volatiles as well.

Tomato volatiles have been divided into six groups: lipid derived, carotenoid related, amino acid related, terpenoids, lignin related, and other misceleneous.[140] This division implies different pathways, substrates, and enzymes for the biosynthesis of the aroma volatiles. It is very important to study the biochemistry of aroma biogeneration in fresh

tomato, since moleculer biologists and plant breeders do not have clear targets for the genetic manipulation of flavor.[141]

Volatiles originated from lipoxygenase activity are considered by far the most important components of tomato aroma, but the exact roles in perception and synthesis patterns require further investigation.

It has been thought that in plant tissues lipid peroxidation occurs through enzymatic processes, such as those of the oxylipin / lipoxygenase (LOX) pathway.[142] The products of lipoxygenase pathway reactions depend upon the nature of the cellular stimulus, the available substrates, and the intracellular site of the reactions. Oxylipin is the generic name for a family of oxygenated compounds formed from fatty acids by enzymatic reactions. They include fatty acid hydroperoxides, hydroxy fatty acids, epoxy fatty acids, keto fatty acids, volatile aldehydes, and cyclic compounds. The oxylipin pathway influences aroma, taste, and possibly the deterioration of fresh plant products.[143,144]

Although some lipoxygenase isoenzymes can oxidize specific glyceride lipids, it is generally acknowledged that free polyunsaturated fatty acids are the preferred substrates. General substrates for lipoxygenase are fatty acids containing a cis, cis-1,4-pentadiene structure. The main substrates for lipoxygenase in plants are linoleic and linolenic acid, while in mammals these are arachidonic and eicosapentaenoic acid.[143-145]

The term lipoxygenase (linoleate:oxygen oxidoreductase) refers to a group of enzymes that catalyze dioxygenation, by use of molecular oxygen, of methyleneinterrupted polyenoic fatty acids into corresponding enantiomeric hydroperoxy fatty acids.

Multiple forms or isozymes exist in many tissues, showing differences in regiospecificity, optimum pH and enzymatic properties. LOXs are inactivated by its substrate analogs or by lipid antioxidants i.e., a-tocopherol, nordihydroguaiaretic acid (NDGA), propyl gallate, hydroquinone, and a-naphthol. LOX is also a self-destructing enzyme, and the velocity of catalysis decreases linearly with substrate utilization.[142,145,146]

Tomato lipoxygenases have been widely studied. Apparent molecular weights of 87 to 95 kDa have been reported.[147-149] Optimum pH can range from 4.2 to 8.0. Although one study reported a linear increase in LOX activity by fruit ripening,[152] others reported that the highest level of activity was at the breaker stage, subsequently decreasing through the full ripe stage.[147,151] Greater amounts of LOX mRNA and protein have been found in

the breaker to ripe and red-ripe stages.[153] LOX activity in tomato fruits has been reported both in membranous systems and in soluble form.[147-150]

The highest level of LOX activity was found to exist between the skin and outer flesh of the fruit.[154] However, the greatest amount of LOX mRNA and protein was found to be in the locular jelly and radial walls of ripe fruit.[153] Lipid oxidation products by the LOX pathway are usually characterized by green, fresh, and grassy notes. These reactions are dominant when tissue is disrupted, upon which the substrates and enzymes are combined in the presence of oxygen. Lipid-derived volatiles occur in intact tissue during fruit ripening, although the concentration of compounds may be much smaller than in macerated tissue. The level of enzyme activity present is certainly a factor in the biogenesis of aroma volatiles. The amounts of substrates available and the sub-cellular conditions are other factors which affect the production of volatile compounds. In addition, there are competing pathways that share the substrates and/or products.

Another important metabolic pathway in tomato aroma formation is the synthesis of terpenoids.

Terpenoids are enzymatically synthesized de novo from acetyl CoA and pyruvate provided by the carbohydrate pools in plastids and the cytoplasm. Although fatty acid oxidation is one of the major pathways producing acetyl CoA, this process probably does not contribute to the formation of terpenoids as it takes place in peroxisomes.

Many of the terpenoids produced are non-volatile and are involved in important plant processes such as membrane structure (sterols), photosynthesis (chlorophyll side chains, carotenoids), redox chemistry (quinones) and growth regulation (gibberellins, abscisic acid, brassinosteroids).[155] The volatile terpenoids – hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15) and some diterpenoids (C20) – are involved in interactions between plants and insect herbivores or pollinators and are also implicated in general defense or stress responses.

1.5 Chemical contaminants in food

Regulation EC 1907/2006[156] introduces several major activities associated with the movement of chemicals, including harmonized measures to ensure the protection of human health and the environment and to promote the development of alternative methods for the assessment of hazards associated with chemical substances. Management of chemicals should be based upon a complete evaluation of the associated risk and the application of proportionate risk management strategies. In order to monitor protection of the environment and of human health, it is necessary to assess exposure of relevant populations. Exposure assessment requires the ability to measure substances of possible concern with adequate confidence, unambiguously and with an uncertainty that is low enough not to bias any related risk assessment. Risk is the product of two factors: the hazard associated with a substance and the exposure to the substance. Many chemicals are hazardous, but risk management is normally applied to limit human exposure and thus to reduce the associated risk to an acceptably low level.

Many chemicals, including pollutants from large-scale use that have been banned or phased out, have been identified in human tissues in recent studies.[157-159] However, it is unclear whether or not the levels observed present a significant risk to human health.

Deploying a high proportion of analytical resources to monitor those compounds with physico-chemical properties that indicate persistence, bioaccumulation and toxicity is therefore a positive strategy. However, POPs may not be the only chemicals of concern as we learn more about the behaviour of other substances.

Human exposure to chemical substances can be via multiple pathways. The major routes of possible human exposure to chemical substances include occupational (*i.e.* the working environment), inhalation of air (outdoor and indoor), ingestion of dust, ingestion of water, ingestion of food and absorption via the skin, for example, application of cosmetics. Depending upon the physicochemical properties of the substance, one exposure pathway may predominate over others but this is not the case for all chemicals. For example, it is considered that human exposure to polybrominated diphenyl ether flame retardants is mediated by both dust ingestion and by the diet.[160] Primary concerns about human or environmental exposure are often directed towards a single, discrete substance. However, in some cases degradation or transformation

products may also be regarded as hazardous and thus a number of related chemicals (starting compound, metabolites and/or degradation products) may require consideration during risk assessment. Additionally, many organic chemicals consist of homologous series or may comprise a number of isomers, requiring the measurement of a suite of similar compounds. The resulting analytical data may need to reflect the combined measurement of large numbers of similar compounds of varying toxicity.

Chemical contamination of the human food chain may arise in several ways. Direct contamination can occur by contact with the food, for example, hygienic cleaning residues can transfer to food from a treated surface if it is not properly rinsed. Indirect transfer can occur from materials used in food manufacture to a finished food product, including transfer between the animal feed and human food chains. However, human dietary exposure to chemicals that pass up the food chain by transfer from the environment is possibly the greatest concern. This pathway into the food chain is typical of the more persistent and potentially bio-accumulative compounds, such as dioxins, PCBs, chlorinated paraffins, phthalates, brominated diphenyl ethers and perfluorinated compounds.

Many chemicals that are released into the environment may have the potential to transfer into food or drinking water. Certain compounds may also be distributed in the environment to the point at which they become ubiquitous. Such compounds may not be classed as persistent but their environmental levels may be maintained by constant addition, for example, by incomplete removal in waste water treatment plants and therefore release into rivers and drinking water, or by wash-off into waterways and thus into the aquatic food chain. Their ubiquity can lead to concern that they may enter the food chain at significant concentrations.

Possible examples include UV blocking agents, residues of pharmaceutical products[161,162] and residues of bacteriostats such as triclosan and triclocarban.[163] These and other similar classes of chemical have been proposed as ‘emerging contaminants’, reflecting concern about their increasing detection in the environment. Synthetic musk fragrances have been historically used in home cleaning and personal care products. They have been detected in the tissues of freshwater and marine biota,[164,165] leading to pressure to limit or end their use in consumer products. For such emerging contaminants, indirect human exposure, in addition to direct exposure during product

use, may be linked to the consumption of drinking water, use of potable water in the manufacture of food or dietary exposure via the aquatic food chain, such as the consumption of freshwater and marine fish.

The combination of different exposure routes (*e.g.*, water, food, indoor environment) significantly increases analytical requirements to encompass different sample matrices and multiple series of analytes (parent compounds, degradation products, metabolites) in order to produce an accurate, holistic assessment of exposure. As a consequence, there is an increasing requirement for more complex analytical methods. However, the validation of expanded methodology becomes problematic because the availability of appropriate, matrix-matched reference materials is limited for many compounds of potential concern. Thus, the expansion of analytical capability may be at the cost of less demonstrable method performance.

For certain classes of chemical that may be present in food, regulatory limits have not been established. A common reason is that the chemical is not expected to be present in food at a level of concern or has not previously been encountered in food at an appreciable level. Consequently, the toxicological information required to decide on an acceptable level of dietary exposure may not exist. However, the purpose of producing exposure data is to assess the related risk, so some form of risk assessment paradigm is needed to place the data in context.

Procedures to measure chemical contaminants in food should ideally provide data that are fully consistent with their intended purpose. Regulatory limits have been defined for certain chemicals in food and other media to ensure human and environmental safety. Such limits are based on comprehensive (or, at least, adequate) toxicological knowledge and they are designed to ensure that human or animal exposure does not approach a level of possible concern. To monitor regulatory compliance, analytical methods must be capable of unambiguous identification and accurate measurement of the target chemical at a decision limit below the level of concern.[166] Appropriate methods must therefore provide high chemical selectivity, for example, optimized extraction, targeted clean-up and high chromatographic separation, and suitable sensitivity (*e.g.*, a limit of detection significantly lower than the level of concern). In practice this frequently results in the specification of hyphenated instrumental techniques including GC/HRMS[167] and HPLC/MS/MS.[168] The demonstration of reliable measurement is

usually linked to inter-laboratory exercises since there is a requirement for laboratories accredited to the ISO/IEC 17025 standard to demonstrate their ongoing analytical proficiency.[169] Such methodology has often been refined through inter-laboratory exercises (*e.g.*, for PCDDs, PCDFs and PCBs) to achieve high accuracy and acceptable precision except when operating close to the limit of quantification. For less well-characterized contaminants, human exposure data are limited and may originate from only a few laboratories, with a consequent possibility that exposure may not be fully assessed due to limited sample capacity. The methods used to measure new contaminants may have undergone internal validation but not necessarily inter-laboratory comparison. This can influence the comparability of data between laboratories and also the analytical (measurement) uncertainty. From the perspective of seeking to obtain reliable data on human exposure in order to provide robust risk assessment, the inter-laboratory evaluation of analytical methods (and linked to this, the development of reference materials for use in analytical quality control) is highly valuable. This has been demonstrated in many areas of food chemical analysis, including the progress made on inter-laboratory agreement of data for PCDDs and PCDFs in food[170] and subsequently the availability of increased analytical capability. The resolution achieved by chromatographic systems has increased enormously in the last fifteen years, both for HPLC and GC. High resolution chromatography has facilitated congener-specific measurement and has reduced limits of detection. As chromatographic resolution increases, sample clean-up procedures can be reduced and are sometimes no longer necessary, leading to faster analysis and improved recoveries of analytes. However, the dwell times available for mass selective detection reduce with peak width, leading to potential problems of reduced sensitivity unless fast detectors (such as micro-ECD or ToF) are used. Thus, such approaches may provide more information but not necessarily the best measurement accuracy. The continuing development of lower-cost analytical solutions (such as the development of GC/GC/ECD as an alternative to GC/HRMS) with the aspiration of achieving benchmark standards of performance is highly encouraging.

For some classes of chemical, robust analytical methodology may not be currently available. The determination of short-chain polychlorinated alkanes (SCCPs) is an example. SCCPs are technical mixtures of C10 to C12 chain length alkanes, of varying

degree of chlorination and with up to 8000 congeners. SCCPs have replaced PCBs in some industrial applications and were subsequently found to be persistent in the environment with the potential to accumulate in organisms. The complexity of the SCCP mixtures found in the environment means that they cannot be chromatographically separated into discrete components.[171] This creates a problem in detector calibration and quantification. Consequently, analytical data are based on a total peak area with relatively high uncertainty.

Developmental toxicity has been observed for SCCPs,[172] but can only be linked to the degree of chlorination rather than to specific chemical entities, because of the complexity of the mixture.

In the same way as analytical methodology for PCBs has evolved from the measurement of Aroclor equivalents to congener-specific determination, future chromatographic developments may resolve complex mixtures such as SCCPs. For the present, measurement uncertainty remains a key limitation in exposure assessment for these compounds.

Although targeted analysis is highly valuable to assess compliance with safety and regulatory standards, it is applied with the premise that the chemical is likely to be present and the purpose of analysis is thus to quantify the target chemical or chemicals. A growing number of routine analytical methods are multi-residue in nature, giving the potential to detect and quantify larger numbers of compounds using non-selective clean-up with increased chromatographic separation and selective (usually MS-based) detection.[173,174] Techniques such as multidimensional gas chromatography (GC×GC)[175,176] can separate very large numbers of compounds by the use of orthogonal stationary phases. The logical extension to these approaches is the application of rapid spectroscopic techniques in exploratory modes, such as time-of-flight MS,[177] to identify chemical residues in food.

1.5.1 Phthalates

Phthalates are phthalic acid esters with a common chemical structure show in figure 1.5.1. Phthalic acid esters (phthalates, PAEs) are used primarily as plasticizers in polymeric materials, such as polyvinylchloride or polyvinylidenchloride copolymers,

increasing their flexibility only through weak secondary molecular interactions with polymer chains. Being not covalently bound to the vinyl polymer matrix, their migration from plasticized products to contact materials may occur through extraction (leaching) or evaporation processes. World wide production of PAEs and their frequent application in different products of everyday use has resulted in their widespread presence in all parts of the environment and, consequently, in food. The most frequently used ester, di-2-ethylhexyl phthalate (DEHP), became practically ubiquitous in the environment as a result of its massive use and persistent character.

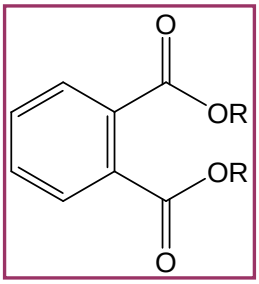

Figure 1.5.1 Common chemical structure of phthalic acid esters.

The quest for a reasonable stand concerning the possible adverse impact of phthalates on human health is still an unresolved issue. In the 1980s the US Environmental Protection Agency (EPA) and several other countries classified the commonly occurring phthalates as priority pollutants[178,179] and recommended maximum admissible concentration in water of 6 •g l^{-1} for the di-(2-ethylhexyl) phthalate.[180] As phthalates are widespread in the environment, a proof was furare nished of oestrogenic properties exhibited by some phthalates in recent years.[181-183]

The presence of phthalates in the environment has been discussed for decades, but a greater interest in phthalates during the eighties was provoked by the suspicion of the carcinogenic effect of di-2-ethylhexyl phthalate. DEHP has been shown to cause liver cancer in rats and mice.[184-185] The finding that some phthalates (benzylbutyl-[BBP], di-*n*-butyl-[DnBP], di-2-ethylhexyl-[DEHP] and diethyl [DEP] phthalate) show the ability to interfere with development of young mammals and to affect their reproductive system[186] has again prompted a growing interest in these substances in recent years.[187] Dimethyl phthalate together with benzylbutyl phthalate have been found to be the esters

with the highest estrogenic potency among phthalates.[188] According to research carried out in this field, it is now clear that some phthalate metabolites, mainly monoesters, play an important role in the toxic action of PAEs.[189]

A list of the target analytes together with selected physico-chemical properties relevant to our experiments is summarized in table 1.5.1.

[a] Partition coefficient octanol–water.

Analyte	Abbreviation	Molecular weight	H_2O solubility (mg L^{-1})	log K_{OW} [a]	Vapour pressure (mPa) [25 °C]
Dimethyl phthalate	DMP	194.19	4200	1.61	267
Diethyl phthalate	DEP	222.24	1100	2.38	133
Di-*n*-butyl phthalate	DnBP	278.35	11.2	4.45	3.60
Butylbenzyl phthalate	BBP	312.39	2.7	4.59	0.67
Di-2-ethylhexyl phthalate	DEHP	390.56	0.003	7.50	0.013
Di-*n*-octyl phthalate	DnOP	390.56	0.0005	8.06	0.013

Table 1.5.1 List of most diffused phthalate esters and their selected physico–chemical properties.[190]

1.5.1.1 Methods used for assaying of phthalates

Several analytical methods are described for the analysis of plasticisers and additives in PVC gaskets and food. Biedermann-Brem et al. used multianalytical GC/MS method for the determination of plastisols in PVC gasket seals.[191]

For analysis of Epoxidized Soy Bean Oil (ESBO) in food, the pioneering work was done by Castle et al.[192] The method included two derivatization steps of epoxy triglycerides and further determination of the derivates by GC/MS. For oily food samples, Fankhauser-Noti et al. proposed the method in which after transesterification of epoxidised triglycerides the methyl esters of diepoxy linoleic acid and two internal standards were isolated by HPLC and transferred on-line to GC/FID using on-column interface with concurrent eluent evaporation.[193] Suman et al. developed the method for analysis of ESBO in foodstuffs, which was based on reversed phase liquid chromatography interfaced with electrospray ion trap tandem mass spectrometry and, which included simple sample preparation procedure with extraction step without any further purification prior the analysis.[194]

For the analysis of PVC plasticizers (other than ESBO) in the food, several authors used GPC clean-up prior the analysis with GC/MS.[195,196-199] Biedermann et al.[200] and Fiselier

et al.[201] proposed the method for analysis of oily samples, which was based on GC/MS or FID by using injector-internal thermal desorption approach in order to avoid GPC clean-up. For the identification and quantification of polyadipates, the methods are described in details by Biedermann and Grob.[202,203]

Extraction and pre-concentration techniques, such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are widely applied to determine phthalates in water samples.[204-207] The EPA has published analytical procedures dealing with the determination of phthalate esters in drinking water and in municipal and industrial wastewater[208,209] based on these pre-concentration techniques. Nevertheless, these methods are expensive, time-consuming, and employ different sorbent materials and solvents, enhancing contamination risks. In the last years, solid phase microextraction (SPME) has acquired an increased importance in the analysis of semivolatile compounds[210-212] including phthalates.[213-217] This technique is an interesting alternative for the determination of phthalates in aqueous samples, because the risk of contamination during sample handling can be significantly reduced. In addition, the elimination of organic solvents in the sample preparation process could reduce phthalate background levels. Nevertheless, the main problem for applying SPME to phthalate analysis is the levels of phthalates found in blanks of laboratory purified water and even commercial water (especially for DBP and DEHP).

In these studies, some factors affecting the extraction efficiency are evaluated. Nevertheless, most papers consider optimization strategies based in the study of one factor at a time. This approach can lead to erroneous conclusions about the importance of certain factors on the extraction process, due to the fact that interactions between factors are not being considered. A multivariate approach to the optimization of the SPME process allows the simultaneous study of various factors and then, it is more advantageous than univariate. To the knowledge of the authors, up to now only one study using a multivariate strategy was applied to the problem of phthalate determination by SPME.[217] In this interesting study, a screening method for the analysis of 16 PAHs, 6 PCBs and 6 phthalate esters has been developed using multi simplex optimization. Due to blank problems, detection limits for some phthalates were quite high, especially for DEHP ($3.15 \bullet gL^{\bullet 1}$). In addition, due to inherent difficulties of

simplex with categorical variables only one fiber (PDMS) was studied, although many others are potentially applicable to phthalate analysis.

Phthalates analysis may generally pose a serious problem due to the high risk of secondary contamination from chemicals, materials and laboratory equipment. Also the difficulty of separating co-extracts (lipids, pigments) makes the determination of low levels of PAEs in some matrices (food matrices above all) almost impossible, namely when capillary gas chromatography is used for the final separation and detection of analytes. Therefore, many procedures use high performance liquid chromatography (HPLC) to determine phthalates in complex matrices, such as plasma[18] or milk,[219] regardless of the fact that lower sensitivity of determination has to be accepted in this case.

Several methods determining PAEs in fatty matrices have been published. Some procedures use adsorption column chromatography on Florisil or alumina and the elution of target analytes using various mixtures of diethyl ether in petroleum ether.[220,221] Page and Lacroix employed a sweep codistillation technique for phthalate separation in matrices, such as animal tissues, fats or cheeses with a high fat content.[222] It should be noted that in most of these methods the separation of PAEs from matrix components was either incomplete or very laborious.

With regard to these difficulties indirect methods consisting of the saponification of the parent phthalic acid esters and fats, and the subsequent determination of the resulting alcohol moiety or phthalic acid, were introduced.[223,224] In the early 1990s gel permeation chromatography (GPC) employing Bio-Beads S-X3 became the most often applied clean-up method. Unfortunately, the complete separation of lipids from the common PAEs presented in sample extracts could not be achieved with any conceivable mobile phase. To solve this problem one of two approaches was applied: either conversion of PAEs into easily separable dimethyl phthalate[225,226] or the so called “Stable Isotope Dilution” approach in which the deuterated analogue of analyte is added to the sample and used for recovery calculation after the intentional narrowing of GPC fraction containing phthalates.[226-228]

Solid-phase microextraction in sample preparation became very popular in the late 1990s. The SPME procedure (sampling, extraction, concentration and sample introduction in one step) significantly reduces the risk of secondary contamination and

substantially simplifies the overall analytical process. Consequently, SPME seems to be an 'ideal' technique for phthalate determination. Several studies employing direct SPME for extraction of phthalates from water have been published.[229-232] These methods have used polyacrylate, Carbowaxdivinylbenzene or polydimethylsiloxane-divinylbenzene fibers.

The SPME technique can be applied not only in water analysis but also, providing that headspace sampling mode is used, in examination of complex liquid samples, such as human body fluids,[233-235] wine[236] or beer[237] and even solid matrices like fruit or vegetables.[238-240] Applications of SPME in food analysis were reviewed by Kataoka et al.[241]

Low volatility of target analytes may be a limiting factor in SPME headspace analysis. In this case, extraction at elevated temperature is recommended to increase the vapour pressure of analytes. However, the negative temperature effect on the coating-headspace partition coefficient of analytes in the system, and the consequent reduction of the extracted amount of analytes, should be considered. Mass transfer from a liquid sample to the headspace can also be enhanced by stirring of the sample. Whenever the matrix retains analytes strongly, a modification of the matrix should be considered to facilitate their transport from the matrix to the headspace. In the case of liquid samples, 'salting-out' and pH adjustment are the most commonly used techniques.[242-246] The addition of organic solvent or water to solid[247-249] or liquid matrices[233,244,250] has also been reported.

More recently a new solvent-free analytical procedure based on headspace solid-phase microextraction (SPME) coupled to gas chromatography employing an electron capture detector (GC/ECD) or alternatively a mass spectrometric detector (GC/MSD) has been developed for the determination of phthalic acid esters (dimethyl-[DMP], diethyl-[DEP], di-*n*-butyl-[DnBP], butylbenzyl-[BBP], di-2-ethylhexyl-[DEHP] and di-*n*-octyl [DnOP] phthalate) in vegetable oils.[251]

1.5.2 Pesticides

Pesticides are used widely in agriculture to maintain and increase crop yields, and they are also applied in homes and gardens. The annual application of synthetic pesticides to

food crops in the EU exceeds 140,000 tonnes.[252] Despite European policies to reduce pesticide use, EU statistics data for 1992–2003 show that the annual pesticide consumption has not decreased.[252] A few hundred different compounds are authorised for use in all EU member states, but a similar number of pesticides is in current use in different EU countries and are being evaluated for possible authorisation in all of EU.

Approximately 300 different pesticides have been reported as contaminants of food products of European origin.[253] Up to 50 percent of fruits, vegetables and cereals grown in the European Union are known to contain pesticide residues, but only a small fraction of pesticides in current use are included in the monitoring programs.

Nonetheless, one out of twenty food items is known to exceed a current EU legal limit for an individual pesticide.[253] Further, over 25% of fruits, vegetables, and cereals contain detectable residues of at least two pesticides. Processed food and baby food are also commonly contaminated. In addition, other sources, such as contaminated drinking water, dusts and spray drift contribute to human exposures. The total level of population exposures to pesticides in Europe is unknown, but data from US population studies show that the majority of the population has detectable concentrations of methyl phosphate, ethyl phosphate, and other pesticide metabolites in the urine.[254]

Many pesticides target the nervous system of insect pests. Because of the similarity of neurochemical processes, these compounds are also likely to be neurotoxic to humans. This concern is of particular relevance to the developing human brain, which is inherently much more vulnerable to injury caused by toxic agents than the brain of adults.[255] During prenatal life, the human brain must develop from the ectodermal cells of the embryo into a complex organ consisting of billions of precisely located, highly interconnected, and specialized cells. For optimum brain development neurons must move along precise pathways from their points of origin to their assigned locations, they must establish connections with other cells, and they must learn to communicate with other cells via these connections.[255-257] All of these processes have to take place within a tightly controlled time frame, and each developmental stage has to be reached on schedule and in the correct sequence. If a developmental process in the brain is halted or inhibited, there is little potential for later repair, and the consequences may therefore be permanent.[255,257]

Concerns in regard to developmental neurotoxicity due to pesticides have been fuelled by recent epidemiologic observations that children exposed prenatally or during early postnatal life suffer from various neurological deficits.[258,259] Urinary pesticide metabolite concentrations associated with adverse effects overlap with the ranges that occur in the general population.[254] Although the identity of the parent pesticides and the exact magnitude of causative exposures are unclear, these observations suggest that developmental neurotoxicity from pesticide exposure is a public health concern.

Despite the increasing recognition of the need to evaluate developmental neurotoxicity in safety assessment,[259-261] only very few of the commercial chemicals in current use have been examined with respect to neurodevelopmental effects.[262] Validated rodent models exist, but they are considered expensive and are only infrequently used. According to the current EU Plant Protection Directive (91-414-EEC), a neurotoxicity test in hens is required only for organophosphates and some carbamates to assess the possible risk of delayed peripheral neurotoxicity following acute exposure.

From a public health viewpoint, the prevention of neurodevelopmental disorders is a priority; these disorders include learning disabilities, attention deficit hyperactivity disorder (ADHD), autism spectrum disorders, developmental delays, and emotional and behavioural

problems. The causes of these disorders are unclear, and interacting genetic, environmental, and social factors are likely determinants of abnormal brain development.[263]

Medical statistics data are difficult to compare between countries, but one report suggests that 17% of US children under 18 years of age suffer from a developmental disability, in most cases affecting the nervous system.[264] In calculations of environmental burdens of disease in children, lead neurotoxicity to the developing brain is a major contributor.[265] Pesticide effects could well be of the same magnitude, or larger, depending on the exposure levels.

A recent review listed 201 chemicals known to be neurotoxic in humans; only 5 of these substances have been firmly documented as causes of developmental neurotoxicity.[262]

Identification of human neurotoxicity was based on available evidence, including poisoning incidents described in the scientific literature, as identified from the Hazardous Substances Data Bank of the U.S. National Library of Medicine. Although

published clinical information may not be representative for the relative neurotoxicity risks due to industrial chemicals, it is noteworthy that a total of 90 (45%) of the neurotoxic substances are pesticides. For these substances, only neurotoxicity in adults had been documented, thereby documenting that access to the brain is possible and may cause toxic effects. Given the vulnerability of the developing brain, it is likely that many of these substances will also be capable of causing developmental neurotoxicity. Indeed, studies in laboratory animals support the notion that a wide range of industrial chemicals can cause developmental neurotoxicity even at low doses that are not harmful to mature animals.[260,266]

In regard to developmental neurotoxicity in humans, in table 1.5.2 are listed the principal pesticides approved for current use in Europe, either authorised or being evaluated for authorization within the European Union.

Pesticide	Annex I status
Organophosphate insecticides	
Chlorpyrifos	In
Dimethoate	In
Ethoprophos	In
Phosmet	In
Fenamiphos (nematicide)	In
Carbamates	
Pirimicarb	In
Methomyl	Application resubmitted
Pyrethroid insecticides	
Cypermethrin (type II)	In
Deltamethrin (type II)	In
Pyrethrum/pyrethrin (natural pyrethrin)	Pending
Other insecticides	
Nicotine	Pending
Dithiocarbamate fungicides	
Maneb	In
Thiram	In
Chlorophenoxy herbicides	
2,4-D	In
Bipyridyl herbicides	
Diquat dibromide	In
Rodenticides	
Warfarin	In
Fumigants	
Phosphides (zinc, magnesium, and aluminum phosphides)	Pending
Sulfuryl fluoride	Pending

Table 1.5.2 Neurotoxic pesticides, which are authorised or pending evaluation for authorisation in the EU. The list includes pesticides, which are registered as "in" or "pending" on the current EU Annex 1 list (as of August 2008), and for which neurotoxicity in humans has been reported in The Hazardous Substances Data Bank and/or the NIOSH Pocket Guide to Chemical Hazards. The full Annex 1 list with the status of active substances under EU review can be downloaded as an Excel sheet at http://ec.europa.eu/food/plant/protection/pesticides/index_en.print.htm.

1.5.2.1 Organophosphate insecticides: Dimethoate

Organophosphorus (OP) compounds have been utilized as therapeutic agents, agricultural chemicals, plasticizers, lubricants, flame retardants, fuel additives, and, most notoriously, as chemical warfare agents, but their most widespread use is as pesticides for the control of insects in many different crops. With the restrictions on use of most of the persistent organochlorine insecticides imposed in the 1970s, the less persistent but highly effective OP agents became the insecticides of choice.

The primary target of organophosphate insecticides is the enzyme acetylcholinesterase (AChE), which hydrolyses the neurotransmitter acetylcholine in both the peripheral and the central nervous system. OPs containing P = O moiety are effective inhibitors of AChE, whereas OPs with a P = S moiety require bioactivation to form an "oxon" or oxygen analogue of the parent compound. Inhibition of AChE by OPs is obtained by the P = O moiety forming a covalent bond with the active site of the enzyme. The enzyme-inhibitor complex can become "aged" by a non-enzymatic hydrolysis of one of the two radical groups in the OP, and once the complex has aged, inhibition of AChE is irreversible.[267] Inhibition of AChE causes accumulation of acetylcholine at cholinergic synapses, leading to over-stimulation of muscarinic and nicotinic receptors. In addition, acetylcholine has important functions during brain development.[268]

In severe cases of OP poisoning in adults (AChE inhibition exceeding 70%), a "cholinergic syndrome" is elicited, including various central nervous system (CNS) effects such as headache, drowsiness, dizziness, confusion, blurred vision, slurred speech, ataxia, coma, convulsions and block of respiratory centre.[269,270] Some OPs can also induce a delayed neuropathy which does not involve inhibition of AChE but rather the neuropathy target esterase (NTE).[271,272] The physiological functions of NTE are still unknown, and it is obscure how phosphorylation and aging of NTE leads to axonal degeneration.[273]

The syndromes described above are observed only following high dose, acute exposures to OPs. Survivors recover from these syndromes, but it is likely that the exposure also produces long-term adverse health effects. In rats, a single high exposure to an OP can cause long lasting behavioural effects, and the same has been reported from several human studies.[274,275]

The concern is growing that also chronic, low exposures to OPs may produce neurological effects, although the evidence remains somewhat equivocal. Most studies have found an association of OP exposure with increased neurological symptom prevalence.[276-278]

Acetylcholine and other neurotransmitters play unique trophic roles in the development of the CNS,[279] and inhibition of AChE by OPs and the resulting accumulation of acetylcholine may then conceivably disturb this development. Still, developing rats recover faster from AChE inhibition than adults, largely due to the fact that developing organisms have a rapid synthesis of new AChE molecules.[280-281] It therefore seems that either developmental toxicity may be unrelated to AChE inhibition, or that even a brief period of AChE inhibition is sufficient to disrupt development.[282]

Dimethoate is an organophosphate insecticide used to kill mites and insects systemically and on contact. It is used against a wide range of insects, including aphids, thrips, plant hoppers, and whiteflies on ornamental plants, alfalfa, apples, corn, cotton, grapefruit, grapes, lemons, melons, oranges, pears, pecans, safflower, sorghum, soybeans, tangerines, tobacco, tomatoes, olives, potatoes, watermelons, wheat, and other vegetables. It is also used as a residual wall spray in farm buildings for house flies. Dimethoate has been administered to livestock for control of botflies.

Not only is dimethoate a commonly used agricultural insecticide and acaricide, but it is also listed (Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) priority list of hazardous substances) as one of the contaminants at hazardous waste sites that pose the most significant potential threat to human health (United States Department of Health and Human Services 1997).[283] In fact, investigators have shown that repeated exposure to dimethoate decreases serum testosterone levels, testicular weight, and sperm motility and increases the percentage of dead and abnormal sperm in rats and rabbits (Salem *et al.* 1988, Afifi *et al.* 1991).[284] Moreover, it accumulates in the testes where it persists for weeks even after its oral administration is stopped (Afifi *et al.* 1991).[285] Since spermatogenesis and fertility are critically dependent upon the maintenance of adequate levels of testosterone, the ability of dimethoate to reduce serum testosterone levels might contribute to the reduction in spermatogenesis and fertility observed in animals exposed to this pesticide.

1.5.2.1.1.Toxicological effects of dimethoate:

- **Acute toxicity**

Dimethoate (figure 1.5.2) is moderately toxic by ingestion, inhalation and dermal absorption. As with all organophosphates, dimethoate is readily absorbed through the skin. Skin which has come in contact with this material should be washed immediately with soap and water and all contaminated clothing should be removed. Organophosphates are easily absorbed through the lungs. Persons with respiratory ailments, recent exposure to cholinesterase inhibitors, impaired cholinesterase production, or with liver malfunction may be at increased risk from exposure to dimethoate.

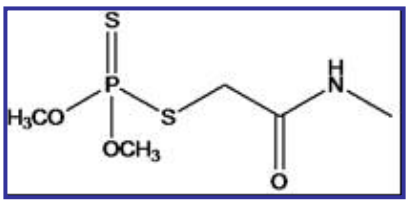

Figure 3.4.1. Structure of dimethoate.

As a cholinesterase inhibitor, it is highly toxic by all routes of exposure. When inhaled, the first effects are usually respiratory and may include bloody or runny nose, coughing, chest discomfort, difficult or short breath, and wheezing due to constriction or excess fluid in the bronchial tubes. Skin contact with organophosphates in general, may cause localized sweating and involuntary muscle contractions. Eye contact will cause pain, bleeding, tears, pupil constriction, and blurred vision. Following exposure by any route, other systemic effects may begin within a few minutes or be delayed for up to 12 hours. These may include pallor, nausea, vomiting, diarrhea, abdominal cramps, headache, dizziness, eye pain, blurred vision, constriction or dilation of the eye pupils, tears, salivation, sweating, and confusion. Severe poisoning will affect the central nervous system, producing incoordination, slurred speech, loss of reflexes, weakness, fatigue, involuntary muscle contractions, twitching, tremors of the tongue or eyelids, and eventually paralysis of the body extremities and the respiratory muscles. In severe cases there may also be involuntary defecation or urination, psychosis, irregular heart beats,

unconsciousness, convulsions and coma. Death may be caused by respiratory failure or cardiac arrest.[286]

Some organophosphates may cause delayed symptoms beginning 1 to 4 weeks after an acute exposure which may or may not have produced immediate symptoms. In such cases, numbness, tingling, weakness and cramping may appear in the lower limbs and progress to incoordination and paralysis. Improvement may occur over months or years, but some residual impairment will remain.[286]

v **Chronic toxicity**

There was no cholinesterase inhibition in an adult human who ingested 18 mg (about 0.26 mg/kg/day) of dimethoate/day for 21 days. No toxic effects and no cholinesterase inhibition were observed in individuals who ingested 2.5 mg/day (about 0.04 mg/kg/day) for 4 weeks. In another study with humans given oral doses of 5, 15, 30, 45 or 60 mg/day for 57 days, cholinesterase inhibition was observed only in the 30 mg/day or higher dosage groups.[287] In many scientific experiments it showed different toxicological effects: reproductive effects, teratogenic effects, mutagenic effects and carcinogenic effects.

Studies on the carcinogenicity of the insecticide dimethoate in animals were reviewed. Examination of histological sections showed that dimethoate is highly carcinogenic in Osborne-Mendel rats.[288] Neoplasms at all sites, as well as malignant neoplasms, were increased in both low and high doses of dimethoate-treated male rats in the National Cancer Institute study. The malignant neoplasms were both carcinomas and sarcomas. Neoplasms of the endocrine organs, particularly carcinomas, were increased in male and female rats given dimethoate. These carcinomas were observed in the adrenal, thyroid, and pituitary glands. Neoplasms were also increased in the liver of male and female rats and in the reproductive organs of female rats given dimethoate. Male and female rats treated with dimethoate developed monocytic leukemia. There also were toxic changes in rats. Male rats had atrophy of the testes, chronic renal disease, parathyroid hyperplasia, and polyarteritis. Wistar male and female rats given dimethoate by gavage or intramuscularly developed a significant increase in malignant neoplasms, mainly sarcomas, and granulocytic leukemia.

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Chapter 2

Mass spectrometry

2.1 Hyphenated separation techniques

The coupling of chromatography with mass spectrometry is an area that has served a critical need in the analysis of real-world samples, such as environmental samples, food samples and biological tissues and fluids. In fact, these samples are complex by nature, as they are made up of hundreds and thousands of components.

Unequivocal characterization of such complex samples is a monumental task for any stand-alone analytical technique. One needs either infinite separation capability of a separation system or infinite selectivity of an identification method. One solution is to subject the sample to an exhaustive purification protocol to isolate the analyte of interest from a complex real-world mixture. Such off-line purification procedures are, however, laborious and time consuming and entail losses of precious samples. A more sensible approach is to combine a separation technique with a compound-specific detector that can identify the separated components.

Mass spectrometry offers an opportunity for such an ideal detection system. A range of separation techniques, including gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), supercritical-fluid chromatography (SFC), size-exclusion chromatography, and thin-layer chromatography (TLC), has been combined with mass spectrometry.

2.1.1 Benefits of coupling separation devices with mass spectrometry

The role of GC, HPLC, and CE in high-resolution separation of complex mixtures is unquestionable. Similarly, mass spectrometry has attained an indisputable position in analytical chemistry as a highly structure-specific technique that can provide structural identity of a wide range of compounds. Chromatography and mass spectrometry both, however, have their limitations in stand-alone operation.

First, the separation power of any chromatography system is finite. It will be nearly impossible to achieve complete separation of all components of a complex mixture. Second, identification of a compound in chromatography is less than reliable because of marginal information content. The basis of identification of the target analyte in chromatography is comparison of its retention time with that of a reference material. Therefore, the common separation techniques of GC, LC, and CE cannot provide

unequivocal identity of the analyte when used with conventional detection systems. Uncertainty may arise because another component of the mixture may elute at the desired retention time. A compound-specific detector is thus an essential adjunct to characterize unambiguously the components that elute from any separation system. In this respect, mass spectrometry offers the unique advantages of high molecular specificity, detection sensitivity and dynamic range. Only mass spectrometry has the ability to provide confirmatory evidence of an analyte because of its ability to distinguish closely related compounds on the basis of the molecular mass and structure-specific fragment ion information. The confidence in identification of a target compound, however, diminishes when it is present in a mixture. Because of the universal nature of mass spectrometry detection, the data obtained might also contain signal due to other components of the mixture. The coupling of a separation device with mass spectrometry thus benefits mutually. The result is a powerful two-dimensional analysis approach, where the high-resolution separation and the highly sensitive and structure-specific detection are both realized simultaneously. Following are some of the benefits that accrue when a separation technique and mass spectrometry are coupled.

- The capabilities of the techniques are enhanced synergistically. As a consequence, both instruments may be operated at subpar performance levels without compromising the data outcome.
- The high selectivity of mass spectrometry detection allows one to identify coeluting components.
- The certainty of identification is enhanced further because, in addition to the structure-specific mass spectral data, the chromatographic retention time is also known.
- Multicomponent samples can be analyzed directly without prior laborious off-line separation steps, resulting in a minimal sample loss and saving of time.
- The sensitivity of analysis is improved because the sample enters the mass spectrometer in the form of a narrow focused band.
- Less sample is required than the amount required for off-line analysis by the two techniques separately.
- Because of the removal of interferences, the quality of mass spectral data is improved and any mutual signal suppression is minimized.

- The confidence in quantitative analysis is increased because mass spectrometry permits the use of a stable isotope analog of the analyte as an internal standard.

2.1.2 Characteristics of an Interface

A chromatography–mass spectrometry system consists of three major components: a separation device, an interface, and a mass spectrometer (Figure 2.1.1).
The purpose of an interface is to transport the separated components into the ion source of a mass spectrometer for their identification. An ideal interface should not affect the performance capabilities of either the chromatography or the mass spectrometry system. Other important characteristics of an interface are that it should have a high sample transport efficiency, should not alter the chemical identity of the analyte, should be mechanically simple and chemically inert and have a low chemical background, should have the ability to remove most of the mobile phase while transferring the maximum amount of eluted component, and should not degrade the ionizing efficiency and vacuum of the mass spectrometry system.

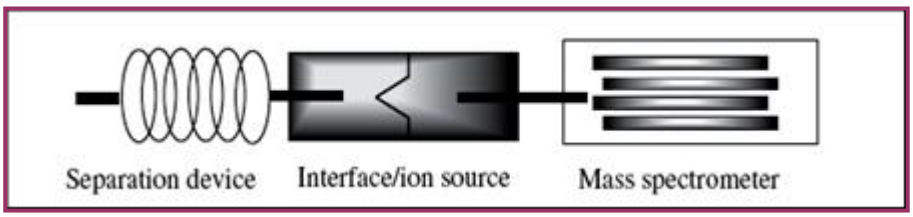

Figure 2.1.1 Conceptual diagram of the coupling of a separation device with a mass spectrometer.

2.1.3 Mass Spectral Data Acquisition

The determination of molecular mass, identification of compounds, and quantification are three broad areas of application of a combined chromatography–mass spectrometry system. To identify the eluting components and to determine their molecular mass, the mass spectrometry data are recorded by scanning a mass analyzer over a wide mass range, with the upper limit dictated by the highest expected molecular mass of the analyte and the lower limit by the background ions from the chromatography mobile phase. The mass spectrometry signal is plotted as an ion chromatogram or mass spectrum.

Three different types of ion chromatograms can be retrieved from the mass spectrometry data:

1. A *total ion current* (TIC) *chromatogram* is a plot of the sum of the signals due to all observed ions in a defined mass range versus either the scan number or time. A peak in this chromatogram indicates the elution of a component. This trace is similar to a conventional UV chromatogram from an LC analysis and can be used to identify compounds provisionally on the basis of their retention times. A mass spectrum can be retrieved for any chosen scan number in the TIC chromatogram to provide qualitative identification of a compound.
2. A *mass chromatogram*, also known by various other names, such as *ion-extraction chromatogram and reconstructed ion chromatogram*, is a plot of the ion current due to a characteristic ion versus either the scan number or time. This chromatogram is generated postacquisition from the TIC trace. It is a useful means to distinguish coeluting components and to identify a homologous series of compounds.
3. For quantitative measurements, a *selected-ion monitoring* (SIM) *chromatogram* is acquired by recording the ion signal due to only one or more compound-specific ions. This trace is also useful to selectively detect a homologous series of compounds. For quantification, the area or height of the analyte peak is compared with that of the internal standard.

The quantitative information can also be obtained from a mass chromatogram. It should, however, be remembered that although a mass chromatogram and an SIM chromatogram are identical in terms of selectivity, the latter provides much higher sensitivity, owing to the increased time spent monitoring each ion selected.

Signal acquisition from a chromatography system is challenging because the volume of data is enormous and the peak width is often very narrow. In addition, the partial pressure of the sample that enters the mass spectrometry ion source changes continuously. Therefore, a rapid scanning mass spectrometry system is very essential so that no useful data point will be missed during the scan.

Acquisition of required data points ensures that there is no distortion in the peak intensities and that the chromatographic peak profile is not skewed (Figure 2.1.2). It also ensures that complete information is obtained for all coeluting components in a narrow peak.

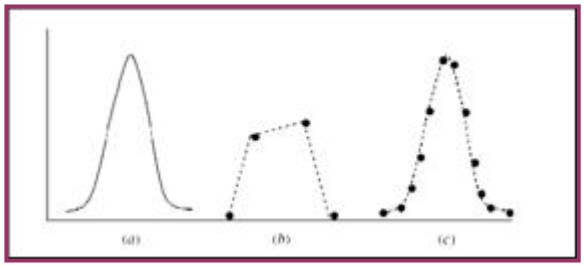

Figure 2.1.2 (*a*) Ideal chromatographic peak; (*b*) peak with insufficient data points; (*c*) peak with large and nearly sufficient data points. This peak profile nearly matches an ideal peak.

The specificity of mass spectrometry data is enhanced further when a spectrum is acquired in the tandem MS (MS/MS) mode. With the current mass spectrometry data systems, it has become feasible to acquire MS/MS spectra during acquisition of the TIC trace.

2.2 Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry was first used in the 1950s.[1] Since then it has acquired an enviable status as a modern analytical technique for the sensitive, high-resolution, and selective identification and quantification of a wide range of compounds. Its use is mentioned frequently in the fields of forensic drug analysis, structural characterization of biomolecules, toxicology, medicinal chemistry, clinical science, and environmental chemistry. GC/MS is considered a standard technique for positive identification of the presence of a target compound. Methods based on the GC/MS protocol are readily acceptable in medical–legal defense. GC/MS is, however, restricted to relatively volatile and thermally stable organic compounds. Some non volatile compounds can be made volatile at the temperatures used for separation after chemical derivatization.

2.2.1 Basic Principles of Gas Chromatography

In gas chromatography, a sample is vaporized in a heated injector block and deposited onto the head of a chromatographic column that contains a nonvolatile liquid stationary phase. The components of a mixture are separated on the basis of their varying affinity for, and solubility in, the stationary phase. Elution of the separated components is effected by the flow of an inert carrier gas (usually, helium). It is a usual practice to perform GC separations at elevated temperatures (150 to 300•C) to bring many not-so-volatile, but

thermally stable compounds under its domain. In early applications, GC analysis was carried out on wide-bore (1 to 3 mm i.d.) packed columns. Modern analytical GC is practiced with more efficient, faster, and longer capillary columns. The inside wall of these fused-silica open-tubular (FSOT) columns is coated with a thin layer of a stationary phase, usually an organosiloxane polymer. Polydimethyl siloxane, •[•O•Si(CH3)2•]*n*•, is a general-purpose nonpolar stationary phase. The replacement of the methyl groups with polar functional groups, such as phenyl, trifluoropropyl, or cyanopropyl, increases the polarity of this phase. Polyethylene glycol is another common polar stationary phase. GC is commonly practiced with flame ionization, electron capture, thermionic, and thermal conductivity detectors; all of them lack the ability to provide the chemical composition or structure of the separated components.

2.2.2 Interfaces for Coupling Gas Chromatography with Mass Spectrometry

The first coupling of GC was demonstrated with a TOF mass spectrometer. Gas chromatography and mass spectrometry are both highly compatible with respect to the sample size, and both deal with the gaseous samples. The only incompatibility between the two systems is the pressure mismatch. For example, the separated components exit from the GC column at atmospheric pressure, whereas the mass spectrometry source operates at 10^{-6} to 10^{-5} torr.

This mismatch is not a serious handicap in the coupling of capillary columns with mass spectrometry. The low gas flow rates (e.g., in the range 1 to 2 mLmin^{-1}) of capillary columns can readily be accommodated by the vacuum system of modern mass spectrometers; no interface is necessary for columns up to 320 •m internal diameter (i.d.); the exit end of these columns is simply inserted into the ion source through a heated sheath (Figure 2.2.1 *a*). The high-capacity pumping systems of modern mass spectrometers can effectively pump away the extra gas load from a capillary column.

Because of the greater gas load (e.g., 10 to 20 mL/min) of larger-diameter open-tubular and packed columns, their coupling requires an appropriate interface.

One such device is an *open-split interface*.[2] It consists of a chamber in which a high flow of the purge gas is maintained at atmospheric pressure (see Figure 2.2.1 *b*). The purge gas

removes most of the carrier gas from the GC effluent and allows only a small fraction to enter the mass spectrometer through a capillary tube.

Another common interface for packed columns is a *jet separator*, shown in Figure 5.4*c*. It consists of a partially evacuated chamber in which a jet of GC effluent is sprayed through a small nozzle.[3] It operates on the principle that the fast-moving lighter-mass components effuse at a faster rate than do the slower heavier-mass species (solid circles in Figure 2.2.1 *c*). Thus, the lighter carrier-gas molecules effuse faster at a wider angle, whereas the heavier sample components travel over a narrow angle around the central axis. The analyte-enriched middle portion is sampled into the ion source of the mass spectrometer through a skimmer placed opposite the emerging jet.

A *molecular-effusion interface* (also known as a *Watson–Biemann interface*) is made of a glass-frit tube that is placed in an evacuated chamber (Figure 2.2.1 *d*). One end of this tube is connected to the GC column and the other to the ion source of a mass spectrometer.[4] The GC effluent is preferentially enriched in the sample molecules by the molecular effusion process; the lighter gas molecules effuse faster through the pores of the fritted tube and are pumped away, whereas the enriched sample molecules enter the ion source. An interface using a *silicon-rubber membrane* for enrichment of the solute molecules has also found applications.[5] This membrane allows nonpolar organic molecules to pass through it selectively, and blocks the passage of the carrier gas.

Electron ionization (EI) and chemical ionization (CI) ion sources are both compatible with the gas-phase GC-eluting components. EI especially provides both molecular ion and fragment ion information that can be used independently or compared with a reference mass spectrum in a spectral library to identify known and unknown compounds. Commercially marketed libraries are available that contain over 100,000 spectra.

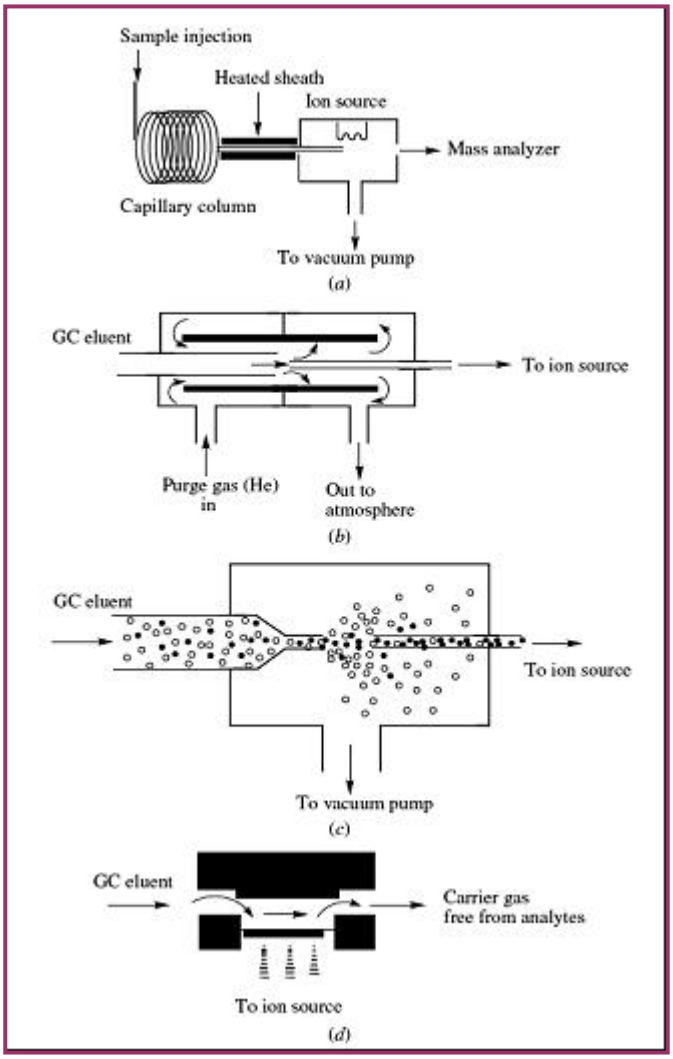

Figure 2.2.1. Various forms of GC/MS interfaces: (*a*) direct coupling of capillary columns with MS; (*b*) open-split interface; (*c*) jet-separator interface; (*d*) molecular-effusion interface.

2.3 Liquid chromatography/mass spectrometry

More than 85% of compounds in nature fall into the category of being polar and thermally labile, which are not amenable to GC/MS. In the past, a strong need for a similar combination of HPLC and MS was felt for the analysis of such compounds.

Although attempts to combine LC with MS were initiated over 30 years ago, a robust and dependable combination that could be adapted for a wide range of applications has emerged

only after the discovery of atmospheric-pressure ionization techniques. Currently, the practice of LC/MS arguably has become the single most widely used analytical technique. A variety of applications has been envisioned for both qualitative and quantitative analysis of complex mixtures of biochemical, inorganic, and organic compounds. HPLC/MS has become the mainstay of proteomics and pharmaceutical laboratories.

2.3.1 Basic Principles of HPLC Separation

HPLC is practiced in the following formats:

- *Adsorption chromatography*, in which the stationary phase consists of a high-surface-area solid adsorbent; the solutes physically adsorb on the stationary phase, while the liquid mobile phase tries to dislodge them.
- *Partition chromatography* involves distribution of the solutes between a liquid stationary phase and a liquid mobile phase.
- *Ion-exchange chromatography*, used for ionic solutes, involves competition for solute ions between immobilized ionic sites on the stationary phase (e.g., ion-exchange resin) and liquid mobile phase.
- *Size-exclusion chromatography* separates solutes on the basis of their size. The stationary phase consists of a polymer matrix of various pore sizes. Solutes are separated according to their ability to penetrate the polymer matrix. The small solutes penetrate more and lag behind the larger solutes.
- *Affinity chromatography* involves specific interaction between one type of solute molecule and a second, complementary type of molecule that is immobilized on a solid support.

Of these, partition chromatography, especially the *reversed-phase (RP) mode*, is the most widely used technique for the separation of several classes of compounds.

In RP–HPLC , the stationary phase is a nonpolar matrix and the mobile phase is a polar solvent (e.g., water mixed with a polar organic modifier such as methanol, isopropanol, or acetonitrile). Mobile phase and stationary phase both play prominent roles in the separation mechanism. A sample is applied onto the head of a column filled with an appropriate stationary phase. The components of a mixture are partitioned differentially between the two phases and are separated when eluted by a stream of a liquid mobile phase. Two common modes of elution are *isocratic*, in which the solvent composition remains constant

during elution, and *gradient*, in which the solvent composition is changed either in steps or in a continuous fashion during elution.

The basis of *RP–HPLC separation* is the hydrophobic interaction between the analyte and the nonpolar matrix of the stationary phase. A typical stationary phase is prepared by chemically bonding a long-chain alkyl group, such as *n*-octadecyl (*C*18) to porous silica. The smaller alkyl groups, such as *n*-octyl, *n*-butyl, and *n*-ethyl, can also be used for specific applications. In practice, the nonpolar, hydrophobic solutes interact strongly with the stationary phase. In contrast, relatively hydrophilic compounds spend more time in the polar aqueous mobile phase and thus elute earlier. Increasing the strength of the eluting solvent enables elution of the more strongly retained hydrophobic solutes. The RP–HPLC separation is further fine-tuned by use of appropriate buffers and ion-pairing reagents in the mobile phase. Although a large number of buffer systems has been used in a conventional ultraviolet–visible detection system, volatile ion-pairing reagents work best for LC/MS operation.

Partition chromatography is also practiced in the *normal-phase mode*, in which the stationary phase is a polar matrix and the mobile phase at the start of the separation is a polar solvent. The polar solutes prefer to remain in the stationary phase and elute late.

2.3.2 Fast-Flow Liquid Chromatography

An important area of research in LC/MS is to reduce the analysis time. The driving force for the development of high-speed separations is the need for high-throughput analysis; such a development can especially speed up the drug discovery process. Several imaginative attempts have been made to develop fast LC. One approach is to use short LC columns (typically, <50 mm long) that are packed with small particles (e.g., these with 3-•m diameter).[6,7] A decrease in the analysis time by a factor of about 7.5 is realized when the column length is reduced from 250 mm to 33 mm. The small stationary-phase packing also shortens the analysis time because of the inverse relation between the optimal linear mobile-phase velocity and the particle diameter.

Another attempt to perform high-speed separation is to use ultrahigh or turbulent flows (over 30 mL/min).[8] At turbulent flow rates, the solvent front profile is plug-like rather than the usual parabolic. Therefore, a considerable improvement in column efficiency is achieved. These prohibitive ultrahigh flow rates are, however, possible when columns are packed with particles over 50 •m.

High-speed chromatography can also be accomplished by the use of special packing materials. Three different types of stationary-phase packing—perfusion, nonporous, and monolithic-have provided fast flows through the LC columns. The perfusion packing is made of polymer particles with a network of larger through-pores (e.g., 6000 to 8000 • in diameter) and small diffusive pores (300 to 1500 •). The through-pores allow 10 to 20-fold faster flow of a mobile phase through the packing, resulting in increased mass transfer rates and consequently, increased separation efficiency. The nonporous silica packing is made from small (e.g., 1.5 •m in diameter) silica or resin particles. With this type of packing, stagnant flow, one of the major causes of band broadening, is eliminated to increase mass transfer rates. Small particle packing, however, results in higher back pressures. Therefore, short columns 2 to 3 cm in length must be used for fast separations. Monolithic columns contain a continuous mass of porous silica rather than conventional particle packing. The silica skeleton of this packing contains two different types of pores, macropores and mesopores, giving lower pressure drops and higher mass transfer rates. Because of low-pressure drops, fast separations are easy to achieve with monolithic columns. Several examples of coupling of these columns with ESI–MS can be found in the literature.[9,10]

Ultraperformance Liquid Chromatography (UPLC) This mode of LC is performed with columns that are packed with very small particles (ca. 1.7 •m in diameter).[11,12] The benefits of these columns are high-resolution separation at exceedingly large theoretical plates. Small-particle packing creates operating pressures that are in the range 6000 to 15,000 psi and requires special pumping system that is capable of operating at such high pressures.

2.3.3 Interfaces for coupling liquid chromatography with mass spectrometry

The coupling of LC with mass spectrometry is not as straightforward as a similar combination of GC with MS. There are several fundamental differences in the operating environment of HPLC and mass spectrometry. The first mismatch is the solvent flow rate. The separation in conventional wide-bore analytical columns is accomplished at liquid flow rates of 0.5 to 1.5 mL/min. Unlike GC/MS, this liquid produces a gas flow too large for safe operation of the mass spectrometry vacuum system ($10^{\bullet 5}$ to $10^{\bullet 8}$ torr). For example, 1.0 mL of water will produce about 1.0 × 105 m^3 of gas load when introduced in a mass spectrometer at $10^{\bullet 5}$ torr pressure (see Example 5.3). Liquid flow rates below 10 •L/min

can be accepted safely by a mass spectrometry system. Another problem is the incompatibility of common HPLC solvents and nonvolatile additives with mass spectrometry operation. The third problem in the early years of LC/MS efforts was unavailability of ionization methods that could be used for thermally labile and nonvolatile compounds.

2.3.3.1 Electrospray Ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces

With the development of an atmospheric-pressure ionization (API) source, coupling of LC with MS has become a routine matter. The ESI format of API is the most appropriate interface for the LC/MS combination thanks to:

- its potential for the analysis of a variety of nonvolatile and thermally labile molecules of low to very high molecular mass at unprecedented low detection sensitivity,
- the ionization occurs at atmospheric pressure,
- its compatibility with RP–LC solvents,
- a range of solvent flow can be accepted.

As a consequence, the LC/ESI–MS combination has gained prominence in several areas of research, such as to sequence proteins; to identify mixtures of compounds, tryptic maps, and posttranslational modifications in proteins; to elucidate structure of metabolic products; to analyze drugs, pesticides, and toxins; and to screen combinatorial libraries. The development of LC/ESI–MS has also greatly advanced the science of quantification. Several reviews of LC/ESI–MS technology have appeared in the literature.[13-16]

The composition and flow rate of the solvent are two variables that are paramount for optimum operation of the ESI system. The flow rate determines the size as well as the size distribution of the droplets formed during ESI. A conventional ESI source operates at a flow rate of 1 to10 •L/min. At higher flow rates, the spray is not stable because of the formation of larger droplets, which lead to electrical breakdown. Similarly, a fluid with high surface tension, such as pure water, is difficult to electrospray, but many polar solvents commonly used in RP–HPLC (e.g., methanol, ethanol, isopropanol, and acetonitrile) are suitable for the electrospray operation. Nonpolar solvents are difficult to disperse; therefore,

normal-phase HPLC is not easy to implement with the ESI process unless a polar solvent is admixed with the nonpolar mobile phase.

HPLC is performed with various size columns that range from 0.1 to 4.6 mm in i.d. The common types of columns and their characteristics are given in table 2.3.1.

LC mode	Packing materials	Mobile phase	Interaction
Normal phase	Silica gel	n-Hexane/IPE	Adsorbtion
Reversed phase	Silica C-18	MeOH/Water	Hydrofobic
Size exclusion	Porous polymer	THF	Gel permeation
Ion excange	Ion excange gel	Buffer sol.	Ion excange
Affinity	Packings with ligand	Buffer sol.	Affinity

Table 2.3.1 The common types of columns and their characteristics.

The electrospray process involves the creation of a fine aerosol of highly charged micro droplets in a strong electric field. Electrospray as an ionization technique for mass spectrometry was developed by Dole and co-workers in the late 1960s[17] and considerably improved upon by Yamashita and Fenn who in 1984 coupled an electrospray source to a quadrupole mass analyser.[18] A continuous flow of solution containing the analyte from a highly charged (2–5 kV) capillary generates an electrospray. The solution elutes from the capillary into a chamber at atmospheric pressure, producing a fine spray of highly charged droplets due to the presence of the electric field (figure 2.3.1), a process called nebulisation. A combination of thermal and pneumatic means is used to desolvate the ions as they enter the ion source.

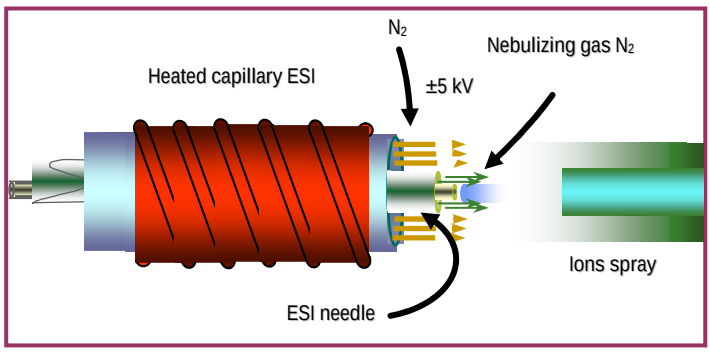

Figure 2.3.1 Electrospray ionization (ESI) interface.

The solvent contained in the droplets is evaporated by a warm counter-flow of nitrogen gas until the charge density increases to a point at which the repulsion becomes of the same order as the surface tension. The droplet then may fragment in what is termed a 'Coulomb explosion', producing many daughter droplets that undergo the same process, ultimately resulting in bare analyte ions (figure 2.3.2).

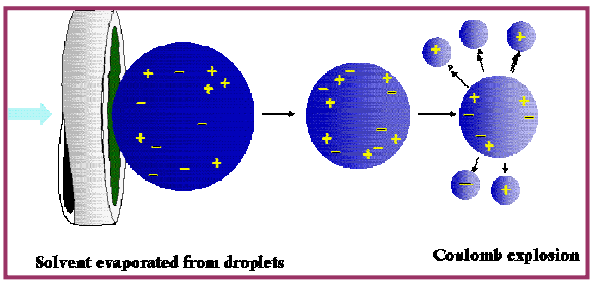

Figure 2.3.2. The desolvation process.

An alternative picture is one in which the ions 'evaporate' from the surface of the droplet. Whatever the exact mechanism, ESI is a very 'soft' means of ionization that causes little or no fragmentation of the sample.[19,20] The electrospray ion source is at very high pressure (atmospheric) with respect to the very low pressure that is required for ion separation by a mass analyzer, so the interface between the two involve a series of skimmer cones (acting as small orifices) between the various differentially pumped regions (figure 2.3.3). Early designs had the capillary exit pointing directly into the mass analyzer but to limit contamination practically all modern designs have an orthogonal (or at least off-axis) spray direction.

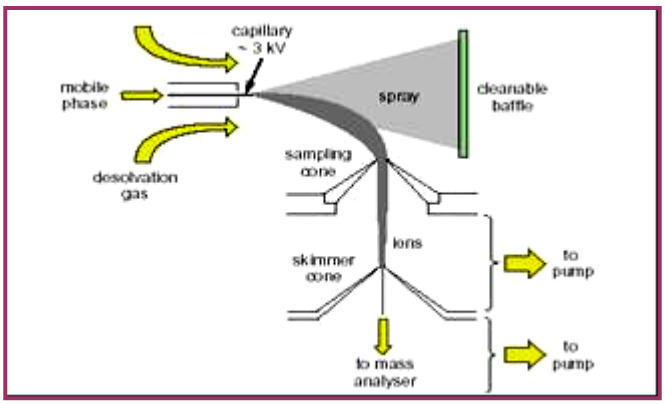

Figure 2.3.3. An electrospray source.

The ions are drawn into the spectrometer proper through the skimmer cones. A voltage can be applied (the cone voltage), which will accelerate the ions relative to the neutral gas molecules. This leads to energetic ion-neutral collisions and fragmentation due to what is termed collision induced dissociation (CID).[21] The remaining bath gas is pumped away in stages (in order to attain the high vacuum necessary for separation of the ions) and the ions are focused through a lensing system into the mass analyzers.

The appearance of multiply charged species enables ESI to characterize compounds whose molecular weight would otherwise be far in excess of that accessible to most mass analysers.[22,23] Biological macromolecules tend to accumulate one unit of charge for every 1 – 2000 Da, so nearly all proteins, for example, produce signals in the region of 1 – 2000 m/z, regardless of their actual molecular weight. Ionization of a neutral analyte often occurs by protonation, or alternatively cationisation, with an adventitious cation present in the solvent used, such as Na^+, K^+ or NH_4^+. In some cases, adduct ions with several cations can occur, giving $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$ and $[M + K]^+$ ions. Atmospheric-pressure chemical ionization (APCI)[24,25] is another of the techniques in which the stream of liquid emerging from an HPLC column is dispersed into small droplets, in this case by the combination of heat and a nebulizing gas. In this case the liquid flow is passed through a pneumatic nebulizer where the droplets are both generated and desolvated. The successive neutral dried spray, obtained by a heated region, passed through a corona discharge were the analyte are ionized. The mechanism is a chemical ionization but an atmospheric pressure were is necessary, for initial gas ionization, utilized a corona discharge(fig. 2.3.4).

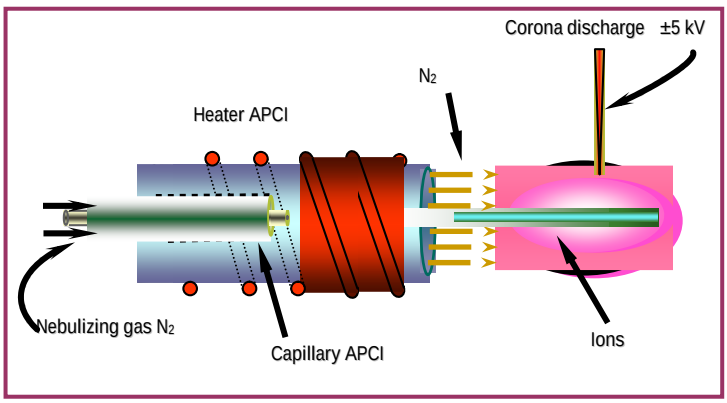

Figure 2.3.4. Atmospheric pressure chemical ionization (APCI) interfaces.

Then, the ions produced by the interaction of the electrons with the surrounding gas, undergo a number of reactions leading to the generation of reactive ions which interact with the analyte molecules present.

The reagent species in the positive-ion mode may be considered to be protonated solvent ions, and in the negative ion mode O_2^-, its hydrates and clusters. Is also possible the formation of cluster involving solvent molecules which can removed with use a "curtain gas". Finally, this technique can be applied both volatile and thermally stable and moderate polar compounds but the ionization regime is much more harsh that ESI and this precludes its use for the study of large biomolecules, with the mass limit for APCI being generally considered as below 2000 Da.

2.4 Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is a technique where structural information on sample molecules is obtained by using multiple stages of mass selection and mass separation.

A prerequisite is that the sample molecules can be transferred into gas phase and ionized intact and that they can be induced to fall apart in some predictable and controllable fashion after the first mass selection step. Multistage MS/MS, or MSn, can be performed by first selecting and isolating a precursor ion (MS2), fragmenting it, isolating a primary fragment ion (MS3), fragmenting it, isolating a secondary fragment (MS4), and so on as long as you can obtain meaningful information or the fragment ion signal is detectable. A variety of imaginative modes of tandem MS is described in the literature, but the four most common are precursor ion mode, product ion mode (also referred to as multiple reaction monitoring, MRM), neutral loss mode, and single reaction monitoring (Table 2.4.1 and Figure 2.4.1).

Mode	Purpose	Experiment
Product ion	To get structural information on ions produced in the ion source	MS1 selects one precursor ion, MS2 acquires a full mass spectrum of the fragment ions produced
Precursor ion	To find compounds that produce a common fragment	MS1 is scanning all precursor ions, MS2 selects one fragment ion
Neutral loss	To find compounds that lose a common neutral species	MS1 and MS2 are both scanning at a fixed m/z difference
Selected reaction monitoring	To monitor a selected reaction	MS1 selects one precursor ion, MS2 selects one fragment ion

Table 2.4.1. The Standard Tandem MS Modes.

1. The *product-ion scan* (the old, now-unaccepted term, still used by some, is *daughter-ion scan*) is the most common mode of MS/MS operation. That spectrum is useful in the structure elucidation of a specified analyte. Information obtained in this scan is similar to that derived from a normal mass spectrum, except that the spectrum contains only those product ions that are formed exclusively from a mass-selected precursor ion. To acquire this spectrum, the first mass analyzer is set to transmit only the precursor ion chosen, and the second mass spectrometer is scanned over a required *m*/*z* range.
2. Another popular MS/MS scan is the *precursor-ion scan* (the past, now unaccepted term is *parent-ion scan*). It provides a spectrum of all precursor ions that might fragment to a common, diagnostic product ion. The spectrum is obtained by adjusting the second mass spectrometer to transmit a chosen product ion (e.g., *m*1) and scanning the first mass analyzer over a certain *m*/*z* range to transmit only those precursor ions that fragment to yield the chosen product ion. This scan is useful for the identification of a closely related class of compounds in a mixture.
3. In a *constant-neutral-loss scan*, all precursors that undergo the loss of a specified common neutral are monitored. To obtain this information, both mass analyzers are scanned simultaneously, but with a mass offset that correlates with the mass of the specified neutral. Similar to the precursor ion scan, this technique is also useful in the selective identification of closely related class of compounds in a mixture.
4. The fourth scan, *selected-reaction monitoring* (SRM), is useful in combination with online chromatographic separation to quantify a specific compound in a complex matrix. Frequently three degrees of separation: elution time, precursor m/z, and fragment m/z are necessary to obtain an unambiguous and quantifiable signal. Conceptually, this scan mode is similar to the product-ion scan. However, instead of scanning the second mass spectrometer in a broad mass range, the two mass analyzers are adjusted to monitor one or more chosen precursor–product pairs of the analyte. This operation is identical to the selected-ion monitoring mode of data acquisition. Monitoring more than one reaction is termed *multiple-reaction monitoring* (MRM).

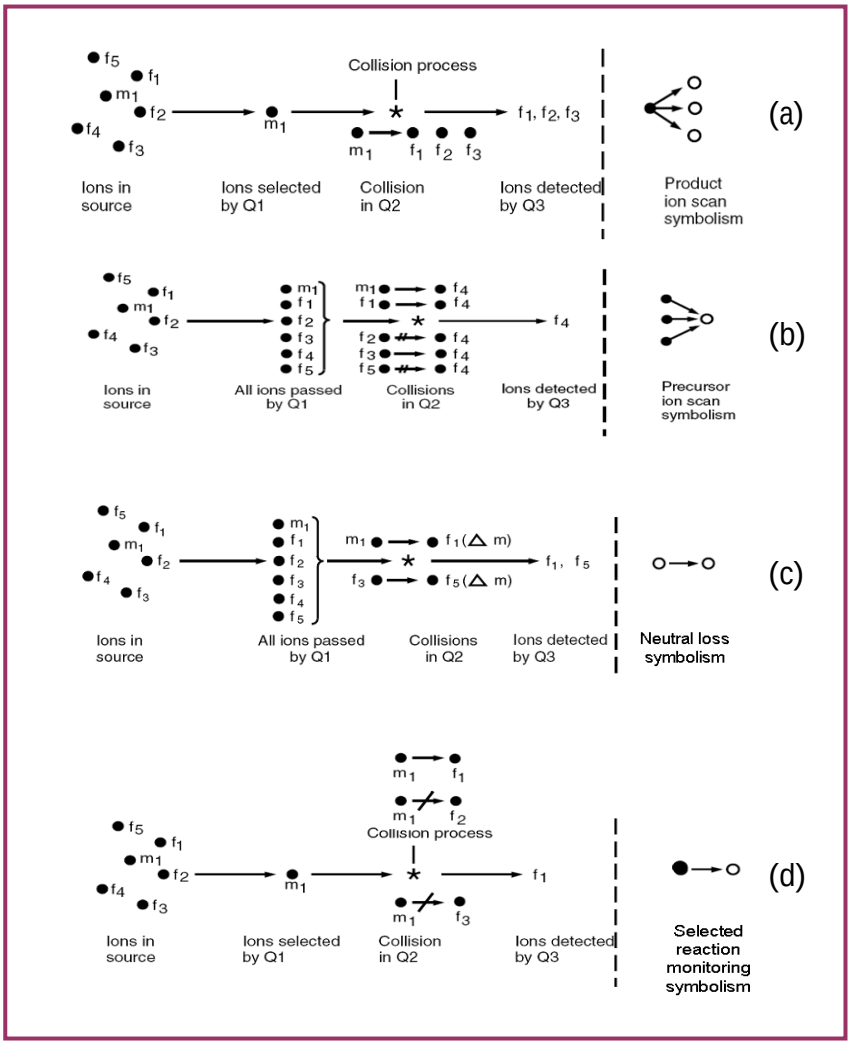

Figure 2.4.1 The principles of (a) product ion scanning, (b) precursor ion scanning, (c) selected reaction monitoring, and (d) neutral loss scanning.

2.4.1 Tandem MS analyzer combinations

Tandem MS has been more or less successfully performed with a wide variety of analyzer combinations. What analyzers to combine for a certain application is determined by many different factors, such as sensitivity, selectivity, and speed, but also size, cost, and availability. The two major categories of tandem MS methods are tandem-in-space and tandem-in-time, but there are also hybrids where tandem-in-time analyzers are coupled in

space or with tandem-in-space analyzers. Moreover, the ongoing development of faster electronics and high-voltage circuits as well as better software tools and hardware control devices constantly open up new possibilities of making innovative combinations of analyzers for specific applications. In this chapter a few examples of commercially available tandem MS instruments are presented. A brief summary of their weaknesses, strengths, and main areas of applications is given.

2.4.1.1 Tandem-in-Space

A tandem-in-space mass spectrometer consists of an ion source, a precursor ion activation device, and at least two nontrapping mass analyzers. The first mass analyzer is used to select precursor ions within a narrow m/z range. Isolated precursor ions are allowed to enter the ion activation device, for example, a gas-filled collision cell, where they dissociate. Created fragments continue on to the second mass analyzer for analysis. The second mass analyzer can either acquire a full mass fragment spectrum or be set to monitor a selected, narrow, m/z range. In principle the second mass analyzer could be followed by more ion activation devices and mass analyzers for MSn experiments. However, due to rapidly decreasing transmission and increasing experimental complexity and instrument size, MS3 experiments and beyond are seldom performed in tandem-in-space instruments. In tandem-in-space MS the ion activation has to be fast enough to produce fragment ions before the precursor ions enter the second mass analyzer.

This means that tandem MS instruments with mass analyzers where precursor ions are accelerated to high kinetic energies, for example, time-of-flight (TOF) and sector analyzers, demand very fast ion activation events, such as in high-energy collision induced dissociation. Triple-quadrupoles, on the other hand, where the precursor ions are comparatively slow, can accommodate slow ion activation techniques, such as low-energy CID. Very slow activation techniques, such as electron capture dissociation and infrared multiphoton dissociation, on the other hand, have so far only been implemented in trapping instruments. Tandem-in-space instruments have a short duty cycle, which makes them highly suitable for coupling with online chromatography and they have found widespread use in high throughput applications.

2.4.1.1.1 Triple-Quadrupole

A widely used tandem-in-space instrument, especially in bioanalytical assays, is the triple-quadrupole.

The QqQ in combination with online LC separation is often used for quantitative analysis of low-abundance molecules in complex mixtures,[26] see also Chapter 8 for an application example. In the SRM mode the first quadrupole of the QqQ is set to transmit a particular m/z. However, when the sample is very complex, for example, body fluids

such as serum or urine, the resolution of the chromatographic separation is often not high enough and the compound of interest may co-elute with a number of molecules within the selected m/z range. These (approximately) isobaric interferences will all continue to the second quadrupole. In most QqQ instruments the second quadrupole is in reality an octopole or a hexapole, but it is nevertheless normally denoted quadrupole for historical reasons. This second "quadrupole" contains a gas-filled collision cell and here fragmentation occurs through low-energy CID. The third quadrupole is also set to transmit a particular m/z, namely a known fragment of the compound that is the object of the investigation. This two-step mass filtering process provides high specificity even for high sample complexity and comparatively fast chromatographic separations. The very rapid duty cycle (10 to 50 ms) of the QqQ also makes it particularly suitable for multicomponent monitoring and high throughput analysis.

Another major advantage of the rapid duty cycle is that many data points can be obtained over one chromatographic peak, thereby facilitating a reliable quantitation. In a recent comparison it was shown that the QqQ (SRM mode) reaches, at least, tenfold higher sensitivity for the majority of the compounds compared to the QIT (MRM mode) and Qq-TOF (MRM mode).[27] Strong differences were also observed in the linear response range between the three mass analyzers. Also here the QqQ, which provided a linear response over three orders of magnitude, was roughly ten times better than its competitors. Even though the QqQ is often used in the SRM mode the instrument also performs very well in the precursor ion, product ion, and neutral loss modes. Then again, since the quadrupole filter is a scanning device the QqQ inherently has a lower sensitivity in the full mass scan than the Qq-TOF and the QIT.

2.4.1.1.2 Tandem Time-of-Flight

For more than a decade the speed, sensitivity, and reliability of MALDI-TOF-MS has made it the favored method for high throughput protein identification and characterization in proteomics. The only major downside has been the difficulty to obtain direct structural information through tandem MS. One method to obtain fragment data, which in principle can be performed in any MALDI TOF instrument equipped with a reflector (RTOF), is termed MALDI post-source decay (PSD). In MALDI-PSD intact ions are produced and accelerated in the MALDI ion source. If the ions have gained excess internal energy during the desorption/ionization or through collisions in the ion source they will decay in the field-free flight tube by post-source or metastable decay.

Fragment ions have approximately the same velocity as the precursor ion, but their kinetic energies are mass dependent. Before the ions enter the reflector they pass a timed ion selector or an ion gate. The ion gate allows unaffected passage for only a brief period of time ideally selecting only ions originating from one precursor ion (in reality the selected m/z range is approximately 10 Th). Fragment ions with different m/z will now penetrate to different depths of the reflector, thus reaching the detector at different flight times (Figure 2.4.2 a). The conversion to a mass scale for the fragment spectrum is more complicated than for a normal spectrum where all ions have the same kinetic energy and travel the same distance. Moreover, the resolution of each fragment peak is strongly mass (energy) dependent. This can be remedied by collecting several fragment spectra, typically about ten, at different reflector potentials. After collection, the parts of the spectra that have best resolution are stitched together. However, stitching is time consuming, reduces sensitivity, and complicates calibration procedures.

By inserting a collision cell in the field-free region of the TOF analyzer high energy CID can also be performed. The only difference from PSD is the type of fragments that are generated.

Another method to obtain MS/MS data with a single MALDI-RTOF is to use a nonlinear field reflector instead of a conventional linear field reflector. In theory a quadratic field reflector can focus fragment ions within a wide mass (energy) range simultaneously and no voltage stepping or stitching is necessary.[28] However, in practice a true quadratic field reflector is difficult to realize. Several RTOF instruments with close to quadratic reflecting fields have been designed.[29-31]

Here the difficulty is to find a satisfactory compromise between the advantage of second-order energy focusing and the disadvantage of a divergent ion beam that is causing low transmission and hence low sensitivity.

Presently the most common tandem time-of-flight (TOF-TOF) configuration consists of a MALDI ion source, a short linear TOF analyzer, an ion gate, a collision cell for (moderately) high-energy CID, and a linear reflector TOF analyzer (Figure 2.4.2 b and c).[32,33] The key feature in these configurations is that the fragments are created with a comparatively moderate kinetic energy (below 10 keV) and afterwards further accelerated (to at least 16 keV) when they enter the second TOF analyzer. When the fragment ions penetrate into the reflector they have a relatively narrow energy spread and can be sufficiently focused by the linear reflector.

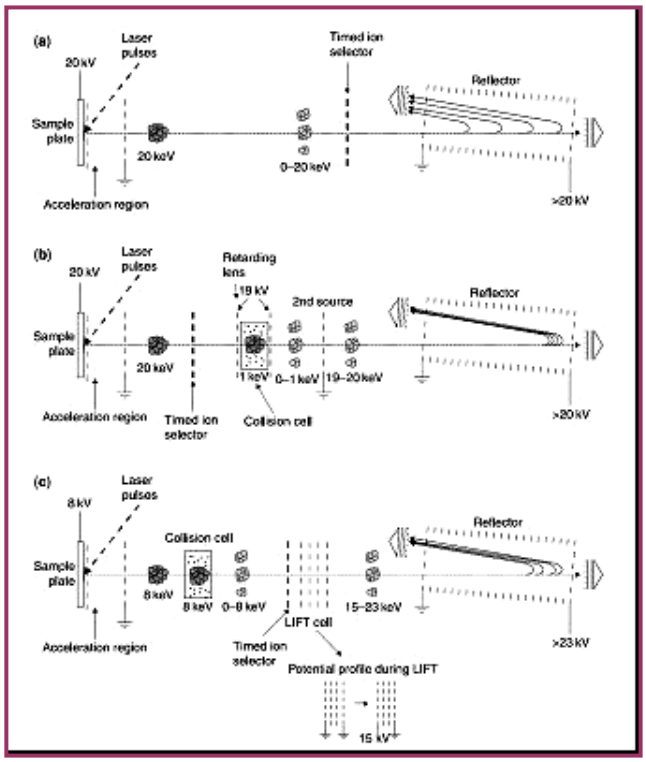

Figure 2.4.2. Schematics of MALDI-TOF tandem MS configurations. (a) Standard MALDI-TOF mass spectrometer equipped with a linear reflector. (b) MALDI-TOF-TOF configuration were the selected precursor ions are decelerated before they enter the collision cell. Product ions (and surviving intact ions) are subsequently accelerated in the second "source" and enter the linear reflector with a relatively narrow energy spread. (c) MALDI-TOF-TOF configuration were the ions are accelerated by an 8 kV potential gap in the source. After the collision cell product ions (and surviving intact ions) pass through a timed ion selector and the selected ions enter

the LIFT cell. The potential in the LIFT cell is rapidly raised to 15 kV a short moment after the selected ions have entered it and these ions are subsequently accelerated. In this configuration the product ions enter the linear reflector with an energy spread, which is greater than for the configuration in (b), but can still be relatively well compensated for by the linear reflector.

2.4.1.1.3 Quadrupole-Time-of-Flight

Hybrid mass spectrometers are instruments equipped with two or more different types of mass analyzers coupled together.

A particularly fruitful hybrid tandem-in-time instrument is the quadrupole mass filter-TOF (Qq-TOF). The Qq-TOF can be regarded as a QqQ with the third quadrupole substituted by an orthogonal TOF equipped with a reflector (Figure 2.4.3). In MS mode the quadrupole mass filter only acts as a passive transmission element and the mass spectrum is acquired by the TOF. In MS/MS mode the quadrupole mass filter is set to transmit only the precursor ion of interest (m/z window typically 1 to 3 Th).

Fragmentation of the precursor ions through low-energy CID occurs in the collision cell and the fragment mass spectrum is acquired by the TOF. Advantages with the TOF compared with a third quadrupole filter include higher resolution, better mass accuracy, and the opportunity to register all ions simultaneously without scanning. However, for quantification of targeted compounds the sensitivity and linear dynamic range of the Qq-TOF (MRM mode) is inferior to the QqQ (SRM mode).[27,34] On the other hand, the higher resolution of the TOF can provide a better selectivity, which can be beneficial in some analytical cases. Another advantage of the Qq-TOF is that since all fragments ions are detected simultanously (without compromising the sensitivity) it is possible to do post analysis of products.

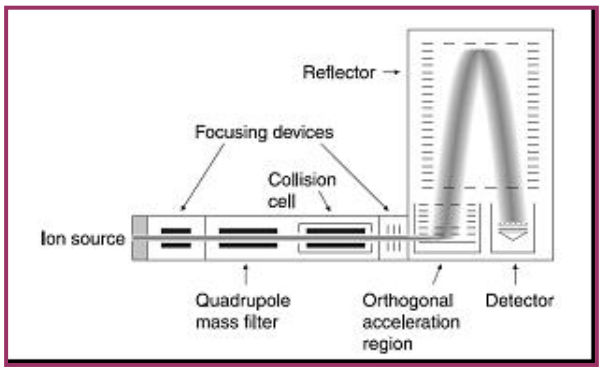

Figure 2.4.3. Schematic of a quadrupole time-of-flight (Qq-TOF) mass spectrometer.

2.4.1.2 Tandem-in-Time

In tandem-in-time MS ions produced in the ion source are trapped, isolated, fragmented, and m/z separated in the same physical device. This is only possible in trapping devices, such as the QIT and the FTICR, where ions can reside in the trap for a long time. Long trapping times make it possible to manipulate the ions several times, thus making tandem-in-time mass spectrometers ideal for MSn measurements. Moreover, since the precursor ions can be trapped for considerable amounts of time it is possible to use very slow activation methods, such as ECD and IRMPD. However, the relatively long duty cycle of tandem-in-time instruments limits the use in high throughput/high complexity applications. Nonetheless, several tandem-in-time mass spectrometers fast enough to enable online coupling to chromatography are commercially available.

2.4.1.2.1 Quadrupole Ion Traps

Wolfgang Paul and co-workers at the University of Bonn, Germany, obtained a patent in the 1950s for a quadrupole mass filter that is the most common analytical mass spectrometer in use today.[35] A mass filter consists of a square parallel array of four round (rather than the ideal hyperbolic form) rods to which a radiofrequency (rf) potential is applied such that opposite rods are held at the same potential and adjacent rods are out-of-phase by 180° (Figure 2.4.4).

The mass filter is a two-dimensional quadrupole device because there is a quadrupole ion trapping field in the x- and y-directions only. Described in the same patent as the mass filter was its three-dimensional (3D) analog, the 3D quadrupole ion trap, wherein three quadrupole fields act. The quadrupole ion trap is an enormously versatile mass spectrometer that is capable of multiple stages of mass selectivity (tandem mass spectrometry, MS/MS and MSn), high sensitivity, high mass resolution, and high mass range. In combination with electrospray ionization, the quadrupole ion trap is applied widely for the study of polar molecules such as peptides and proteins.

In recent years, there has been relatively little further development of the quadrupole ion trap; rather, the range of applications has expanded by making use of the versatility of the instrument. Bruker Daltonik has reported recently on a high charge ion trap wherein the hyperbolic angle of the ion trap has been modified. This change in geometry, combined

with resonant ion ejection under the influence of combined dipolar, hexapolar, and octopolar fields, permits rapid mass scanning at high mass resolution of a greater ion charge in the absence of space charge perturbation.

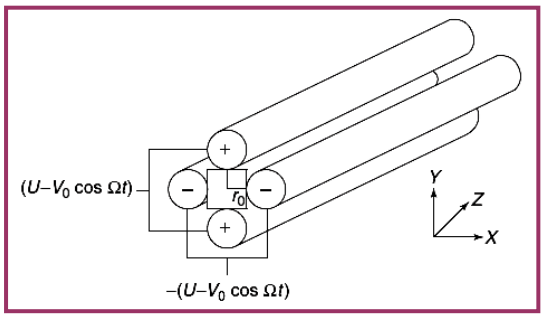

Figure 2.4.4 An ensemble of circular rods that forms a quadrupole mass filter.

The mathematics of ion trajectory stability within a quadrupole field follows the following Mathieu second-order differential (equation 2.4.1):

$$\frac{d^2u}{d\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0, \quad \xi = \frac{\Omega t}{2} \qquad \text{(eq. 2.4.1)}$$

where the subscript u refers to ion motion in the x, y, and z coordinates, x is a dimensionless quantity, O is the radial frequency of the rf potential, and t is time.

A great strength of quadrupolar devices is that the quadrupole fields are independent of one another. Thus, we can apply solutions of the Mathieu equation to 2D or 3D devices provided the weighting factors in each dimension sum to zero. For a 2D device, the trapping parameters are given as (equation 2.4.2)

$$a_x = -a_y = \frac{8eU}{mr_0^2 2\Omega^2}; \quad q_x = -q_y = -\frac{4eV_{0-p}}{mr_0^2\Omega^2} \qquad \text{(eq. 2.4.2)}$$

where U is a dc voltage, V_{0-p} is the zero-to-peak amplitude of the rf potential, m is the ion mass, e is the electronic charge, and r_0 is the radius of the inscribed circle of the rod array.

In the operation of 2D and 3D quadrupole instruments, the modification of ion trajectories by ion/neutral collisions must be considered. In rf quadrupole fields, an ion/neutral collision reduces both ion kinetic energy and ion excursions such that the ions are cooled and focused to the center of each field.

Collisional cooling is an important aspect of the behavior of ions in a quadrupole field; for example, in a 2D quadrupole device, collisional cooling is employed to limit the excursions of ions so as to form a tightly focused ion beam of diminished kinetic energy constrained close to the central axis. A focused beam of ions may be transmitted through a relatively small orifice from one section of an instrument to the next such that pumping requirements are reduced, and a focused ion beam can be accelerated with reduced ion loss. When the axial motion of a focused ion beam is arrested within a rod array such that the confined ions can be excited resonantly, a linear ion quadrupole trap is obtained.

In 2002, two linear ion traps were reported; they had the basic structure of a quadrupole mass filter. The first linear ion trap instrument was described by J.W. Hager of MDS SCIEX and a second linear ion trap instrument was described by J.C. Schwartz, M.W. Senko, and J.E.P. Syka of Thermo Finnigan.

Both instruments employ mass-selective ion ejection. Axial ion ejection is employed in the SCIEX instrument while in the Thermo Finnigan instrument ion ejection occurs radially. In addition, ion trapping in the SCIEX instrument can occur either in a pressurized collision cell region or in a low-pressure quadrupole rod array downstream of the collision cell.

The principal advantage of a linear ion trap is that more ions can be confined in the larger device than in a 3D ion trap. Thus, the onset of space-charge repulsion is experienced only at greater ion loading of the trapping device. Virtually all of the operating characteristics of the 3D ion trap are retained in the linear device. Both linear ion trap instruments were developed using existing experimental platforms that permitted prior collisional cooling of the ion beam.

SCIEX linear ion trap The SCIEX instrument is based on a triplestage quadrupole (TSQ) mass spectrometer wherein, normally, the ion beam travels from left to right. Ions are focused collisionally close to the axis in Q_0, mass selected in Q_1, and collisionally dissociated to fragment ions in the collision cell Q_C. Q_C, is a quadrupolar array of round rods of length 127mm enclosed in a pressurized container to which a collision gas is introduced. Q_2 is a quadrupole mass filter that has rods of length 127mm also. The round rod arrays were fabricated with a field radius, r_o, of 4.17 mm.

When Q_C or Q_2 is operated as a linear ion trap, an rf drive voltage of 1MHz is applied. An auxiliary ac potential is applied in quadrupolar fashion to the rod arrays of either Q_C or Q_2 so as to excite ions at secular frequencies. Ions are trapped radially in the linear ion trap Q_C by the rf potential, and are confined axially within the rod array by dc potentials applied to the aperture plates, IQ_2 and IQ_3. Thus, a linear ion trap resembles a bathtub having a

parabolic cross-section and near vertical ends. Ions enter the linear ion trap close to the zero-field centerline of the device and encounter a series of momentum dissipating collisions with collision gas.

When an auxiliary ac field is applied to induce radial resonant excitation, coupling of radial and axial motions effected axial ion ejection when the ion radial secular frequency matched that of the ac field.

In the Thermo Finnigan instrument an ion beam from an electrospray source is directed through a heated capillary, two successive rod arrays, a front lens, and into a linear ion trap composed of three sections of quadrupole hyperbolic rod arrays with r_o=4 mm. Radial confinement of ions is effected by the trapping potential well in the center section and axial confinement by dc potentials applied to the front and rear lenses. The basic design of the linear ion trap is shown in Figure 2.4.5 where the front and rear sections are of length 12mm and the center section is of length 37mm. An rf potential at 1MHz is applied to the linear ion trap, and an auxiliary ac potential is applied in dipolar mode across the rods in the x-direction of the center section. Ions are directed radially from the center section; in order to detect the ions externally, a 0.25 mm high slot was cut along the middle 30mm of the center section of one rod as shown in Figure 2.4.5; a standard detector system is mounted in front of the slot.

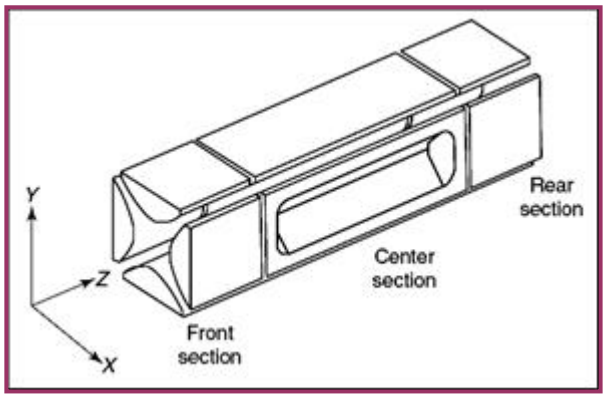

Figure 2.4.5 An angled view of the three sections of the two-dimensional linear ion trap. The detector faces the center section.

2.4.2 Ion activation methods

No tandem MS experiment can be successful if the precursor ions fail to fragment (at the right time and place). The ion activation step is crucial to the experiment and ultimately

defines what types of products result. Hence, the ion activation method that is appropriate for a specific application depends on the MS instrument configuration as well as on the analyzed compounds and the structural information that is wanted. Various, more or less complementary, ion activation methods have been developed during the history of tandem MS.

- **In-Source Decay**

In-source decay occurs when the precursor ions gain sufficient internal energy, either while being created or through early collision events, that they decay before exiting the source. The likelihood for in-source decay depends strongly on the type of ion source and the operating parameters chosen. One example of in-source decay is nozzle-skimmer dissociation (NSD), where fragment ions are generated in an electrospray ionization (ESI) source by accelerating the precursor ions as they pass through the atmospheric pressure/vacuum interface.[36] A modification of this method has recently been used to obtain top-down sequence information on purified 200 kDa proteins.[37] However, in-source decay does not allow for separation of precursor ions with different m/z before dissociation and is thereby not truly a tandem MS method.

- **Post-Source Decay**

The main difference between postsource decay (PSD, also referred to as metastable ion dissociation) and in-source decay is that metastable ions are stable enough (or the time they spend in the source is short enough) for them to leave the source intact. Hence, they can be m/z separated before they fragment and a proper tandem MS measurement can be performed. An example where metastable fragmentation is used is vacuum MALDI-PSD in combination with a reflectron TOF or TOF-TOF analyzer. Postsource decay of vacuum MALDI produced precursor ions is probably a combination of contributions from primary laser induced processes and secondary processes, for example, high-energy collisions with the matrix plume during extraction.[38] Several factors influence the fragment spectrum, including the laser intensity and the effective temperature of the matrix.

- **Collision Induced/Activated Dissociation**

In collision induced/activated dissociation (CID/CAD) precursor ions collide with gas atoms or molecules, such as nitrogen, argon, or helium, and fragment. In the collision a part of the kinetic energy is converted into vibrational/rotational energy of the parent ion. If the

internal energy gained is high enough the precursor ion will fragment fast enough for the fragment ions to be observed in the mass spectrometer. Depending on the type of mass analyzer, either high-energy CID (kiloelectronvolt collision energy) or low-energy CID (<100 eV) is performed. Low-energy CID is common in quadrupole and ion trap instruments and high-energy CID is typical for sector and TOF instruments.

- **High-Energy CID**

High-energy CID is performed by acceleration of the precursor ions to kiloelectronvolt energies and subsequent collision with gas atoms or molecules in a collision cell. One collision is enough to cause electronic excitation of the precursor ion and subsequently virtually all structurally possible fragmentations occur that have some probability of occurring. Fragment-ion mass spectra are often complex, with abundant low mass and internal fragment ions. The complexity of the MS-MS spectra can make the elucidation more challenging, but also increase confidence in the identification of the precursor ion. For example, high-energy CID often produces side-chain cleavage of peptides that makes it possible to distinguish between the amino acids leucine and isoleucine, which have the same elemental composition. High-energy CID is considered as very reproducible. This is due to the high kinetic energy of the accelerated precursor ion. Compared to the kiloelectronvolt energy of the precursor ion the kinetic energy of the gas atom is negligible in the collision. This means that changes in collision conditions, such as type of target gas, gas pressure, and temperature, do not largely influence the appearance of the fragment-ion spectrum.

- **Low-Energy CID**

In low-energy CID, only a small amount of energy is added to the internal energy of the precursor ion in each collision and energy from hundreds of collisions will have to be accumulated in order to induce fragmentation. Here, the time between the collisions is long enough for the internal energy to redistribute on all degrees of freedom thereby causing low-energy fragmentation pathways to dominate.

In linear quadrupoles the CID activation time is equal to the time the accelerated precursor ion (<100 eV) takes to pass through the collision cell. The primary fragments produced will continue to undergo collisions that cause them to fragment as well, and so on, thus producing richer and more complex fragment-ion spectra.

In a QIT the precursor ion is selectively accelerated via resonance excitation and collided with the background gas. Since the ion acceleration is m/z dependent, no additional fragmentation occurs of the primary fragments. Hence, if the precursor ion contains groups that dissociate at a markedly lower internal energy than other groups (e.g., peptides containing phosphorylated serine or threonine residues) the resulting MS/MS spectra contain only a few intense fragment ions and thereby insufficient information for unambiguous precursor identification. An additional limitation is that it is not possible to simultaneously accelerate the precursor ion and trap the lightest fragment ions. In practice fragment ions with m/z below about one third of the m/z of the precursor ion are absent from the MS-MS spectra of a QIT.

In the ion cyclotron resonance (ICR) cell CID is performed by resonant excitation of the precursor ion and subsequent collisions with the background gas. One serious drawback with on-resonance excitation in the ICR cell is that the risk for ion losses due to ejection increases with increasing activation time. Several alternative ICR-CID methods with enhanced MS-MS sensitivity have been introduced, for example, sustained off resonance (SORI)-CID, very low energy (VLE)-CID, and multiple excitation collisional activation (MECA).[39]

2.4.3 Tandem Mass Spectrometry Applications

MS/MS applications are plentiful, for example in elucidation of structure, determination of fragmentation mechanisms, determination of elementary compositions, applications to high-selectivity and high-sensitivity analysis, observation of ion–molecule reactions and thermochemical data determination (kinetic method).

a ***Structure Elucidation***

Tandem mass spectrometry allows more structural information to be obtained on a particular ionic species, because the used ionization method yields relatively few structurally diagnostic fragments, or because its fragmentation is obscured by the presence of other compounds in the mixture introduced in the source, or because it is obscured by other ions generated from the matrix in the course of ionization.

Tandem mass spectrometry also allows the determination of isomers and diastereoisomers.

- ***Selective Detection of Target Compound Class***

  The following example explains the development of a rapid selective analysis method for the components of a complex mixture based on MS/MS. The aim is to find an easy way of detecting a class of compounds, the carnitines, in biological fluids. This compound participates in carrying fatty acids with various chain lengths through cell membranes; it contributes to eliminating excess fatty acids in urines. It is therefore of interest to detect them in order to arrive at a diagnosis.

  Elaborating a selective detection method starts with a study of the fragmentation reactions.

  This general methodology for the selective detection of compounds or compound classes can be combined with a chromatographic separation, allowing selective detection of one compound in a complex mixture. Tandem mass spectrometry also allows the signal-to-noise ratio to be substantially improved in the detection of selected compounds or compound classes.

  The possibility to obtain both high selectivity and high sensitivity by tandem mass spectrometry is largely used in the pharmaceutical industry to monitor and quantify a selected compound in pharmacokinetics studies, which is the field that uses the largest number of tandem mass spectrometers.

- ***Ion–Molecule Reaction***

  If a reactant gas is introduced into the collision cell, ion–molecule collisions can lead to the observation of gas-phase reactions. Tandem-in-time instruments facilitate the observation of ion–molecule reactions. Reaction times can be extended over appropriate time periods, typically as long as several seconds. It is also possible to vary easily the reactant ion energy.

  The evolution of the reaction can be followed as a function of time, and equilibrium can be observed. This allows the determination of kinetic and thermodynamic parameters, and has allowed for example the determination of basicity and acidity scales in the gas phase. In tandem-in-space instruments, the time allowed for reaction will be short and can be varied over only a limited range. Moreover, it is difficult to achieve the very low collision energies that promote exothermic ion–molecule reactions. Nevertheless, product ion spectra arising from ion–molecule reactions can be recorded. These spectra can be an alternative to CID to characterize ions.

a ***The Kinetic Method***

The kinetic method[40,41] is a relative method for thermochemical data determination which is based on measurement of the rates of competitive dissociations of mass selected cluster ions. This method was introduced by Cooks[42] for proton affinity determination.

Later, an extension of this method was proposed by Fenselau.[43]

For proton affinity determination, the kinetic method involves the formation of the proton bound heterodimer between the two bases whose affinities are to be compared. By tandem mass spectrometry, the appropriate cluster ion $[B_1HB_2]^+$ is selected and its spontaneous or collisional dissociation is observed. As shown in figure 2.4.6, the competitive dissociation leading to the two protonated monomers is analysed and the relative abundances of the

monomers $[B_1H]^+$ and $[B_2H]^+$ are measured. From these abundances, the relative proton affinities of the two bases B_1 and B_2 can be calculated and the proton affinity of one of the two bases can be determined, if the proton affinity of the other is known.

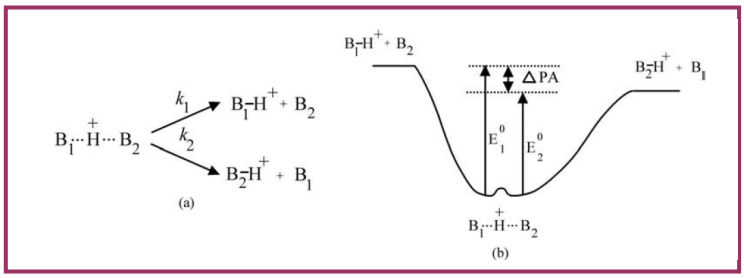

Figure 2.4.6 Proton affinity determination by the kinetic method. (a) This method is based on competitive dissociation of heterodimer clusters. (b) Potential energy diagram for proton-bound dimmer dissociation.

The kinetic method provides an alternative to equilibrium measurements for the determination of gas–phase thermochemical properties. It has been applied more and more in thermochemical data determination mainly because of its ability to measure very small energy differences and its simplicity. Indeed, it can be executed easily on any tandem mass spectrometer. Furthermore, this method is sensitive and is applicable with impure compounds.

Its applications are broad, covering thermochemical properties in the gas phase such as proton affinity,[44] electron affinity,[45] metal ion affinity,[46] ionization energy,[47]

acidity[48] or basicity.[49] In addition to the determination of thermochemical data, the kinetic method has also been applied in structural and chemical analysis such as chiral distinctions. This method is able to distinguish enantiomers and to measure precisely enantiomeric ratios.[50]

2.5 Quantitative analysis: advantages of mass spectrometry

Using MS detectors, with or without GC or LC separation, it is possible to carry out precise and accurate quantification of analytes. MS quantification is more usually based on the peak area for specific ion fragments, called SIM.

The aim of quantitative analysis is to provide accurate and reliable determination of the amount of a target analyte in a real-world sample. Quantitative analysis is the cornerstone of many health-related fields, such as clinical chemistry, pharmaceutical science, forensic science, and environmental science. Knowledge of the accurate amount of a biological compound in a cell culture or in a particular organ provides information that leads to an understanding of its functional role in various neurological and pathophysiological events. The development of a drug and its clinical trial heavily rely on accurate quantitative analysis. The management of various forms of illnesses requires quantitative analysis of endogenous biomolecules in extracts of body tissues and fluids. Quantification of drugs of abuse and their metabolites is a key factor in the management of an overdose patient. From a health point of view, it is important to know the accurate amounts of toxic chemicals in the air that we breath, the water we drink, and the food we consume.

Quantitative analysis is performed to provide absolute quantification or relative quantification. In absolute quantification, the aim is to determine the amount or concentration of the analyte in absolute terms (i.e., in terms of per unit mass or per unit volume of the sample). In contrast, the aim of relative quantification is to determine the amount or concentration of the analyte relative to another analyte or of the same analyte relative to another sample.

Mass spectrometry has attained a unique position in analytical chemistry as a quantitative analysis tool, especially when coupled with high-resolution separation devices. Quantitative analysis of a variety of molecules is performed routinely with mass spectrometry–based methods at unprecedented high-sensitivity. It has several desirable features that make it the most sought-after analytical technique for quantitative analysis. These features include:

- Applicability to most types of compounds
- High level of sensitivity, including the capability to detect a single ion and detection limits in the attomole to femtomole range.
- High level of specificity because measurements are made on molecular and fragment ions and both are highly compound-specific parameters
- Coupling with high-resolution separation devices, providing an opportunity to analyze real-world samples
- Use of stable isotope-labeled internal standards of the same structure
- Wide dynamic range
- High speed of analysis
- Automation and high-throughput analysis.

Quantitative analysis by mass spectrometry involves establishing the accurate correlation of the compound-related ion signal with the amount of analyte. Although quantitative analysis has been performed by acquiring mass spectrometry data in the narrow-mass-range and full-scan modes, most applications have used either the selected-ion monitoring (SIM) or selected-reaction monitoring (SRM) modes of data acquisition. The sensitivity, specificity, type of information desired, and cost of instrumentation are the criteria that influence the choice of a particular technique. The detection sensitivity of a full-scan mode, in which the data are acquired in a wide mass range, is compromised significantly because large amounts of scan time and sample ion current are wasted. This scan mode, however, has the advantages that the identity of the analyte ion can be confirmed readily, and the presence and absence of any interfering signal can be ascertained.

Scanning in the narrow mass range improves the detection sensitivity to some extent and still provides the isotopic pattern of the sample ion. This scan mode is a compromise between the full-scan and SIM modes.

2.5.1 Selected-Ion Monitoring

The SIM mode is a technique frequently used to record the sample ion current in quantitative analysis. In this procedure, the ion current from only one or a few selected ions, rather than a complete mass spectrum, is repetitively recorded and accumulated. The data system spends more time in recording the ion current at the m/z values selected to impart a significant improvement in detection sensitivity versus a full scan. A 1000-fold

gain in detection sensitivity can be realized in switching from a full-scan mode to SIM, but at the expense of specificity because the identity of the ion selected may be questionable. An other benefit of the SIM mode is that a chromatographic peak shape can be defined more accurately because of the acquisition of more data points.

The type and number of ions that are selected for the SIM procedure depend on the nature of the compound. An important criterion in choosing the ion is its abundance. In most cases, the molecular ion, which usually is the most abundant ion, satisfies this criterion. In the absence of a prominent molecular ion, any other high-mass fragment ion next in abundance could serve the purpose. The ion chosen for SIM measurements must originate exclusively from the analyte; otherwise, the molecular specificity of the procedure would be severely compromised. This situation is a common occurrence with biological and environmental samples, which often are heterogeneous even after extensive chromatographic separation.

Furthermore, the background signal due to matrices and solvents used in some ionization techniques might also produce an interfering signal. The selectivity of the SIM procedure can be improved when:

(1) m/z the value of the selected analyte ion is away from that of the interfering ion(s),

(2) more than one analyte ion is monitored,

(3) measurement is done at higher mass resolution to eliminate the contribution from any interfering ions,

(4) SIM is coupled with a chromatography separation device.

Monitoring of more than one ion and increasing the resolution, however, would lower the detection sensitivity. If these changes are of no avail, the SRM approach must be used in place of SIM.

2.5.2 Selected-Reaction Monitoring

Conceptually, SRM is similar to acquiring a product-ion scan spectrum in tandem mass spectrometry (MS/MS). Instead of recording the complete spectrum, however, the ion current due to a specific precursor–product pair is monitored. As with the product-ion scan, the molecular ion of the analyte is mass-selected by MS-1 and allowed to undergo collision-induced dissociation (CID) to a unique product ion; the ion current due to this exclusively derived ion is monitored by MS-2. The term *multiple reaction monitoring*

(MRM) refers to the monitoring of more than one reaction, either from the same precursor or from more than one precursor. The following three criteria are applied in selecting a precursor–product reaction in SRM analysis:

(1) a fragmentation reaction chosen for quantification must be unique to the analyte;

(2) the precursor–product reaction should provide a strong ion current signal;

(3) an isotope-labeled internal standard of the same compound should be available in which the stable isotope is present at a site that yields the labeled fragment ion.

An obvious advantage of SRM over SIM is the increased level of detection specificity because of the exclusive structural link that is maintained during the analysis between the mass-selected precursor ion and its product ions. Also, in SRM, chemical noise is virtually eliminated. The latter aspect might improve the detection sensitivity in some cases. In general, the detection sensitivity of SRM is less than that of SIM because of the distribution of the precursor ion current into several of its CID products. The higher cost of the tandem instrumentation is another discouraging factor in favor of SRM. Increased specificity, however, outweighs these disadvantages.

Instrument operation in SRM measurements is exactly identical to that used in the product-ion analysis except that instead of acquiring a full scan spectrum, only the products selected are monitored. A triple-quadrupole instrument is ideally suited for SRM experiments, although magnetic-sector and QIT instruments have also been used.

2.5.3 Specificity, Sensitivity and Detection Limit

The degree of specificity of a quantitative analysis using mass spectrometry depends on how the spectrometer is used, and even more on the signal that is used during the correlation.

Many methods exist that improve the specificity of quantitative analysis using mass spectrometry. These methods can be classified into either of two categories: those that act upon the sample and those that act on the spectrometer.

The first type of method that increases the specificity is based on a simple preliminary purification which can be an extraction into a solvent that is less polar, an acid–base separation, and so on, in order to eliminate all possible interference. Another possibility is to use different characteristic ions with higher masses after having formed a higher mass derivative from the compound. A further approach consists of making the spectrometer

more selective, for example by increasing the resolution, or by resorting to a different ionization technique, or by applying another data acquisition mode such as SRM.

All of these techniques can, of course, be used simultaneously with chromatographic separation techniques. Methods that allow an improvement in the mass spectrometer specificity can thus be worthwhile alternatives to long procedures for sample preparation. However, quantitative analysis carried out directly on neat samples may undergo a matrix effect characterized by a variation in the sample response because of the effect of the matrix on the abundance of ions within the source.

Sensitivity in mass spectrometry is defined as the ratio of the ionic current change to the sample change in the source. It is important that the relevant experimental conditions corresponding to sensitivity measurement should always be stated.

The detection limit should be differentiated from sensitivity. It is the smallest sample quantity that yields a signal that can be distinguished from the background noise (generally a signal equal to 10 times the background noise). It should be noted that this minimum quantity is not enough to obtain an interpretable mass spectrum. The limit of detection depends considerably on the abundance of the ionic species that is measured. The more abundant the measured ionic species is with respect to all of the ions derived from the analysed molecule, the higher is the limit of detection. The goal is thus to produce a signal that is as intense as possible. Several methods allow this goal to be reached, such as the modification of the ionization conditions, reversal into the negative mode, the use of other softer ionization techniques or the derivative of the sample, in order to increase the number of ions produced in the source or to reduce their fragmentation.

Another factor influencing the sensitivity corresponds to the length of time of signal integration. Of course, the longer that time is, the more intense is the signal. The data acquisition mode thus influences the sensitivity by this factor. Three acquisition modes exist: the scan, SIM and SRM modes. These three modes are cited in ascending order of their effect on the sensitivity.

As a reminder, the scan consists of measuring complete spectra between two limit masses several times. The detection of selected ions consists of tuning the analyzer so as to focus on to the detector only those ions with a specific m/z ratio. If several ions with different directly m/z ratios have to be detected, the analyzer goes rapidly from one mass to another. This method is clearly more sensitive than the scan mode as the length of time of signal integration is greater and the increase in the resulting signal-to-noise ratio allows a sensitivity gain of up to a 1000-fold.

The detection of selected reactions is a technique that requires tandem mass spectrometry, which makes the SRM even more sensitive and more selective than the SIM. In order to carry out this type of acquisition, the instrument is tuned to transmit only the ions derived from a fragmentation reaction in the chosen reaction region. The sensitivity gain obtained by using this method compared with SIM is due to the increase in the signal-to-noise ratio that is characteristic of tandem mass spectrometry.

The increased sensitivity of the SIM and SRM data acquisition results in lower flexibility. The full-scan mode furnishes complete data so new structural information can be extracted in order to answer questions that may arise after the initial experiment. This is not possible for data acquired in the SIM or SRM modes.

2.5.4 Calibration methods

1. External Standard Method

The role of a standard is to determine the mathematical relationship, in the concentration range to be measured, between the selected signal intensities and the mixture composition.

This external standard method consists of preparing a synthetic sample containing a known quantity of the molecule to be measured (M_{ste}), then introducing a precise volume of this solution into the spectrometer and recording the intensity of the response signal (I_{ste}). Then, without any modification of the analytical conditions, an equal volume of the solution containing the molecule to be quantified (M_x) is introduced into the spectrometer and the intensity of its response signal (I_x) is measured. Since the volumes that are introduced are equal, there is a proportionality between the response intensities and the quantities as long as the response signal intensity remains linear with respect to the concentration and as long as the signal intensity is zero at zero concentration. That is shown in equation 2.5.1.

$$M_x = I_x \times \frac{M_{ste}}{I_{ste}} \qquad \text{(eq. 2.5.1)}$$

or $M_x = I_x \times RF_x$ if the response factor is defined as (equation 2.5.2)

$$RF_x = \frac{M_{ste}}{I_{ste}} \qquad \text{(eq. 2.5.2)}$$

In electron ionization, the response is normally linear with respect to the concentration over a wide range, often six orders of magnitude. This is not true for the other ionization techniques because of the influence which the sample quantity can have on the number of ions that is produced and on the fragmentation, and thus on the production yield of the various ionic species. For instance, in the chemical ionization mode the formation of adducts appearing at higher sample pressures changes the relative intensities in the spectrum. Thus, verification by a calibration curve is necessary. This calibration curve allows one to calculate the quantities of a compound to be measured in the unknown samples, to confirm the method specificity and also to define its sensitivity. In order to do this, equal volumes of a series of synthetic samples containing an increasing quantity of the molecule to be measured are introduced into the mass spectrometer and the intensity of their response signal is recorded.

This allows the determination of the mathematical relationship, in the concentration range to be measured, between the selected signal intensity (I_x) and the quantity of the molecule to be measured (M_x) that is present in the mixture. Ideally this relationship should correspond to the equation of a straight line with a slope equal to one in order to ensure maximum precision. Figure 2.5.1 shows an example of a quantitative analysis of phenobarbital within a mixture using GC/MS.

Experimental procedures for quantitative mass spectrometric analysis usually involve several steps. The final error results from the accumulation of the errors in each step, some steps in the procedure being higher error sources than others. A separation can be made between the errors ascribable to the spectrometer and its data treatment on the one hand and the errors resulting from the sample handling on the other.

Normally, the sample handling errors are higher than those due to the mass spectrometer and thus make up the greater part of the final error. Handling errors can be numerous, such as the error in measuring a sample volume and the error in the introduction into the spectrometer.

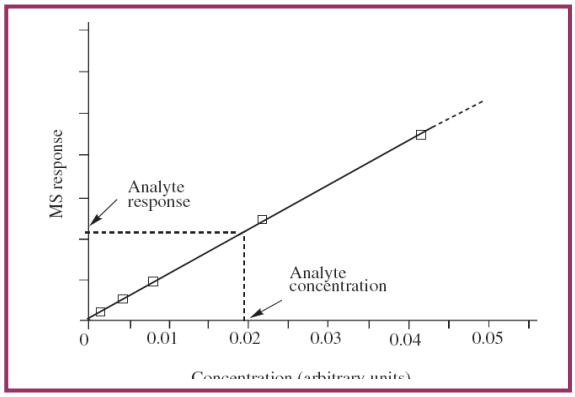

Figure 2.5.1 Typical external calibration curve.

The errors due to the mass spectrometer are also numerous, such as the variation in the source conditions and the instability of the mass scale. For statistical reasons, every measurement of a signal intensity carries a minimal intrinsic error. This error is inversely proportional to the square root of the number of ions detected for that signal. In order to optimize the reproducibility or the precision of the measurements, a maximum number of ions must thus be detected for every ionic species.

All of these error sources, other than the minimal intrinsic error, can be reduced by using the internal standard method. The absolute measurement of the signal is replaced by the measurement of the signal ratio for the molecule that is measured and for the internal standard. The same compound can play the role of the internal standard for the quantification of various compounds within the mixture.

2. Standard Addition Method

The *standard addition method* must be adopted when the sample matrix is very complex and the instrumental fluctuations are difficult to control. In this method, the unknown sample is first analyzed. A known concentration of the standard solution of the analyte is added to this unknown sample, and the mass spectrometry response is measured again to provide the response factor (i.e.; the response per unit concentration). The concentration of the unknown is calculated by multiplying the signal intensity of the unknown with the response factor. This single-point calibration is, however, less precise. To enhance the precision of this method, several increments of the standard solution of the analyte are added to a fixed amount of the unknown sample. After each addition, the mass spectrometry response is also measured, and the calibration curve is obtained as in the

external standard method (Figure 2.5.2). The *x*-axis intercept of the calibration curve provides the concentration of the analyte in the unknown sample.

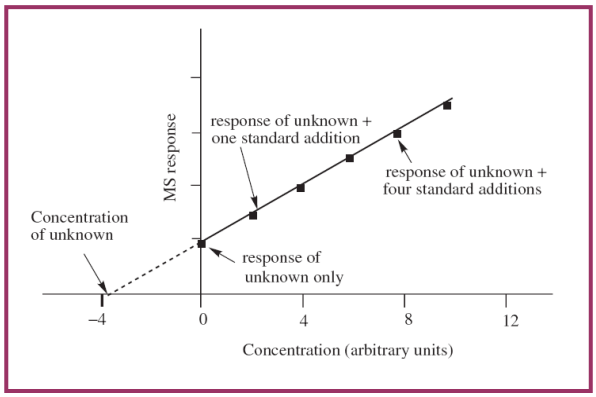

Figure 2.5.2. Calibration by standard addition method.

3. Internal Standard Method

This method is based on a comparison of the intensities of the signal corresponding to the product that has to be quantified with the one of a reference compound called the internal standard. This method allows the elimination of various error sources other than the minimal intrinsic error due to statistical reasons. In fact, if we choose as an internal standard a molecule with chemical and physical properties as close as possible to the properties of the molecule to be measured, the latter and the internal standard undergo the same loss in the extraction steps and in the derivative or the same errors in the introduction of the sample into the mass spectrometer, when the source conditions are varied.

As both compounds undergo the same losses and the same errors, their ratio remains unchanged during the procedure. Knowing the quantity of the internal standard that is added from the start and the relative proportion of the quantity of both compounds allows these losses and errors to be neglected. It is important to add the internal standard as early as possible in the procedure, in order to obtain the maximum precision.

The method consists first of carrying out measurements on synthetic samples containing the same known quantity of the internal standard and increasing quantities of the compound to be measured. With these results a calibration curve is constructed. This allows a mathematical relationship to be obtained between the intensities of the signals corresponding to the compound to be analysed and the internal standard (I_x/I_{sti}) and the quantity of compound present in the sample (M_x). As a reminder, maximum precision is obtained if the relationship corresponds to the equation of a straight line with a slope equal

to one. The measurements are then carried out on the unknown samples that had a constant quantity of internal standard added to them before they were treated according to the experimental procedure. The quantity of compound in each unknown sample can be measured using the built calibration curve.

The internal standard should show physical and chemical properties that are as close as possible to those of the molecule that has to be measured. It must be pure, absent from the sample and, of course, inert towards the compounds in the sample. The internal standards can be classified into three categories: structural analogues that are labelled with stable isotopes, structural homologues and compounds from the same chemical family. These various types of internal standards are classified here in descending order according to their usefulness and their price. In fact, the starting material for labelled compounds is fairly cheap, but most require many steps in their total synthesis and are thus very expensive. In the case of standards corresponding to structural homologues or in the case of compounds belonging to the same chemical family, the ions that are used must have masses differing from that of the compound that must be measured if direct introduction is used. However, if the introduction is carried out by chromatographic coupling and if the retention time of the compound is different from that of the internal standard, then the ions can have identical masses.

4. Isotopic Dilution Method

Isotope dilution mass spectrometry (IDMS) can be considered as a special case of the internal standard method: the internal standard that is used is an isotopomer of the compound to be measured, for example a deuterated derivative. Note that an internal standard is necessary for every compound to be measured. This internal standard is as close as possible to perfection since the only property that distinguishes it from the compound to be measured is a slight mass difference, except for some phenomena that involve the labelled atoms, such as the isotopic effect. In that case, we have an absolute reference, that is the response coefficients of the compound and of the standard are identical. This method is often used to establish standard concentrations. The basic theory of this method rests on the analogy between the relative abundance of isotopes and their probability of occurrence.[51]

The method consists of examining the spectrum of the compound that must be measured in order to select an intense characteristic peak which is used to measure the analyte. A known, exact quantity of labelled internal standard is added to the sample with an unknown

concentration. When the labelled internal standard is added, the peak corresponding to the characteristic peak is moved to a different position in the spectrum, according to the number and nature of the atoms that were used in the labelling. The ratio of these two signal intensities is used to measure their relative proportion.

2.5.5 Validation of a quantitative method

The process of method validation (i.e., evaluation of the assay) affects the quality of the quantitative data directly. Through method validation, it is assured that the method developed is acceptable. Issues involved in the validation of a mass spectrometry method for quantitative analysis are similar to those in any other analytical technique. The validation involves undertaking a series of studies to demonstrate the limit of detection (LOD); limit of quantitation (LOQ); linear range; specificity; within-day precision and accuracy; and day-to-day precision and accuracy, specificity, and robustness of the method. All of these parameters must be determined with those commonly accepted good laboratory practices criteria that are applicable in the validation of analytical methods.

- *Limit of detection:* LOD is defined as the lowest analyte concentration that can be detected in the sample (Figure 2.5.3). It is the analyte concentration that yields an S/N ratio •3.
- *Limit of quantitation:* LOQ is the lowest analyte concentration that can be determined at an acceptable level of accuracy and precision. It is usually the analyte concentration that yields S/N = 10.

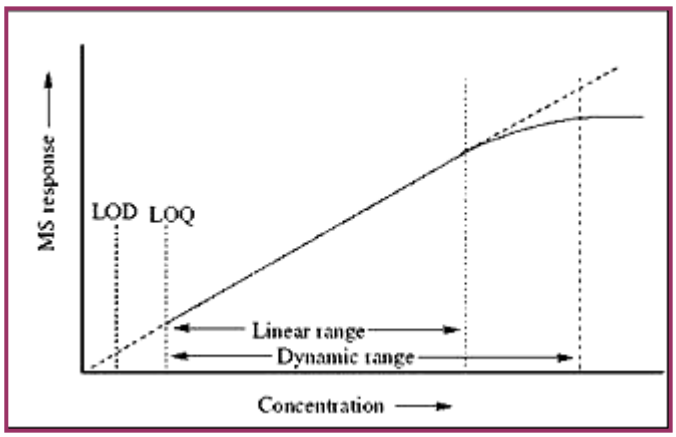

Figure 2.5.3. Graphical depiction of limit of detection (LOD), limit of quantification (LOQ), linear range, and dynamic range.

- *Linear range:* The linear range is defined as the concentration range where the analyte response is directly proportional to concentration. To determine this range, a calibration curve is prepared by analyzing five concentrations of standard solutions between 50 and 150% of the target analyte concentration. For an acceptable linearity range, the correlation coefficient (r^2) should be greater than 0.999 and the y-intercept less than 2% of the target concentration response.
- *Accuracy:* Accuracy is the measure of the closeness of the measured value to the true value. Accuracy is determined by comparing the test result with that obtained for a standard reference material or comparison to an existing well-characterized method.
- *Precision:* The precision of a method is the degree of mutual agreement among a series of measurements from multiple sampling of the sample. The method should be validated for short-term repeatability, day-to-day reproducibility, instrument-to-instrument reproducibility, and laboratory-to-laboratory reproducibility. The precision is expressed as variance, standard deviation, or coefficient of variance.
- *Specificity:* The specificity of a method is its ability to measure only the target analyte accurately in the presence of any other compounds in the sample matrix.
- *Robustness:* Robustness is the ability of the method to remain unaffected by small changes in method parameters (e.g., pH, temperature.).

2.6 Applications of Mass Spectrometry in Food Analysis

In order to protect human health, governments and international organizations are regulating the use of food contaminants such as alkaloids, pesticides, marine toxins, mycotoxins, arsenosugars, antibiotics, dioxins and polychlorinated biphenyls, setting maximum residue levels (MRLs) in foods.

The detection of food contaminants is the most important factor for protecting the health of the population. Several analytical methods have been reported for the determination of food contaminants, such as nuclear magnetic resonance, X-ray crystallography, gas chromatography, high performance liquid chromatography, and immunoassay techniques.

The use of classical detection systems does not often cover the identification criteria required for toxin detection. For example, measuring polychlorodibenzodioxins/ polychlorodibenzofurans (PCDD/Fs) background concentrations in food requires methods

that provide extremely high sensitivity (low-picogram to low femtogram range) as is possible with gas (GC) or high performance liquid chromatography (HPLC).[52] Both techniques have been widely used with class-selective detection methods; for instance GC has been used with electron capture (ECD), flamephotometric (FPD) and nitrogenphosphorus (NPD) detection. However, none of these detectors are confirmatory and all are subject to matrix interference. This is overcome by mass spectrometry and consequently, it has become very popular in the analysis of food contaminants.

MS has proved to be a useful technique for analysis of food contaminants, since it is a highly sensitive method for structural elucidation of chemical compounds. The variety of techniques applied, depending on the ionization mode, allow the analysis of numerous types of compounds, such as volatile or nonvolatile substances, substances with low or high mass ions, with high or low melting points and of compounds that do not contain ionizable groups. Ionization of the sample can be achieved by a number of ways; these include, collision with electrons (EI, Electron Impact), chemical ionization with the aid of a reactant gas (CI, Chemical Ionization or DCI, Desorption Chemical ionization which is a variation of CI), and high energy atoms or ions (FAB, Fast Atom Bombardment). Other techniques employed are electrospray (ESI) and Atmospheric Pressure Chemical Ionization APCI, where the dissolved sample subjected to a high difference of voltage is sprayed through a capillary at a tip where multiply-charged ions are produced, allowing the observation of ions with very high masses.

Many studies have reported the use of GC/MS or HPLC/MS, with either full scan or selected ion monitoring (SIM). However, GC/MS or HPLC/MS in full scan mode provides low sensitivity due to interferences by food matrices and SIM gives poor spectral information. On the other hand, low resolution electron impact multiple ion detection mass spectrometry (EI-LRMS) and methane-enhanced electron capture negative chemical ionization (NCI) with multiple ion detection (MID) do not provide very high sensitivities for all the food contaminants, especially for some types of polychlorinated biphenyls.[52]

Introduction of a second analyzer led to the development of tandem mass spectroscopy (MS/MS), which allows detailed structural information of a fragment ion or the identification of compounds found in complex mixtures such as food. This offers the advantage of selectively examining the fragmentation patterns of particular ions in a mixture of ions.

Gas chromatography or HPLC coupled with tandem mass spectrometry (MS/MS) offers numerous advantages with respect to selectivity and sensitivity in low quantities of “dirty”

food extracts. The increased selectivity of these techniques reduces the influence of the matrix and also lowers the limits of the detection.

MS/MS has developed significantly since the 1970s when it was first introduced in the analytical community. It has found application in a wide range of areas such as protein identification, trace analysis of biological tissue, complex hazardous waste site samples and structural studies, as well as in drug testing. During the last decade, the evolution of MS/MS has offered a strong and useful tool for the determination of food contaminants, a problem of great nutritional and health significance. Tandem MS is very specific in its identification of the compounds of interest, very accurate and can measure very small amounts of material with excellent precision. Tandem mass spectrometry coupled with chromatographic techniques combines the separation capabilities of chromatography, and the power of MS/MS as an identification and confirmation method. It is a promising analytical method with numerous applications in the analysis of complex mixtures, identification of the separated compounds, and structural elucidation of fragmentation pathways.

Products present at trace levels in food samples can now be qualitatively and quantitatively identified and analytically confirmed. Pesticides,[53,54] toxins,[55-57] alkaloids and food additives[58] have been effectively traced in foods with the use of MS/MS coupled to simple chromatographic instruments. Furthermore, tandem mass spectrometry provides superior linear response and dynamic range. Although instrumentation tends to be rather expensive, it can provide a routine analytical technique for a wide variety of samples in food.

Proteins are constituents of cells and play a crucial role in most of the biological processes. Their building blocks are 20 types of amino acids[59] among which, eight are called essential amino acids (the human body cannot synthesize them from other compounds at the level needed for normal growth), which must be obtained from food. Analysis of amino acids is of great importance in food analysis because it can provide relevant information on food quality (i.e. like food quality assessment of the final product, correlating flavor trends, etc.), processing, adulteration, protein composition, etc. Due to enantiomeric ratio of certain amino acids, chiral analysis can be used as a reliable parameter to evaluate food quality.[60] Analysis of amino acids by CE can be done as underivatized compounds or, more frequently, after derivatization (especially if LIF detection is used), which is also more sensitive also in CE-MS.[61]

In the last decade, antioxidants have been the object of increasing interest because of their good biological properties. Although they are important natural constituents of foods, they

are also widely used as food additives. They are found in varying amounts in vegetables, fruits, grain cereals, legumes and nuts.

Some antioxidants such as lycopene and ascorbic acid can be destroyed by long-term storage or prolonged cooking.[62] Other antioxidant compounds (i.e. polyphenols) are more stable in foods such as whole-wheat cereals, wine, chocolate, olive oil, bee pollen and tea.[63,64] In general, the antioxidant content in processed foods is lower than that found in fresh and uncooked foods. Many food substances such as lignans and other phenolic compounds (i.e. flavonoids such as anthocyanins and others) can also act as antioxidants. MS plays a crucial role as a sensitive, selective and accurate method for the determination of all of these compounds in foods. It can also be deduced from the relatively large number of applications on phenols and polyphenols analysis.[65-71]

Anthocyanins, the most abundant flavonoid constituents of fruits and vegetables,[72] are natural water-soluble pigments found in many plants and foods with a strong antioxidants activity. They are therefore valuable for human health,[73] for example by showing preventive action against cancer[74] and by improving visual functions.[75]

Besides the analysis of proteins/amino acids and antioxidants, some other interesting applications have also been carried out in food quality, like the analysis of vitamins, organic acids and cytokinins.

Recently, CE-MS has been applied to the analysis of vitamin B12 (cyanocobalamin) in nutritive supplements using a rapid microwave-assisted extraction procedure and a CE-ICP-MS method for the speciation analysis of cobalt in nutritive supplements and in chlorella samples.[76]

The use of geographical indications allows producers to obtain market recognition and often a premium price. False use of geographical indications by unauthorized parties is detrimental to consumers and legitimate producers. From this point of view, the development of new and increasingly sophisticated techniques for determining the geographical origin of agricultural products is highly desirable for consumers, agricultural farmers, retailers and administrative authorities. It is an analytically challenging problem that is currently the focus of much attention within Europe and the USA. Reports on analytical methods for determining the geographical origin of agricultural products have been increasing since the 1980s. The initial focus was on processed agricultural products such as wine, honey, teas, olive oil, and fruit juice, while later studies examined fresh products such as potatoes, Welsh onions, pistachios, and garlic, chiefly because world-wide trade in fresh agricultural products has increased year by year and the law now enforces

labelling of their geographical origin. Various techniques have been studied based on organic constituents, mineral contents or composition, light- or heavy-element isotope ratios, or combinations thereof.

Chemometric analysis of the data provided by analytical instruments which have the ability to determine more than one component at a time in a sample can be a support to establish links to the food origin. If the components have sufficient discriminatory power, the set of their concentrations will form a characteristic pattern or 'fingerprint' relating to the geographical origin of the sample. Chemometrics provides the ability to detect these patterns, and is essentially helpful when the number of components necessary to differentiate samples from different geographical origins increases. Several analytical approaches developed for determining the geographical origin in combination with or without chemometrics used mass spectrometry techniques and separation techniques.

Mass spectrometry (MS) is a powerful analytical technique for measuring the mass-to-charge ratio of ions.[77]

One example is isotope ratio mass spectrometry (IRMS) that is a technique that can distinguish chemically identical compounds based on their isotope content. The two most common types of IRMS instruments are continuous flow (CF-IRMS) and dual inlet (DI-IRMS).[78-81]

Inductively coupled plasma mass spectrometry (ICP-MS) is a powerful tool for the quantitative determination of a range of metals and non-metals (inorganic elements) in a wide variety of samples at trace (ppb–ppm) and ultra-trace (ppq–ppb) concentration levels.[82-85]

Also GC-MS is one of the most widely used techniques for the determination of the geographical origin of food products and represents the method of choice for the analysis of food volatiles because of its high reproducibility. However, this technique is rather expensive and time-consuming. This concerns mainly dairy products.[86,87]

2.7 Multivariate statistical analysis

Multivariate analysis consists of a collection of methods that can be used when several measurements are made on each individual or object in one or more samples. Commonly in multivariate analysis the measurements represent *variables* and the individuals or objects are *units* (research units, sampling units, or experimental units) or *observations*. In practice,

multivariate data sets are common, although they are not always analyzed as such. But the exclusive use of univariate procedures with such data is no longer excusable, given the availability of multivariate techniques and inexpensive computing power to carry them out.
The concept and tools for multivariate data analysis are collectively referred to as chemometrics in the context of chemistry. Chemometrics, being a juxtaposition of *chemo* (*latin*, chemistry) and *metrics* (*greek*, measure) is the common denominator of all possible tool applied to make rational analysis of chemical measurements.
Historically, the bulk of applications of multivariate techniques have been in the behavioral and scientific fields. However, interest in multivariate methods has now spread to numerous other fields of investigation. For example, problems of education, chemistry, physics, geology, engineering, law, business, literature, religion, public broadcasting, nursing, mining, linguistics, biology, psychology, and many other fields.
Ordinarily the variables are measured simultaneously on each sampling unit. Typically, these variables are correlated. If this were not so, there would be little use for many of the techniques of multivariate analysis. It's necessary to untangle the overlapping information provided by correlated variables and peer beneath the surface to see the underlying structure. Thus the goal of many multivariate approaches is *simplification* through the expression of what is going on in terms of a reduced set of dimensions.

2.7.1 Discriminant Analysis

Most traditional approaches to classification in science are called *discriminant analysis* and are often also called forms of 'hard modelling'.
Discriminant analysis is used in situations where the clusters are known a priori. The aim of discriminant analysis is to classify an object, or several objects, into these known groups evaluating the risk of a possible "wrong decision".
The term *group is used* to represent either a population or a sample from the population.
There are two major objectives in separation of groups:

1. Description of group separation, in which linear functions of the variables (discriminant functions) are used to describe or elucidate the differences between two or more groups. *Discriminant functions* are linear combinations of variables that best separate groups. The goals of descriptive discriminant analysis include identifying the relative contribution of the p variables to separation of the groups and finding the

optimal plane on which the points can be projected to best illustrate the configuration of the groups.

2. Prediction or allocation of observations to groups, in which linear or quadratic functions of the variables (classification functions) are employed to assign an individual sampling unit to one of the groups. The measured values in the observation vector for an individual or object are evaluated by the classification functions to find the group to which the individual most likely belongs.

Unfortunately, there is no general agreement with regard to usage of the terms discriminant analysis and discriminant functions. Many writers, perhaps the majority, use the term discriminant analysis in connection with the second objective, prediction or allocation. The linear functions contributing to the first objective, description of group separation, are often referred to as canonical variates or discriminant coordinates.

The simplest form of classification is *univariate*, where one measurement or variable is used to divide objects into groups. More often, several measurements are required to determine the group to which a sample belongs. Consider performing two measurements and producing a graph of the values of these measurements for two groups, as in figure 2.7.1. The objects represented by circles are clearly distinct from those represented by squares, but neither of the two measurements alone can discriminate between these groups, and therefore both are essential for classification. It is possible, however, to draw a line between the two groups. If above the line, an object belongs to class A, otherwise to class B.

Graphically this can be represented by *projecting* the objects on to a line at right angles to the discriminating line, as demonstrated in the figure. The projection can now be converted to a position along line 2 of the figure. The distance can be converted to a number, analogous to a ‘score’. Objects with lower values belong to class A, whereas those with higher values belong to class B.

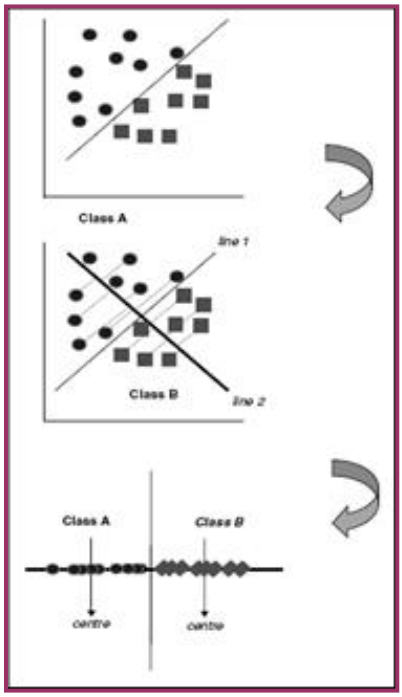

Figure 2.7.1 Discrimination between two classes, and projections.

It is possible to determine class membership simply according to whether the value is above or below a divisor. Alternatively, it is possible to determine the centre of each class along the projection and if the distance to the centre of class A is greater than that to class B, the object is placed in class A, and vice versa, but this depends on each class being roughly equally diffuse.

However, the raw data actually consist of more than one measurement, and it is possible to calculate the Euclidean class distance using the raw two-dimensional information, by computing the centroids of each class in the raw data rather than one-dimensional projection. Now the points can fall anywhere on a plane, as illustrated in figure 2.7.2.

This graph is often called a class distance plot and can still be divided into four regions:

1. top left: almost certainly class A;
2. bottom left: possibly a member of both classes, but it might be that we do not have enough information;
3. bottom right: almost certainly class B;
4. top right: unlikely to be a member of either class, sometimes called an outlier.

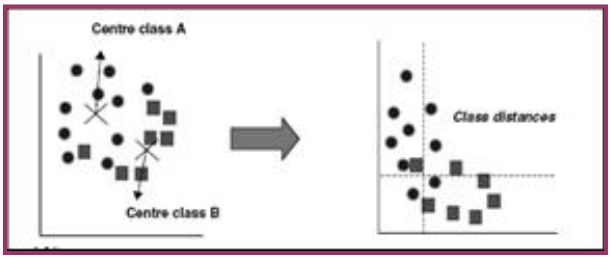

Figure 2.7.2 Class distance plot.

In chemistry, these four divisions are perfectly reasonable. For example, if we try to use spectra to classify compounds into ketones and esters, there may be some compounds that are both or neither. If, on the other hand, there are only two possible classifications, for example whether a manufacturing sample is acceptable or not, a conclusion about objects in the bottom left or top right is that the analytical data is insufficiently good to allow us to assign conclusively a sample to a graph. This is a valuable conclusion, for example it is helpful to tell a laboratory that their clinical diagnosis or forensic test is inconclusive and that if they want better evidence they should perform more experiments or analyses.

2.7.1.1 Mahalanobis Distance and Linear Discriminant Functions

It's possible to use various approaches for determining the distance between objects. Many chemometricians use the Mahalanobis distance, sometimes called the 'statistical' distance, between objects.

In areas such as spectroscopy it is normal that some wavelengths or regions of the spectra are more useful than others for discriminant analysis. This is especially true in near-infrared (NIR) spectroscopy. Also, different parts of a spectrum might be of very different intensities. Finally, some classes are more diffuse than others.

The Mahalanobis distance takes this information into account. Using a Euclidean distance each measurement assumes equal significance, so correlated variables, which may represent an irrelevant feature, can have a disproportionate influence on the analysis.

In supervised pattern recognition, a major aim is to define the distance of an object from the centre of a class. There are two principle uses of statistical distances. The first is to obtain a measurement analogous to a score, often called the linear discriminant function, first

proposed by the statistician R A Fisher. This differs from the distance above in that it is a single number if there are only two classes. This distance is defined by equation 2.7.1

$$f_i = (\overline{\boldsymbol{x}}_A - \overline{\boldsymbol{x}}_B).\boldsymbol{C}_{AB}^{-1}.\boldsymbol{x}_{i'} \qquad \text{(eq. 2.7.1)}$$

where

$$\boldsymbol{C}_{AB} = \frac{(N_A - 1)\boldsymbol{C}_A + (N_B - 1)\boldsymbol{C}_B}{(N_A + N_B - 2)}$$

which is often called the *pooled* variance–covariance matrix, and can be extended to any number of groups; N_A represents the number of objects in group A, and $\boldsymbol{C}_A$ the variance–covariance matrix for this group (whose diagonal elements correspond to the variance of each variable and the off-diagonal elements the covariance – use the population rather than sample formula), with $\boldsymbol{x}_A$ the corresponding centroid. Note that the mathematics becomes more complex if there are more than two groups. This function can take on negative values.

The second is to determine the Mahalanobis distance to the centroid of any given group, a form of *class distance*. There will be a separate distance to the centre of each group defined, for class A, by equation 2.7.2.

$$d_{iA} = \sqrt{(\boldsymbol{x}_i - \overline{\boldsymbol{x}}_A).\boldsymbol{C}_A^{-1}.(\boldsymbol{x}_i - \overline{\boldsymbol{x}}_A)'} \qquad \text{(eq. 2.7.2)}$$

where $\boldsymbol{x}_i$ is a row vector for sample i and $\boldsymbol{x}_A$ is the *mean* measurement (or centroid) for class A. This measures the scaled distance to the centroid of a class analogous to figure 2.7.2, but scaling the variables using the Mahalanobis rather than Euclidean criterion.

An important difficulty with using this distance is that the number of objects much be significantly larger than the number of measurements. Consider the case of Mahalanobis distance being used to determine within group distances. If there are J measurements than there must be at least J + 2 objects for there to be any discrimination. If there are less than J + 1 measurements, the variance–covariance matrix will not have an inverse. If there are J + 1 objects, the estimated squared distance to the centre of the cluster will equal J for each object no matter what its position in the group, and discrimination will only be possible if the class consists of at least J + 2 objects, unless some measurements are discarded or combined. It is important always to understand the fundamental properties of this distance measure, especially in spectroscopy or chromatography where there are usually a large number of potential variables which must first be reduced, sometimes by PCA.

2.7.1.2 Selection of variables

In many applications, a large number of dependent variables is available and the experimenter would like to discard those that are redundant (in the presence of the other variables) for separating the groups. This discussion is limited to procedures that delete or add variables one at a time, emphasizing that there are selecting *dependent* variables (y's), and therefore the basic model (one-way MANOVA) does not change. In subset selection in regression, on the other hand, selecting *independent* variables with a consequent alteration of the model are present. The principal useful methods to reduce variables are:

- A *forward selection* method beginning with a single variable, the one that maximally separates the groups by itself. Then the variable entered at each step is the one that maximizes the partial F-statistic based on Wilks' •, thus obtaining the maximal additional separation of groups above and beyond the separation already attained by the other variables. Since the choice of the variable is that with maximum partial F at each step, the proportion of these maximum F's that exceed F• is greater than •.
- *Backward elimination* is a similar operation in which the selection begins with all the variables and then at each step, the variable that contributes least is deleted, as indicated by the partial F.
- *Stepwise selection* is a combination of the forward and backward approaches. Variables are added one at a time, and at each step, the variables are reexamined to see if any variable that entered earlier has become redundant in the presence of recently added variables. The procedure stops when the largest partial F among the variables available for entry fails to exceed a preset threshold value. The stepwise procedure has long been popular with practitioners.

After the subset selection is completed, it's possible to calculate discriminant functions for the selected variables.

2.7.2 Soft Independent Modelling of Class Analogy (SIMCA)

The SIMCA method, first advocated by the S. Wold in the early 1970s, is regarded by many as a form of soft modelling used in chemical pattern recognition. Although there are some differences with linear discriminant analysis as employed in traditional statistics, the

distinction is not as radical as many would believe. However, SIMCA has an important role in the history of chemometrics so it is important to understand the main steps of the method.

The acronym SIMCA stands for *soft independent modelling of class analogy*. The idea of soft modelling is illustrated in figure 2.7.3. Two classes can overlap (and hence are 'soft'), and there is no problem with an object belonging to both (or neither) class simultaneously.

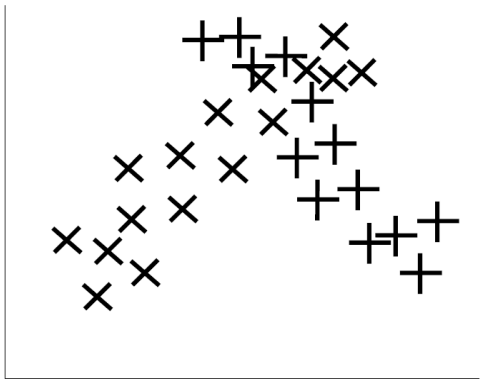

Figure 2.7.3 Two overlapping classes.

The philosophy of soft modelling is that, in many situations in chemistry, it is entirely legitimate for an object to fit into more than one class simultaneously, for example a compound may have an ester and an alkene group, and so will exhibit spectroscopic characteristics of both functionalities, so a method that assumes that the answer must be either an ester or an alkene is unrealistic. In practice, though, it is possible to calculate class distances from discriminant analysis that are close to two or more groups. Independent modelling of classes, however, is a more useful feature.

In contrast, using classical discriminant analysis the entire modelling procedure must be repeated if extra numbers of groups are added, since the pooled variance–covariance matrix must be recalculated.

The main steps of SIMCA are as follows:

a ***Principal Components Analysis.*** Each group is independently modelled using PCA. Note that each group could be described by a different number of PCs. Figure 2.7.4 represents two groups, each characterised by three raw measurements, e.g. chromatographic peak heights or physical properties. However, one group falls mainly on a straight line, defined as the first principal component of the group, whereas the other falls roughly on a

plane whose axes are defined by the first two principal components of this group. When perform discriminant analysis is performed, it's possible to use PCs prior to classification, but the difference is that the PCs are of the entire dataset, which may consist of several groups, rather than of each group separately.

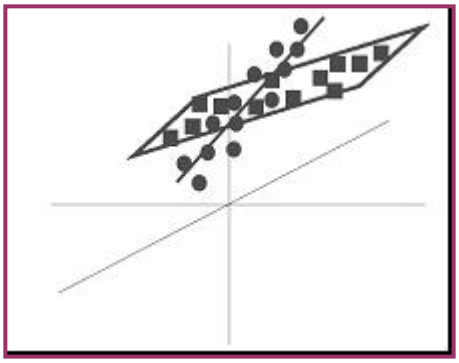

Figure 2.7.4 Two groups obtained from three measurements.

It is important to note that a number of methods have been proposed for determining how many PCs are most suited to describe a class. The original advocates of SIMCA used cross-validation, but there is no reason why one cannot 'pick and mix' various steps in different methods.

- ***Class Distance***. The class distance can be calculated as the geometric distance from the PC models. The unknown is much closer to the plane formed than the line, and so is tentatively assigned to this class. A more elaborate approach is often employed in which each group is bounded by a region of space, which represents 95% confidence that a particular object belongs to a class. Hence geometric class distances can be converted to statistical probabilities.

- ***Modelling Power.*** The *modelling power* of each variable for each separate class is defined by equation 2.7.3

$$M_j = 1 - s_{jresid}/s_{jraw} \qquad \text{(eq. 2.7.3)}$$

where s_{jraw} is the standard deviation of the variable in the raw data and s_{jresid} the standard deviation of the variable in the residuals given by equation 2.7.4.

$$E = X - T.P \qquad \text{(eq. 2.7.4)}$$

which is the difference between the observed data and the PC model for the class.

The modelling power varies between 1 (excellent) and 0 (no discrimination). Variables with M below 0.5 are of little use. This information can be used to reduce the number of measurements.

a ***Discriminatory Power.*** Another measure is how well a variable discriminates between two classes. This is distinct from modelling power, being able to model one class well does not necessarily imply being able to discriminate two groups effectively. In order to determine this, it is necessary to fit each sample to both class models. The residual matrices are then calculated, just as for discriminatory power, but there are now four such matrices:

1. samples in class A fitted to the model of class A;
2. samples in class A fitted to the model of class B;
3. samples in class B fitted to the model of class B;
4. samples in class B fitted to the model of class A.

We would expect matrices 2 and 4 to be a worse fit than matrices 1 and 3. The standard deviations are then calculated for these matrices to give equation 2.7.5.

$$\boldsymbol{D}_j = \sqrt{\frac{\text{class}A\,\text{model}B\,s_{jresid}{}^2 + \text{class}B\,\text{model}A\,s_{jresid}{}^2}{\text{class}A\,\text{model}A\,s_{jresid}{}^2 + \text{class}B\,\text{model}B\,s_{jresid}{}^2}} \qquad \text{(eq. 2.7.5)}$$

The bigger the value, the higher is the discriminatory power. This could be useful information, for example if clinical or forensic measurements are expensive, so allowing the experimenter to choose only the most effective measurements. Discriminatory power can be calculated between any two classes.

SIMCA gives also informations about other two important statistical parameters of a class: the *sensitivity* and the *specificity*.

The *sensitivity* of a class is referred to the number of objects belonging to this class that are correctly classified (equation 2.7.6)

$$SENS = \frac{\langle n_A \rangle}{n_A} = 1 - \text{a} \qquad \text{(eq. 2.7.6)}$$

Where $\langle n_a \rangle$ is the number of objects correctly classified, n_a is the total number of objects of this class and • is an error that represents the probability to wrongly exclude a member of this class and to consider it a no-member (false negative case).

The specificity of a class corresponds to the number of objects not belonging to this class that are correctly classified as belonging to different classes (equation 2.7.7)

$$SPEC = \frac{\langle \overline{n_A} \rangle}{\overline{n_A}} = 1 - \beta \qquad \text{(eq. 2.7.7)}$$

Where $\langle n_a \rangle$ is the number of objects correctly classified as not belonging to that class, $\overline{n_a}$ is the number of objects belonging to different classes and • is an error that represents the probability to wrongly classify a no-member of a class as an its member (false positive case).

[a] ***Validation.*** Like all methods for supervised pattern recognition, testing and cross-validation are possible in SIMCA. There is a mystique in the chemometrics literature whereby some general procedures are often mistakenly associated with a particular package or algorithm; this is largely because the advocates promote specific strategies that form part of papers or software that is widely used. It also should be recognized that there are two different needs for validation: the first is to determine the optimum number of PCs for a given class model, and the second to determine whether unknowns are classified adequately.

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Scope of the thesis

The scope of this thesis was to apply the hyphenated techniques GC/MS, GC-ITD tandem mass spectrometry and LC-MS/MS to perform methods useful and usable for the traceability and safety of some foodstuffs.

In order to characterize the volatile fraction of olive oil and tomato to trace their origin two SPME-GC/MS methods were performed.

While, exploiting the selectivity and sensitivity of tandem mass spectrometry, two methods for the determination of phthalates in olive oil by GC-MS/MS and dimethoate in different vegetable matrixes by LC-MS/MS were performed.

Food quality and safety and their legislations has been introduced in chapter 1. While in chapter 2, the hyphenated techniques, such as GC/MS and LC/MS were described with particular consideration for tandem mass spectrometry techniques and quantitative analysis.

In the next chapters will be shown the used procedures and the obtained results of four research works:

1. A metabolomic approach to the evaluation of the origin of extra virgin olive oil: A convenient statistical treatment of mass spectrometric analytical data.
2. Geographical characterization of tomato by SPME-GC/Ion Trap Mass Spectrometry analysis of volatile components and chemometric treatment.
3. Tandem mass spectrometry in food safety assessment: The determination of phthalates in olive oil.
4. Screening of dimethoate in food by isotope dilution and electrospray ionization tandem mass spectrometry.

Chapter 3

Results and discussion

A metabolomic approach to the evaluation of the origin of extra virgin olive oil: A convenient statistical treatment of mass spectrometric analytical data.

Geographical characterization of tomato by SPME-GC/Ion Trap Mass Spectrometry analysis of volatile components and chemometric treatment.

Tandem mass spectrometry in food safety assessment: The determination of phthalates in olive oil.

Screening of dimethoate in food by isotope dilution and electrospray ionization tandem mass spectrometry.

3.1 A metabolomic approach to the evaluation of the origin of extra virgin olive oil: A convenient statistical treatment of mass spectrometric analytical data

Virgin olive oil represents the most consumed edible fat in the food intake of the Mediterranean basin, obtained by physical methods from the pulp of the stone fruit of the olive tree *Olea europaea* L. More than 90% of the world's olive harvest comes from the Mediterranean region, primarily in Italy and Spain. Olive tree plantations are found to a smaller extent in Japan, Australia, California and South America.

Fresh and good quality virgin olive oil is highly appreciated by consumers for its delicious flavour and aroma. For this reason its flavour is of special interest and often used as important criterion for the quality of virgin olive oil and.

The benefits of virgin olive oil (VOO) consumption are related to protection against cancer and cardiovascular diseases due to its fatty acid profile and the presence of minor constituents such as phenolic compounds.[1,2] However, the increase in the demand for high-quality VOO can be attributed not only to its potential health benefits but also to its excellent organoleptic properties.

Protected Designation of Origin, Protected Geographical Indication, and others are official European Union classifications, which should guarantee both the quality and the origin of the foodstuff.[3,4]

Only some works have been conducted on the identification of markers for the origin of monovarietal oils by analytical methods.[5-7]

Many and different are the factors affecting the profiling of the volatile components, a peculiar role is, in fact, played by the cultivar, the atmospheric, pedologic, and fostering conditions, the ripening degree, the olive and oil storing procedure, and the technology of oil extraction from drupes.[8-16]

The genetic effect related to the cultivar is one of the most important aspects of volatile composition of olive oil. However, climatic and agronomic conditions of olive growing can affect volatile composition of olive oils obtained by the same cultivar, because these conditions have heavy influence on enzymatic activities. Also the geographic origin of

oils plays a fundamental role in defining the volatile compounds profile of virgin olive oils.[17]

In conclusion we can consider that several agronomic and climatic parameters can affect the volatile composition of olive oils. For this reason volatile compounds can be considered as markers, with sensory impact, that can be used to differentiate them.

It is well established that those volatile compounds, which are the constituents of aroma of many fruits and vegetables, are produced from polyunsaturated fatty acids through a cascade of enzymatic reactions known as the lipoxygenase pathway (figure 3.1.1).[18-22]

In a recent work, we have correlated some typical parameters, such as cultivar and ripening stage, of southern Italian olive oils with the distribution of five biomarkers, present in the volatile fraction, originating from this metabolic pathway.

The principal components of this aerobic secondary metabolism being mainly represented by aliphatic C6 species.[8, 23-25]

In this work a statistical method based on the metabolomics of monovarietal olive oils was explored to recognize their origin. In the case of Tunisian oils, the evaluation of the effect of irrigation was possible thanks to the substantial lack of raining in this area, in which the irrigation is almost exclusively artificial.

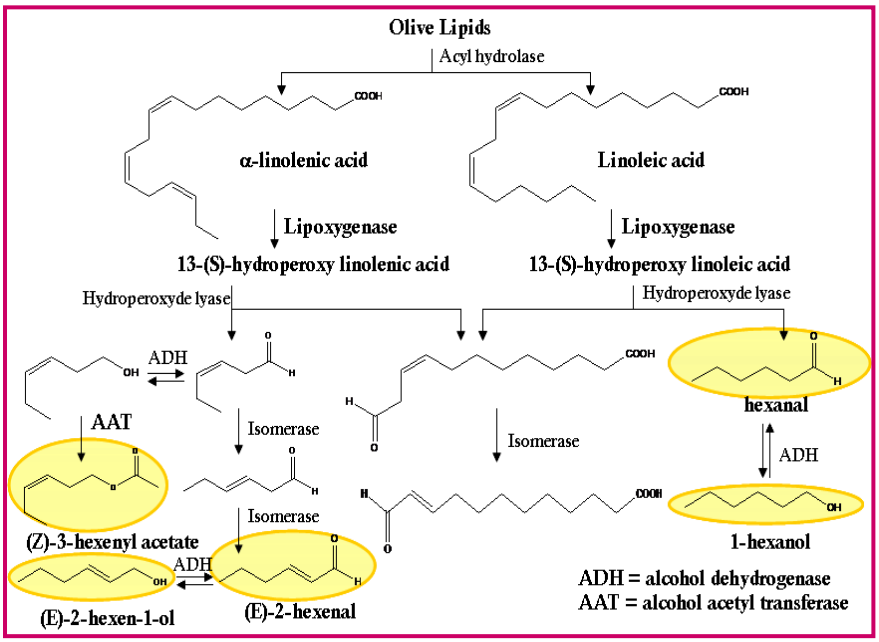


Figure 3.1.1 Lipoxygenase pathway.

Different methods for the identification of olive oil aroma components have been devised, most of which are based on dynamic headspace and SPME experimental approaches.[26] In particular, our group has proposed that the concentrations of the five biomarkers: hexanal, (*E*)-2-hexenal, 1-hexanol, (E)-2-hexen-1-ol, (Z)-3-hexen-1-yl acetate, five secondary metabolites out of the number of species present in the volatile fraction of olive oil and originating from lipoxygenase pathway, could provide clues for cultivar and ripening phase discrimination of different oils.[16]

Accordingly, we are now exploring the correlation, by statistical methods, of the geographical origin of the oil with the concentrations of these biomarkers monitored by GC-CI-ITD mass spectrometry.

3.1.1 Experimental conditions

The experimental conditions for the preparation sample by SPME were optimized in the previous work.[16] The volatile compounds absorbed on carbowax/DVB fibers and desorbed, by an autosampler device, into the mass spectrometer previously described were tested in both electron ionization (EI) and CI modes. A typical reconstructed chromatogram obtained in CI mode is shown in figure 3.1.2 where the ion current profile due to the five markers and to the IS is indicated.

A further disadvantage of the EI method is represented by the lack of structural specificity of the ions, which can be used to build up the chromatogram of the oil samples under investigation. On the contrary, the ionization with isobutane provided diagnostic fragments for each analytes. Isobutane, in fact, favors the obtainment of highly specific mass spectra, preventing also, at a great extent, interferences with coeluted analytes.

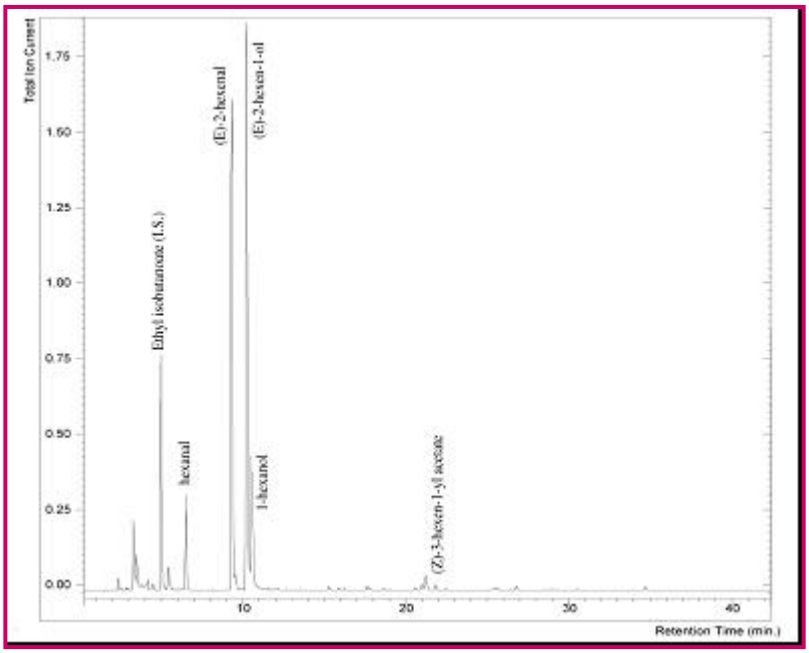


Figure 3.1.2 Typical total ion chromatogram in CI mode of a Tunisian oil obtained from trees grown in the irrigated farmlands.

In particular, a more satisfactory integration was obtained in CI mode for (*E*)-2-hexenal, even though its retention time, 9.30, is close to that of (*Z*)-3-hexen-1-ol, 9.41 min. In CI mode, in fact, the ion at *m/z* 99 chosen for assay of (*E*)-2-hexenal because of its relative intensity is absent in the spectrum of (*Z*)-3-hexen-1-ol spectra obtained in the same ionization conditions (Figure 3.1.3 A).

On the contrary, in EI mode (Figure 3.1.3 B), any abundant ion selected for the assay of the (*E*)-2-hexenal may suffer from interferences with similar, or identical, ionic species present in the EI spectrum of (*Z*)-3-hexen-1-ol.

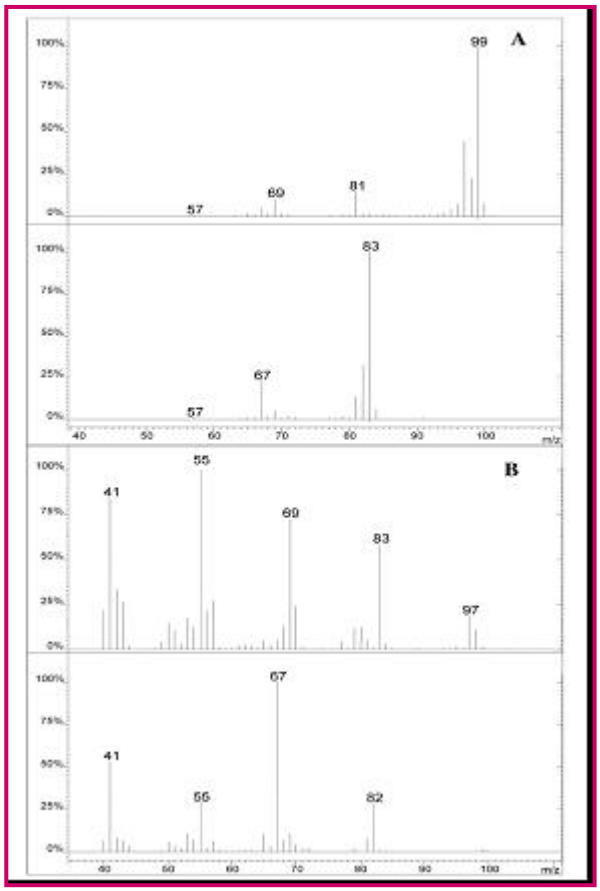


Figure 3.1.3 Spectra of (E)-2-hexenal and (Z)-3-hexen-1-ol in CI (**A**) and EI (**B**) modes.

The CI (isobutane) mass spectra of the internal standard (a) used for quantitative measurements and those of the five biomarkers previously described (b-f) are shown in Figure 3.1.4. It can be easily verified that the total ion current of each spectrum is associated mainly to a single cluster of ions. Moreover, the selection of identical ionic species, i.e., those at *m/z* 83 for b, c, and f, refers to peaks eluted at different times and does not cause any overlapping problem (Figure 3.1.4).

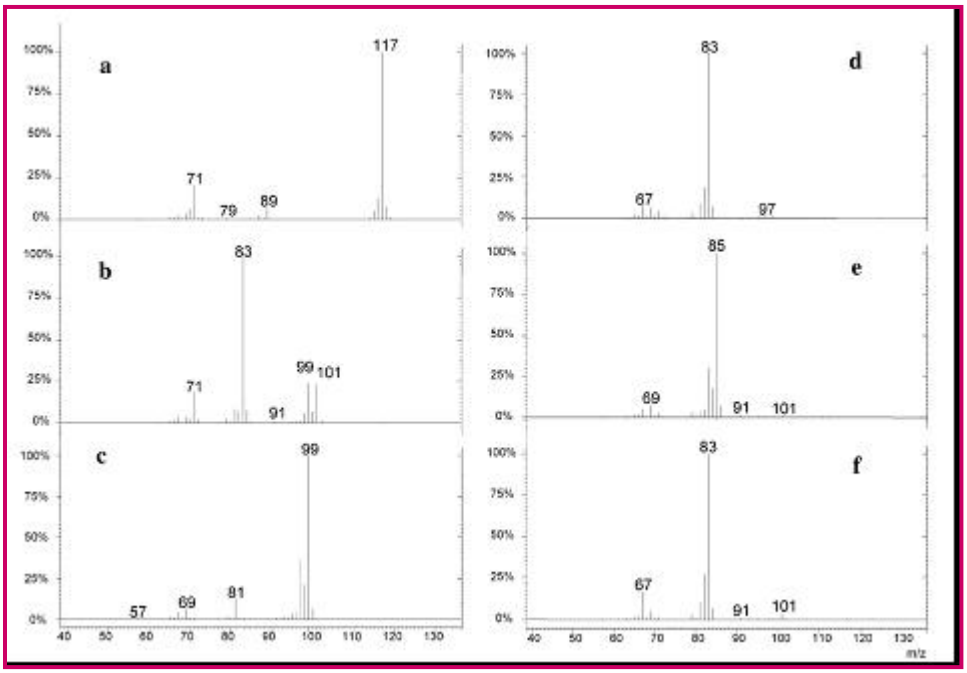


Figure 3.1.4 CI spectra of (**a**) ethyl isobutanoate (IS), (**b**) hexanal, (**c**) (E)-2-hexenal, (**d**) (E)-2-hexen-1-ol, (**e**) 1-hexanol, and (**f**) (Z)-3-hexen-1-yl acetate.

The experimental olive oil samples were produced from drupes harvested in different areas of the Italian Calabria region and different regions of Tunisia (Figure 3.1.5).

The quantitative data of calabrian olive oil samples were subjected to linear discriminant analysis, whereas the Tunisian data were treated by means of other two statistical tools, i.e., the Kruskal-Wallis test and the Wald-Wolfowitz test and the effect of irrigating farmlands was also considered to account for severe drought periods that may be encountered in this country.

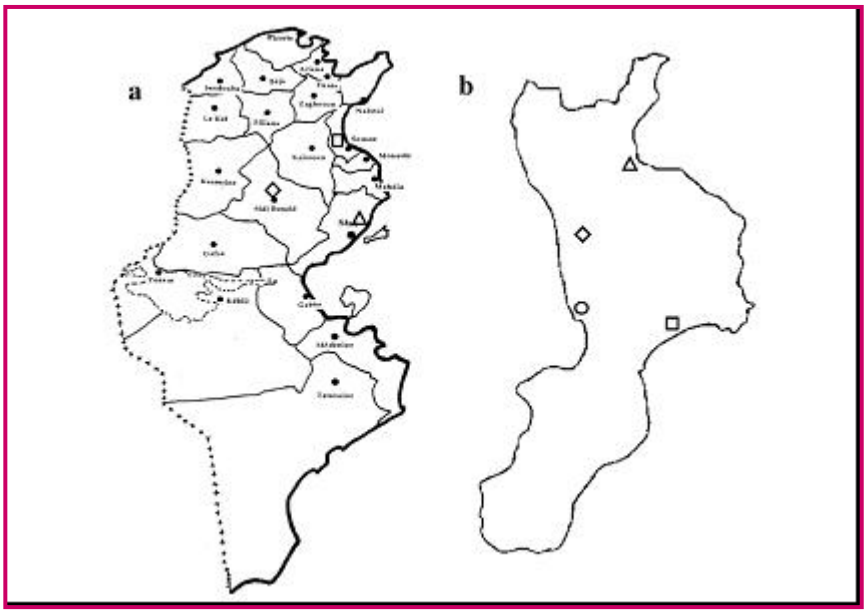


Figure 3.1.5 (a) Tunisia map [Agro-Combinat Enfidha (Sousse), •; Domaine Ettaous (Taous, Sfax), •; Agro-Combinat Touila (Sidi Bouzid), •]. **(b)** Calabria map (Nocera Terinese, •; Catanzaro, •; Rende, •; and Corigliano, •).

3.1.2 Results and discussion

Calabrian Oils. Experimental Calabrian oils were produced from drupes of the same cultivar, Carolea, and harvested in four areas of Calabria: Nocera Terinese, Corigliano, Catanzaro and Rende. The relative ratios of the five markers obtained by the method previously described (Table 3.1.1) show that substantial differences can be observed among the oils produced in the selected different regions. Samples C-1 to C-4, from Nocera Terinese area show, on the average, a higher concentration of alcohols with respect to aldehydes, in particular hexanal, which might be due to an overexpression or overactivity of the dehydrogenase (ADH) enzymes. This behavior is even more pronounced in the oils produced in Corigliano (C-9 to C-12); in this case, in fact, the concentration of the two alcohols is more than doubled as compared to the concentration of the aldehydes. Moreover, the much higher absolute content of the four analytes than in the Nocera Terinese oils could be either due to higher activity of LOX enzymes or to a higher concentration of linolenic and linoleic acids, substrates of

lipoxygenase enzyme, in the drupes harvested in Corigliano area. The detection of hexenyl acetate, even if as a minor component, in the C-9 to C-12 samples, only, could be related to the relative concentration of the alcoholic components in the aroma of the drupes harvested in the Corigliano area.

Similar observations can be drawn for the oils produced from drupes of the other Calabrian areas. Different activity or different expression of the LOX enzymes as a function of the different pedoclimatic parameters might affect the observed behavior.

A statistical evaluation of the data could provide better tools for interpreting the meaning of the results, providing clues to identifying the origin of production.

In order to perform statistical multivariate analysis, data in table 3.1.1 were used as a data matrix in which each row is a sample and each column is quantitative data for each analyte of interest.

sample	origin	hexanal	(*E*)-2-hexenal	(*E*)-2-hexen-1-ol	1-hexanol	(*Z*)-3-hexen-1-yl acetate
C1	Nocera T.	0.080	0.256	0.296	0.412	ND
C2	Nocera T.	0.072	0.218	0.275	0.324	ND
C3	Nocera T.	0.090	0.374	0.331	0.308	ND
C4	Nocera T.	0.062	0.305	0.214	0.454	ND
C5	Catanzaro	0.745	0.379	0.175	0.315	ND
C6	Catanzaro	0.72	0.392	0.083	0.174	ND
C7	Catanzaro	0.657	0.298	0.112	0.250	ND
C8	Catanzaro	0.569	0.302	0.287	0.241	ND
C9	Corigliano	0.758	1.105	1.863	1.802	0.001
C10	Corigliano	0.625	0.892	1.750	1.825	0.015
C11	Corigliano	0.703	0.748	1.678	1.786	0.009
C12	Corigliano	0.108	0.670	1.480	1.813	0.028
C13	Rende	0.498	2.203	0.264	0.299	ND
C14	Rende	0.712	1.785	0.122	0.240	ND
C15	Rende	0.767	1.938	0.096	0.240	ND
C16	Rende	1.065	1.161	0.214	0.799	ND

Table 3.1.1 Calabrian Olive Oils; Markers Concentration (ppm) in the Volatile Fraction of the Oils Produced in Four Different Selected Areas.

3.1.3 Statistical analysis

The first statistical approach was Principal Component Analysis (PCA) in order to obtain a first information about the discriminant power of the selected analytes and the real possibility of considering them as markers.

The second statistical approach selected for Calabrian oils was the Linear Discriminant Analysis (LDA), which could allow the classification of unknown samples after having

verified the possible differences among samples of known origin. Also in this case, data treatment has been applied to the concentrations of the five markers, using the four groups, corresponding to the four selected areas, as a priori input.

In the two-dimensional plot of the first two roots, the clear separation of the four clusters representing the four areas of production is clearly displayed (Figure 3.1.6). From a statistical point of view, this differentiation is also very significant since the very low Wilks • value (0.0000146) shows that the model is highly discriminating, whereas the high value (83.23) of the F (15.22) parameter indicates a significant difference among the means of the groups. Finally, the information from data treatment is characterized by a high degree of reliability since the p level is extremely low (<0.00001).

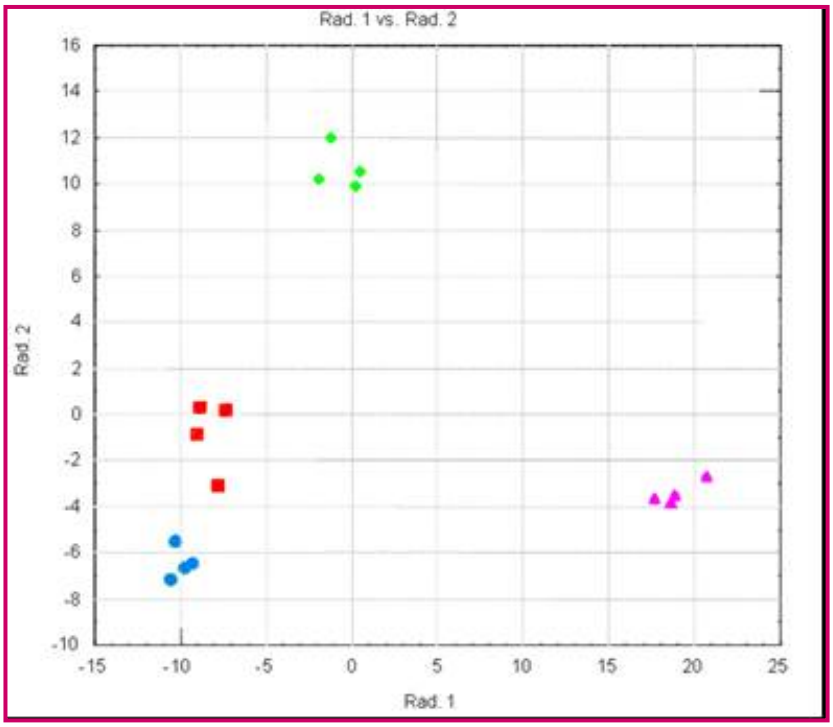


Figure 3.1.6. LDA plot of 16 olive oil samples based on quantitative values of the five selected compounds (Nocera Terinese, • ; Catanzaro, • ; Rende, • and Corigliano, •).

To verify the stability of the model, the method was checked with unknown samples. In particular, one set of four samples, containing one sample for each production zone, was randomly removed in three independent runs and the model was recalculated.
In all cases, the samples were correctly classified, thus showing the stability of the system.
The LDA (the obtained statistical parameters are shown in table 3.1.2) shows that a major contribution to the differentiation is provided by the aldehydes as compared to alcohol and ester components even if all of the components contribute in a significant manner to group separation and identification.

	Wilks' λ	partial Wilks' λ	*F* remove	*p* level
hexanal	0.000188	0.077425	31.77538	0.000086
(*E*)-2-hexenal	0.000491	0.029639	87.30497	0.000002
(*E*)-2-hexen-1-ol	0.000097	0.150666	15.03254	0.001189
1-hexanol	0.000050	0.292956	6.43596	0.015853
(*Z*)-3-hexen-1-yl acetate	0.000087	0.166399	13.35907	0.001756

Table 3.1.2. Linear Discriminant Analysis of the Five Selected Compounds.

Tunisian Oils. Experimental Tunisian oils provided by the Institut de l'Olivier of Sfax (Tunisia) were produced from drupes of the same Chemlali cultivar, harvested in the three different regions. The effect of irrigation on the profiling of the volatile components was checked for oil produced from drupes harvested in Enfidha and Sidi Bouzid regions. Quantitative data (Table 3.1.3) show that alcohols are nearly absent whereas (*Z*)-3-hexen-1-yl acetate is present at very low amounts. This result is in part contradictory to the data obtained by Marzouk et al.[27] that showed that a percentage of alcohols accounted for about 15% of the whole C6 fractions of Chemlali cultivar. This discordance could be explained by the different production area of analyzed oil samples (locality of Bouargoub in the region of Cap-Bon) or by different ripening degrees of olives.

sample	irrigation	origin	hexanal	(*E*)-2-hexenal	(*E*)-2-hexen-1-ol	1-hexanol	(*Z*)-3-hexen-1-yl acetate
E1	no	Enfidha	0.905	8.314	ND	ND	ND
E2	no	Enfidha	0.223	9.311	ND	ND	ND
E3	no	Enfidha	0.353	18.056	ND	ND	0.04
E4	no	Enfidha	0.885	4.036	ND	ND	ND
E5	no	Enfidha	0.669	7.398	ND	ND	ND
E6	yes	Enfidha	1.124	3.815	11.94	4.966	ND
E7	yes	Enfidha	1.492	6.008	10.73	11.28	0.024
E8	yes	Enfidha	2.203	6.225	47.38	8.854	0.029
E9	yes	Enfidha	1.962	12.12	25.81	3.82	0.024
E10	yes	Enfidha	0.821	0.507	29.63	19.01	ND
E11	yes	Enfidha	0.817	1.369	13.18	12.19	ND
E12	yes	Enfidha	0.663	1.728	7.457	4.146	ND
S1	no	Sidi Bouzid	1.121	16.48	ND	ND	0.009
S2	no	Sidi Bouzid	1.456	26.55	ND	ND	0.002
S3	no	Sidi Bouzid	1.239	4.033	ND	ND	0.022
S4	no	Sidi Bouzid	1.725	13.48	ND	ND	ND
S5	no	Sidi Bouzid	1.447	5.882	ND	ND	ND
S6	yes	Sidi Bouzid	0.185	1.157	3.262	0.227	ND
S7	yes	Sidi Bouzid	1.871	4.54	0.44	0.143	ND
S8	yes	Sidi Bouzid	1.663	13.08	0.239	ND	0.006
S9	yes	Sidi Bouzid	1.54	3.808	0.328	ND	ND
S10	yes	Sidi Bouzid	1.086	4.419	0.738	ND	0.02
T1	no	Taous	0.253	13.21	ND	ND	0.001
T2	no	Taous	0.585	15.591	0.106	ND	ND
T3	no	Taous	0.804	13.62	ND	ND	0.001
T4	no	Taous	0.597	4.674	ND	ND	0.02
T5	no	Taous	0.517	12.32	1.24	0.462	0.004
T6	no	Taous	0.794	9.06	ND	ND	0.024
T7	no	Taous	0.484	3.073	ND	ND	ND
T8	no	Taous	0.109	3.156	ND	ND	ND
T9	no	Taous	0.008	1.074	ND	ND	0.028
T10	no	Taous	0.493	3.995	ND	ND	ND
T11	no	Taous	0.542	8.742	ND	ND	ND

[a] ND, not detected.

Table 3.1.3. Concentrations (ppm) of Selected Compounds from LOX Pathway of Samples for Areas of Tunisian.

For these samples there is another variable: the irrigation. To evaluate this effect on the profiling of the volatile components were performed two different statistical analyses.

For the non-irrigated samples the Kruskal-Wallis test to hexanal and (*E*)-2-hexenal variables was performed (Figures 3.1.7 and 3.1.8). Figure 3.1.8 shows that there are not significant differences among concentrations of (*E*)-2-hexenal in olive oil produced in different geographical areas of Tunisia. On the contrary, hexanal seems to be a suitable variable to distinguish oils produced in the Sidi Bouzid region from samples from Enfidha and Taous (Figure 3.1.7). In fact, the Kruskal-Wallis test applied to the three groups of oils showed a highly significant difference between Sidi Bouzid and Taous (p=0.002268) and a less marked difference between Sidi Bouzid and Enfidha (p=0.074797). Enfidha and Taous did not result different (p=1.00000).

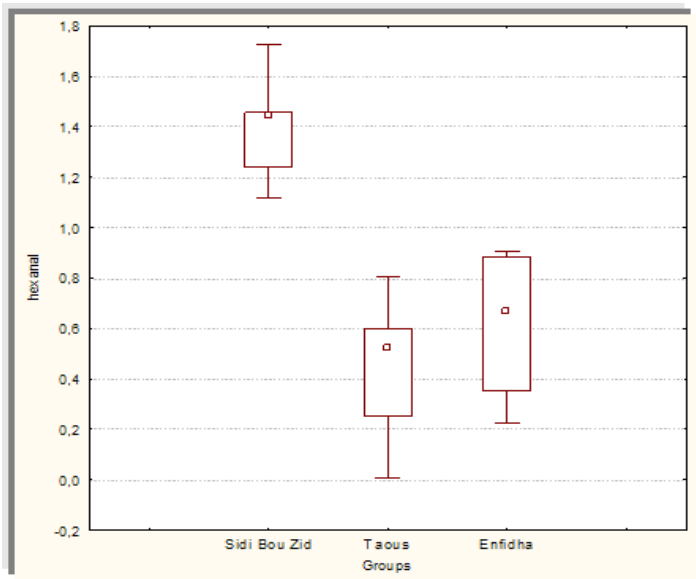


Figure 3.1.7. Box and Whisker plot of the quantitative data of hexanal in Tunisian oils (medians, small squares; 25-75%, large bars; and min-max, lines).

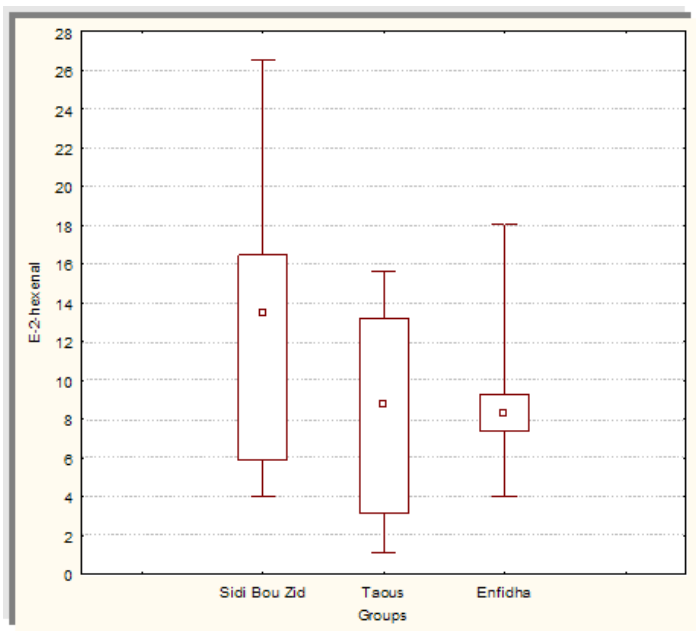


Figure 3.1.8. Box and Whisker plot of the quantitative data of (E)-2-hexenal in Tunisian oils (medians, small squares; 25-75%, large bars; and min-max, lines).

Peculiar features of samples from Sidi Bouzid could be ascribed to different geographical positions in Tunisia (Figure 3.1.9). In fact, the region of Sidi Bouzid lies inland, whereas Sfax and Sousse are near the coast. This fundamental difference could mean similar pedologic and climatic conditions in Sfax and Sousse regions but different to Sidi Bouzid. Furthermore, it could suppose a lower amount of linoleic acid (precursor of hexanal) in olives from Sfax and Sousse with respect to Sidi Bouzid.

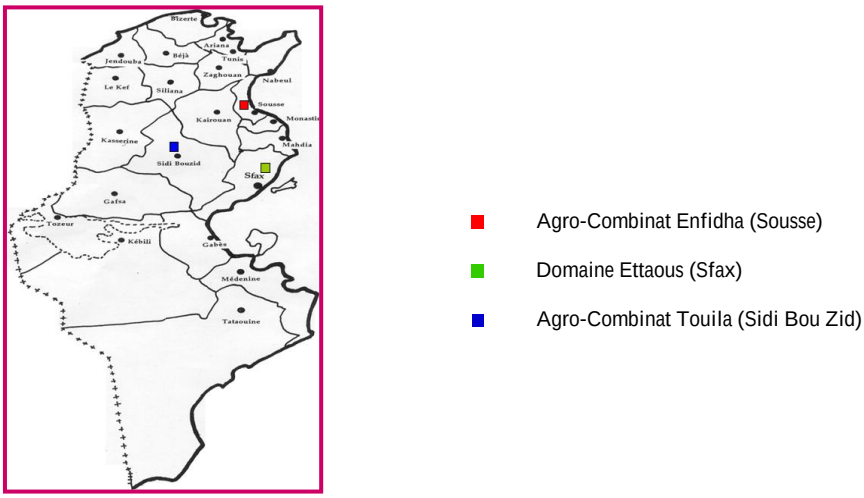


Figure 3.9. Map of Tunisia.

While for the irrigated samples we had two groups of samples (Enfida e Sidi Bouzid) and three variables (hexanal and the two alcohols, 1-hexanol and (*E*)-2-hexen-1-ol).
Seven samples from Enfidha and five samples from Sidi Bouzid, all obtained from trees grown in the irrigated farmlands, were then analyzed. In all 12 cases, it was noticed that the samples from Enfidha contained a very high amount of 1-hexanol as compared to the other samples, which also contained a considerable amount of 1-hexanol. In this case, it could be assumed that the lack of water affects to some extent the activity (or the concentration) of alcohol dehydrogenase enzymes important in the LOX pathway and, thus, results in lowering the content of (*E*)-2-hexen-1-ol and 1-hexanol. The characterization of production areas was carried out by the Wald-Wolfowitz test because, in this case, the comparison concerned two groups of samples. The results are shown in Figure 3.1.10.

The statistical tool has demonstrated that hexanal was not any longer an appropriate variable (p=0.60270; Figure 3.1.10 A), since its amount was enormously increased in the sample grown in irrigated farmlands from Enfidha. The latter, therefore, has become undistinguishable from those produced from trees not grown in irrigated farmland. On the contrary, the same test applied to (*E*)-2-hexen-1-ol and 1-hexanol has proved the possibility of distinguishing sample with remarkable certainty (p=0.00253; Figure 3.1.10 B,C).

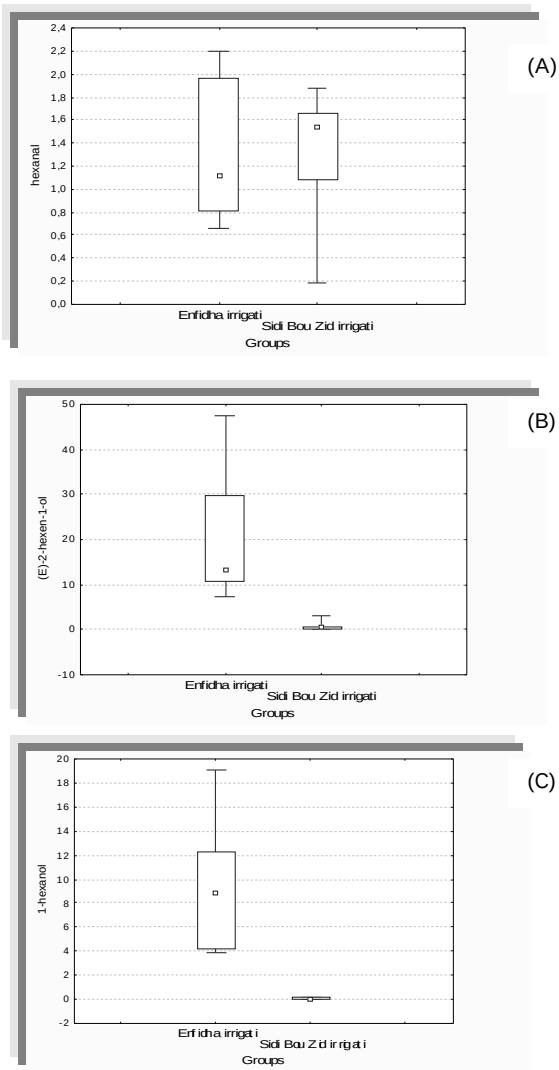


Figure 3.1.10 Box and Whisker plot of the quantitative data of hexanal (**A**), (E)-2-hexen-1-ol (**B**), and 1-hexanol (**C**) in Tunisian oils obtained from olive fruits of irrigated trees (medians, small squares; 25-75%, large bars; and min-max, lines).

In conclusion, the metabolomic approach is currently considered in our and other groups as reliable means to evaluate the authenticity of foodstuff, since the number and relative concentration of components produced by any secondary metabolism pathway could store information related to the story of the aliment.

In the case here examined, we have been able to distinguish olive oils produced in very close areas of the same region. This result opens the opportunity of building-up useful databases to recognize the origin of this important foodstuff. Moreover, it represents the first step for the solution of the old problem of olive oil authenticity, which is associated, nowadays, to the bottling sites only.

3.2 Geographical characterization of tomato by SPME-GC/Ion Trap Mass Spectrometry analysis of volatile components and chemometric treatment.

During the second year of PhD the same method for assaying of olive oil aroma was opportunely modified and applied for assaying of aroma of tomato. Also in this case the aim was to establish correlations between areas of origin of the fruit and its aroma in order to protect this important product from food frauds.

Tomato (*Lycopersicon esculentum* MILL.), species belonging to family of Solanacee, is characterized by unique and complex flavor. Analysis of volatile compounds of tomato appears to be difficult in fresh fruits because the composition of their aroma is unstable due to the presence not only of the most important flavor compounds that origin during the ripening of fruits, but also to the presence of other important flavor compounds that are formed enzymatically in appreciable amounts only when the tissue is disrupted by slicing, chewing, or blending.

The main precursors of volatile compounds in tomato are free amino acids, fatty acids and carotenoids. Over 400 volatile components have been identified in fresh tomatoes, while, during processing, the endogenous enzymes catalyze the formation of other important volatile compounds. In fact saturated and unsaturated C6 and C9 alcohols and aldehydes, that are impact compounds of fresh tomato, are originated through lipoxygenase activity, while terpene and carotenoid derivatives can be released from odourless glycosidic compounds by glycosidase activities.[28,29]

It's known that the flavor of fresh fruits and vegetables, and also the flavor of fresh tomato, is strictly influenced from the characteristics of soil and of climate of the growing areas.[30]

The aim of this work was to value the possibility to characterize the volatile compounds of the fresh and processing tomato and to try to trace their origin from the obtained data.

This work takes place in a very timely because recently the fight the import of foodstuffs from outside the UE that are not complied with safety standards and hygiene is becoming increasingly fierce in order to protect consumer health and ensure the originality of the Italian product fighting fraudulent attribution of origin.

Imported food products can severely damage the health of consumers because, in many countries outside the EU, many of the laws which require the immediate removal of pesticides, herbicides and pesticides which proved harmful to humans, already adopted within the EU, are not yet applicable,.

In particular, in 2007 the tomato concentrate imports from China are tripled (+163%) for an amount of 160 million kilograms which equates to about a quarter of the entire production of tomatoes grown in Italy. To achieve this, as from 1 January 2008, the measure on the origin of fresh tomatoes used in the preparation of tomato puree became mandatory, without exception, in our country. The rules governing the labeling of prescription and tomato sauce makes reference to the DLG of 17 February 2006 of MIPAF.

From 1 January 2008 so all foods marketed as "tomato sauce" must specify the geographical source of the tomato used.

3.2.1 SPME optimization

The analytical method used was chosen referring to the many sources available in literature concerning the analysis of volatile components of fresh tomato and its derivatives using SPME-GC-MS used to establish quantitative and qualitative volatile compounds that constitute it.

The sample preparation, still frozen, was homogenized and sealed in a vial equipped with a stopper pierceable septum together with the internal standard and $CaCl_2$ as soon as possible before thawing to avoid loss of volatile components easily degradable.[31] Regarding the quantity of sample introduced into the vial, some tests were performed to evaluate the response of the analytes respect to sample volume. Optimum amount was 2 g of fresh tomato samples and 3g for paste tomato samples.

The quantity of $CaCl_2$ to add to sample, in order to deactivate the enzyme system that leads to the degradation of volatile compounds, was evaluated by reference to studies of Beltran et al.[31] and Bezman et al.[32] In these studies, the amount of $CaCl_2$ was added for 5% (w / w) compared to the weight of the sample introduced into the vial and a volume in ml equal to the sample weight in grams. The our best results were obtained adding an equal volume of $CaCl_2$ to the weight in grams of sample.

The internal standard used for semiquantitative analysis of analytes of interest was the ethyl isobutanoate in reference to studies conducted by Sindona et al.[16]

Choosing the most suitable SPME fiber was made in reference to studies conducted by Beltran et al.[31] based on the comparison of the efficiency of extraction from three different SPME fibers used in the extraction of volatile compounds present in the space of testing of samples of fresh tomato. These studies showed that Carboxen/PDMS fiber is the more efficient then PDMS/DVB and CW/DVB fibrs, as shown in Figure 3.2.1, in which the absolute peak areas of analytes extracted are shown.

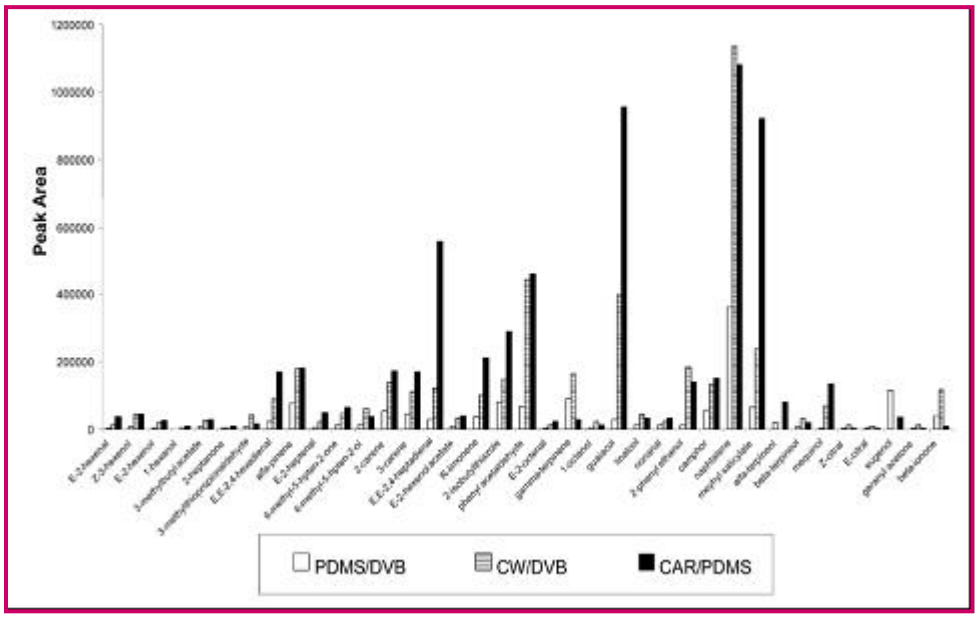


Figure 3.2.1 Comparison of the efficiency of extraction between three different fibers.[31]

All other parameters such as pre-heading, the conditions of adsorption and desorption were chosen in reference to the study of Beltran et al.[31] The only changed parameter was the time of desorption, which was increased to 15 minutes instead of 5 minutes because only after this time release of the analytes was found optimal.

In order to test the possibility of using the autosampler, several tests were performed to evaluate peak areas of analytes of interest after 8, 10, 12 hours after the initial analysis.

Since there was not a significant loss of analytes in this range of time, we decided to perform the analysis with autosampler.

The analysis were performed on ten samples of fresh tomato growing in each of five different areas of Italy and ten samples of concentrated tomato originating from three of the same five areas of production (Table 3.2.1).

Number of Samples	Areas of Origin	Region	Fresh tomato	Concentrated tomato
10	Isola Capo R.	Calabria	X	X
10	Crotone	Calabria	X	X
10	Matera	Basilicata	X	
10	Mesagne	Puglia	X	
10	Collecchio	Emilia R.	X	X

Table 3.2.1. Samples of fresh tomato and paste tomato collected in five different areas of Italy.

The acquisition of the mass spectra was conduced in electronic impact mode according Beltran et al.[31] A typical chromatogram of volatile components of tomato obtained with these conditions is shown in figure 3.2.2.

The chromatograms were integrated and the ratios areas of analytes to areas of internal standard corresponding to every peak were submitted to statistical analysis.

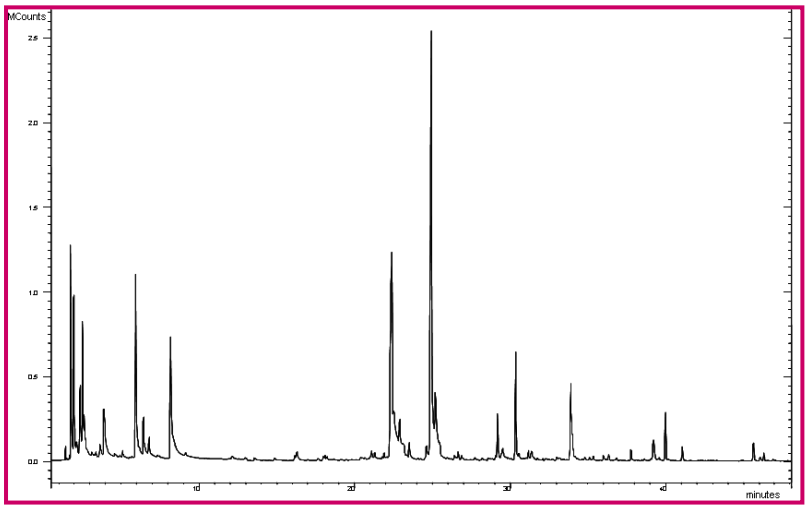


Figure 3.2.2. Typical chromatogram of a fresh tomato sample obtained with the experimental conditions previously described.

3.2.2 Statistical analysis

Fresh tomato samples. The first two methods are methods of classification which allow to classify unknown samples attributing the clusters that arise from the model. While the SIMCA analysis is a method of modeling that considers the characteristics of independent samples from the clusters that are formed without forcing the assignment in one of them.

The analysis was performed by taking as variables the concentrations of analytes for each sample and using 5 groups, corresponding to the five areas of origin, as input *a priori*.

Initially, the obtained data were subjected to Principal Component Analysis (PCA) in order to assess the data structure, the importance of variables and their possible correlations. Figure 3.2.3 shows the two-dimensional plot of scores built according to the first two principal components.

In the plot of the scores it's possible to note a differentiation of the samples on the first principal component for all classes, except Mesagne for which there is no clear trend.

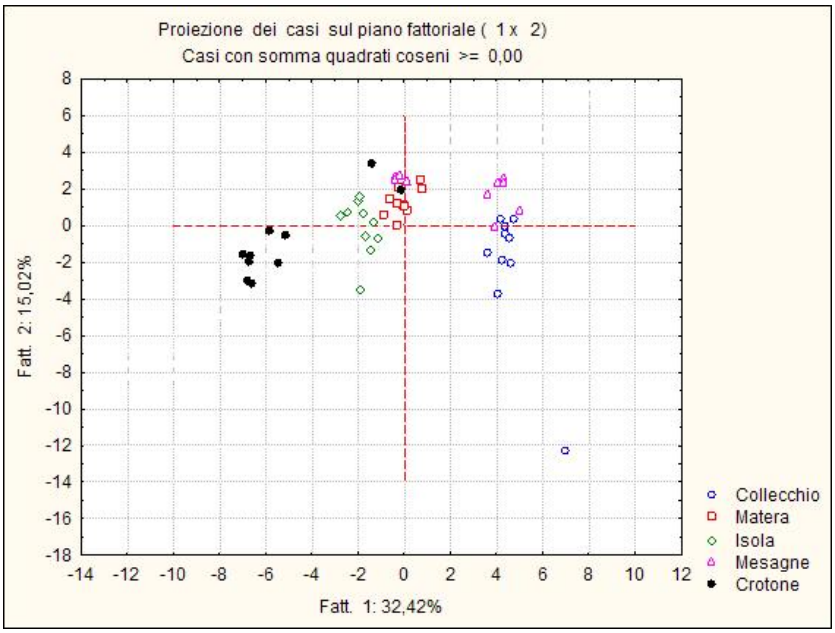


Figura 3.2.3. Two-dimensional plot obtained by PCA for fresh tomato samples.

The obtained data were then subjected to three different multivariate statistical analysis in order to assess the ability to discriminate between different classes and the contribution of variables to the differentiation between objects. These approaches were:

- Stepwise Linear Discriminant Analysis (Stepwise LDA)
- LDA applied on the Principal Components
- Soft Indipendent Modelling of Class Analogy (SIMCA)

In the case of stepwise LDA, the selected variables were the 15 most discriminating of a total of 43 variables (Table 3.2.2).

Analytes	Wilks	Parziale	F-rimoz.	p-level
o-cymene	0.000037	0.114261	60.07735	0.00000
D-limonene	0.000020	0.209548	29.23436	0.000000
4,5-dimethyl-1-hexene	0.000019	0.220570	27.38624	0.000000
linalyl acetate	0.000016	0.259927	22.06610	0.000000
γ-terpinene	0.000014	0.300256	18.06133	0.000000
isobutylthiazole	0.000011	0.374091	12.96686	0.000003
3-methyl-2-heptanone	0.000009	0.455151	9.27731	0.000047
2-pentanone	0.000009	0.488155	8.12611	0.000133
unknown (rt 1,95)	0.000008	0.545788	6.44964	0.000675
2-methoxy-2-methylpropane	0.000008	0.547680	6.40060	0.000709
geranyl acetone	0.000007	0.576179	5.70068	0.001471
unknown (rt 1,77 min)	0.000007	0.585332	5.49034	0.001843
1-(2,6,6-trimethyl-1,3-cyclohexadienil)-2-buten-1-one	0.000007	0.585838	5.47892	0.001866
tetrahydrothiazolo	0.000006	0.675340	3.72571	0.013743
hexenyl acetate	0.000006	0.693238	3.42943	0.019666

Table 3.2.2. Variables selected by stepwise LDA.

In two-dimensional plot of the first two roots is clear the separation of the five clusters that represent areas of origin (figure 3.2.4).

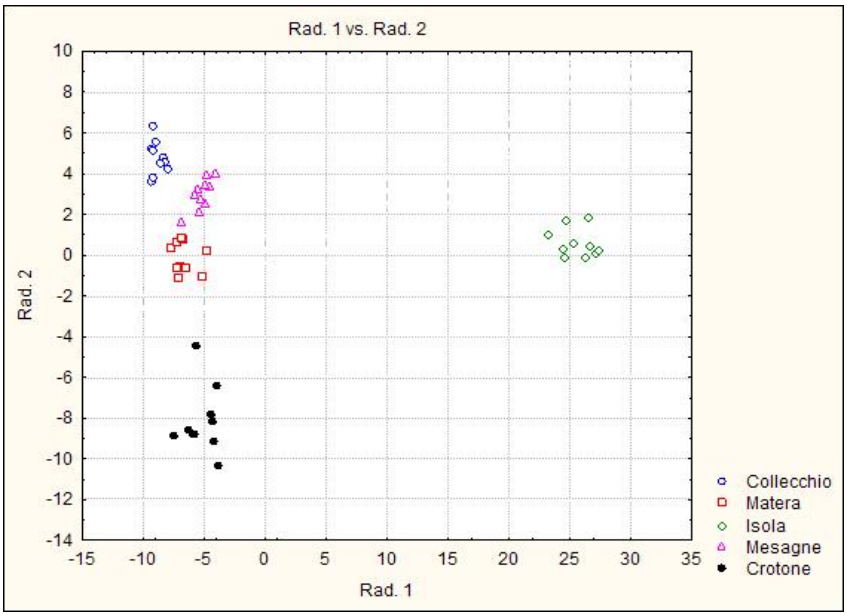


Figure 3.2.4. Two-dimensional plot obtained by stepwise LDA for fresh tomato samples.

This difference is statistically significant and the value • of Wilks very low (<0.000001) confirms this result and shows that the model is highly discriminatory. Moreover also the value F=46.797 is indicative of a difference enough significant between the avarages of groups. Finally, the result can be considered highly reliable because the p-level is very low (<0.00001).

In order to test the stability of the obtained model, the cross validation was performed using unknown samples. In particular, a set of 5 samples, consisting of one sample for each area of origin, was removed randomly ten times, and the model was recalculated. The model can be considered rather stable because it provided 98% of prediction ability. In particular, as shown in the matrix prediction (Table 3.2.3), there was only one erroneous attribution of a sample belonging to the class of Crotone in the class of Matera.

Real Classes \ Assigned Classes	Collecchio	Matera	Isola C. R.	Mesagne	Crotone
Collecchio	10	0	0	0	0
Matera	0	10	0	0	0
Isola C. R.	0	0	10	0	0
Mesagne	0	0	0	10	0
Crotone	0	1	0	0	9

Table 3.2.3. Cross validation performed on stepwise LDA on fresh tomato samples.

However, the stepwise LDA does not guarantee the selection of the optimal combination of original variables because it considers the discriminant power of individual variables without taking into account possible correlations between them that can play an important role in the differentiation of classes.
For this reason we decided to perform LDA considering as variables the top ten components of the Principal Component Analysis (PCA), previously performed, that account for a total variance of 85.36%.
Also in this case in order to test the stability of the resulting model cross validation was carried out and the obtained prediction ability was 92%. This result is due, as shown in table 3.2.4, to false attributions of two samples, belonging to the class Crotone, to the class of Matera, and other two samples of Mesagne wrongly classified in other two classes.

Real Classes \ Assigned Classes	Collecchio	Matera	Isola C. R.	Mesagne	Crotone
Collecchio	10	0	0	0	0
Matera	0	10	0	0	0
Isola C. R.	0	0	10	0	0
Mesagne	1	1	0	8	0
Crotone	0	2	0	0	8

Table 3.2.4. Cross validation performed on LDA applied on the first ten principal components for fresh tomato samples.

The last statistical analysis carried out on obtained data chemometric was Soft Independent Modeling of Class Analogy (SIMCA), which is a technique for modeling of class. Unlike the two previously described methods, with the SIMCA there are no

assumptions about the distribution of variables and each class is represented by a model made by its significant principal components. The objects, unlike LDA, can belong to multiple classes simultaneously.

SIMCA was performed on 42 variables and provided a statistical model enough reliable and stable characterized by three important statistical parameters:

- A capacity of prediction of 96% obtained by cross validation (Table 3.2.5);
- A medium sensitivity of 86%. This parameter describes the number of objects correctly classified to the class in cross validation;
- A medium specificity of 100%. This parameter indicates the number of objects that do not belong to a class and are correctly attributed to different classes.

		Assigned Classes				
		Collecchio	Matera	Isola C.R.	Mesagne	Crotone
Real Classes	Collecchio	9	0	0	1	0
	Matera	0	10	0	0	0
	Isola C.R.	0	0	10	0	0
	Mesagne	1	0	0	9	0
	Crotone	0	0	1	0	9

Table 3.2.5. Cross validation performed on SIMCA for fresh tomato samples.

With the SIMCA analysis we also obtained a two-dimensional plot, "Coomans plot", that put graphically in evidence the distances of the objects belonging to each class from the obtained model. Figure 3.2.5 shows the Coomans plot for the classes Mesagne and Crotone.

The objects of both classes are close to the model built with a confidence level greater than 95%, except for an object of the class of Mesagne and two objects of the class of Crotone with a lower confidence level and for this reason they represent false negative cases, being however correctly classified.

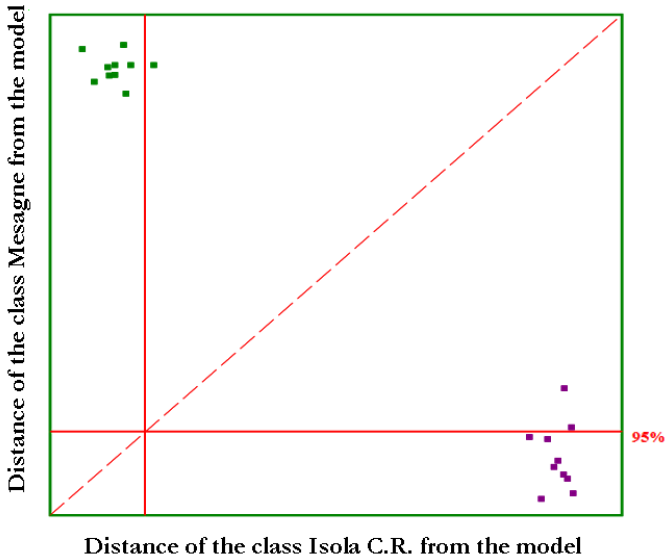


Figure 3.2.5. Coomans plot for fresh tomato samples.

Paste tomato samples. Data obtained from samples of tomato paste were subjected only to stepwise LDA. The variables selected were the seven most discriminant in a total of 47 (Table 3.2.6).

Analytes	Wilks	Parziale	F-rimoz.	p-level
2,2,4-trimethyl-5-Hexen-3-ol	0.008647	0.059225	166.7890	0.000000
benzaldehyde	0.001935	0.264685	29.1698	0.000000
1-methoxy-2-propanol	0.001926	0.265870	28.9930	0.000001
Unknown (rt 1.77 min)	0.001768	0.289672	25.7479	0.000002
2,4,4-trimethyl pentanoate	0.001435	0.356780	18.9299	0.000020
heptanal	0.001138	0.449962	12.8353	0.000228
linalool	0.001062	0.482057	11.2817	0.000470

Table 3.2.6. Variables selected by stepwise LDA for paste tomato samples.

The obtained plot (Figure 3.2.6) shows the clear separation of the three clusters characterized by the following parameters: the Wilks • <0.00051, F equal to 129.56 and p-level <0.0000. The Cross validation has shown a prediction ability of 100%.

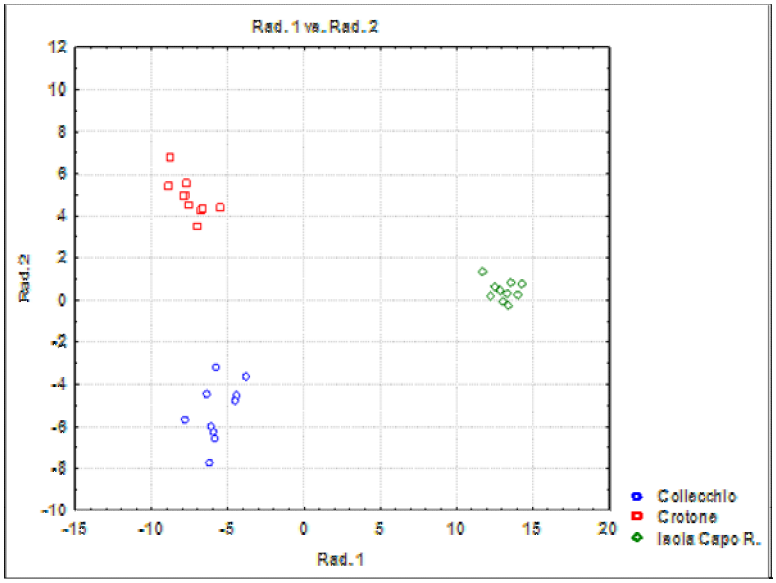


Figure 3.2.6. Two-dimensional plot obtained by stepwise LDA for paste tomato samples.

The Coomans plot, shown in Figure 3.2.7, evaluates the distance of the object, belonging to the classes of Crotone and Isola Capo Rizzuto, from the obtained model.

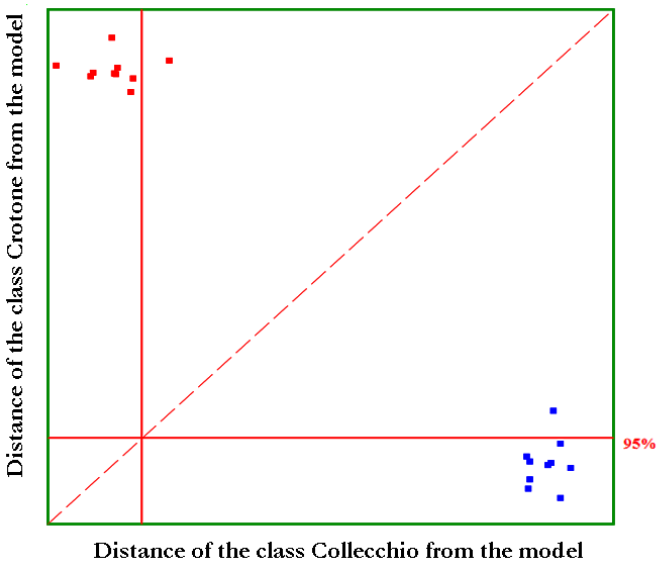


Figura 3.2.7. Coomans Plot for the classe of Crotone and Isola Capo Rizzuto for tomato paste samples.

It's possible to note, observing the plot, that all objects belonging to the two classes are close to the model built with a confidence level greater than 95%, except for two samples that result to be false negatives but correctly classified.

In this work the ability to differentiate fresh tomatoes and tomato concentrate from different zones of Italy according to the distribution of components of the volatile fraction of the samples was evaluated.

The best conditions for SPME-GC-MS analysis were extrapolated from several studies in literature.

The obtained data were submitted to various techniques of multivariate analysis.

The models obtained from both LDA and SIMCA have proved highly reliable and robust, underlining the possibility to use the volatile fraction of tomato, fresh and concentrate paste, as a suitable marker for identification of the origin and the authenticity of this important food.

3.3 Tandem mass spectrometry in food safety assessment: The determination of phthalates in olive oil.

Phthalates are a family of industrial compounds with a common chemical structure, dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid (figure 3.3.1). Since about the 1930s phthalates have been used for a variety of purposes, including personal-care products (e.g. perfumes, lotions, cosmetics), paints, industrial plastics, and certain medical devices and pharmaceuticals.[33-41]

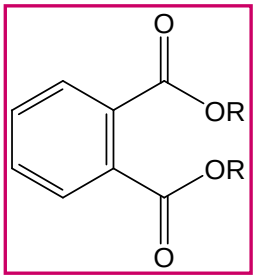


Figure 3.3.1 Common chemical structure of phthalic acid esters.

Physical properties and therefore their field of application depend on the length and branching of the dialkyl or alkyl/aryl side chains (the alcohol portion of the ester).
Some phthalates are commonly added to these commercial products to hold color or fragrance, to provide a film or gloss, or, in the case of some pharmaceuticals, to provide timed releasing. However, phthalates are primarily used as plasticizers to impart flexibility to an otherwise rigid polyvinylchloride (PVC).[33-36]
Since phthalates does not form stable bonds with polymer to which they are added, they tend to migrate in contact materials, especially in the case of oily and fatty foodstuff.[42]
Humans are exposed to these compounds through ingestion, inhalation, and dermal exposure for their whole lifetime, since the intrauterine life.[43-45]
It has been estimated to be in the range of 3 to 30 •g/kg of body weight/day (excluding occupational exposure, medical exposures, and nondietary ingestions in children), the major source being from residues in food.[40,46-48]
The most frequently used ester, di-2-ethylhexyl phthalate (DEHP), became a ubiquitous pollutant in the environment and, particularly, in foods.

The possible adverse health effects and environmental damage potential of phthalates is presently a topic of discuss and interest and, from this point of view, many articles have been published.

Some phthalates and/or their metabolites are suspected human cancer-causing agents, and endocrine disruptors.[49-51] Due to their potential risks to human health and the environment, certain phthalates have been included in the priority list of pollutants in several countries. Possible endocrine disrupting properties of up to 12 PAEs, including di-*n*-butyl phthalate (DBP), butylbenzyl phthalate (BBP), and di-2-ethylhexyl phthalate ester (DEHP), were also reported by European Union (EU).[52] Moreover, their potential dangerousness has prompted the United States Environmental Protection Agency (EPA) to list phthalates as priority contaminants, warranting tighter regulation of these compounds.[53]

In general, since phthalates are lipophilic, these compounds tend to be distributed mostly in fatty foods and this can cause the presence of remarkable amounts of PAEs in olive oil. Therefore, determination of phthalates in olive oil and, in general, in fatty matrices represents a very important goal for the consumers' health and confidence.

Several methods for determination of PAEs in fatty matrices have been published. Some procedures use adsorption column chromatography on Florisil or alumina,[54,55] sweep co-distillation techniques for phthalate separation in matrices, such as animal tissues, fats or cheeses with a high fat content.[56] Many procedures use high performance liquid chromatography (HPLC) to determine phthalates in complex matrices, regardless of the fact that lower sensitivity of determination.[57]

Most of the clean-up steps currently applied are based on gel permeation chromatography (GPC), where separation is performed according to molecular dimensions of analytes.

This methodology is relatively effective at removing fats and oils and is applicable to a wide range of analytes such as pesticides, PAH and phthalates. This chromatographic technique has been utilized for determining PAEs in several foodstuff as milk, cream, butter and cheese.[58,59]

Recently, Holadová et al. have developed a method for assay of PAEs in olive oil by HS-SPME employing solvent-based matrix modification.[60]

In any case, the majority of methods for the determination of PAEs involve gas chromatography coupled with several detector as flame ionization detector (FID), electron capture detector (ECD)[61,62] or mass spectrometry (MS).[58,59,63-66] The former techniques do not provide unequivocal confirmation of identity and are often subject to matrix interferences, whereas MS acquisition was usually carried out in single ion monitoring (SIM) mode.

The main purpose of this work was to develop and validate a simple and rapid method for the analysis of phthalates in olive oil by GC–MS/MS after a GPC clean-up. In particular, it was investigated the capability of tandem mass spectrometry (GC–MS/MS) for the unequivocal confirmation and accurate quantification of PAEs at low limits of detection (LOD) levels in fatty matrices without the need for a liquid–liquid extraction prior to GPC and for SPE clean-up following GPC. The resulting tandem mass spectrometry method was compared to SIM acquisition mode.

In many cases, in fact, a further SPE clean-up step or a liquid/liquid extraction step prior to GPC purification is often required to remove any remaining lipid and other matrix components.[67]

European directives on food safety and quality have been issued by national and international official board, however the best results can be achieved by adopting measures on the basis of a mutual consensus between consumers and producers within the European framework of "the farm to the fork" approach.[68] In this regards the QS system[69] launched by a German producers association could represent a reference for applied research in this particular field.

A further drawback is associated to the assay of PAEs in fatty foods since they are structurally related to the components of the lipidic fraction, thus showing similar retention times between polluting and natural constituents of the aliments. The solution to the problem is, at the moment, represented either by the conversion of PAEs into easily separable dimethyl phthalate[70] or by the "Stable Isotope Dilution" approach after the on purpose narrowing of GPC fraction containing phthalates.[58,59,71]

We have preferred the MS/MS approach, that it has never been applied in the determination phthalates in any food matrix, as a suitable second dimension when the first chromatographic stage is difficult or impossible to be implemented. In this work, therefore, a reasonable improvement of chromatographic parameters combined with the

optimal exploitation of MS/MS facilities have allowed the achievement of satisfactory values of sensitivity, accuracy and precision in the assay of PAEs in olive oil.

3.3.1 Sample extraction and clean-up step

In order to optimize experimental variables of GPC system, a calibration solution was prepared in dichloromethane containing corn oil at 25 mgl$^{\bullet 1}$ and DEHP at 1 mg l$^{\bullet 1}$.
For removal of fatty and high-molecular mass compounds, GPC step was applied directly to the sample dissolved in dichloromethane (500 mg of olive oil up to 5 ml of solvent).
The same solvent was used as mobile phase as previously suggested by other authors[72] and by EPA Method 3640a. In these conditions, the separation of the low-molecular mass PAEs from the high-molecular mass lipidic fraction was not satisfactory, so other mixtures were tested as mobile phase:

- Cyclohexane : ethyl acetate (1:1)
- Cyclohexane : dichloromethane (1:1)

The better result in terms of separation and resolution was achieved by mobile phase cyclohexane:dichloromethane, so several ratios of this mixture were tested. The optimum was obtained using cyclohexane:dichloromethane in ratio 7:3.

3.3.2 GC-ion trap MS analysis

The second step, after the optimization of GPC conditions, was the GC/MS analysis.
The reliability of any analytical approach to pollutant evaluation strongly depends on the possibility of identifying without any ambiguity the structure of the analytes of interest, especially in the case of complex matrices. Since the retention time alone does not often provide reliable results, GC/MS has represented the method of choice either in the selected ion monitoring (SIM) approach or better by the use of mass spectrometry/mass spectrometry (MS/MS) methodology. This bidimensional mass spectrometric analysis which can be performed “in time” or “in space”, increases the sensitivity by drastically reducing the background without loosing its specificity in

analyte identification.[73-79] In analysis of contaminants and pesticides, especially in food matrices, this technique is often preferred respect to others, because it offers an higher selectivity and specificity, and moreover, the possibility to obtain lower detection and quantification limits.

In this work, MS/MS acquisition was performed either in electron impact (EI) ionization mode or in chemical ionization (CI) mode (with isobutane as reagent gas) in order to compare the two methodologies and achieve better analytical performances.

For MS/MS conditions, the resonant method for collision induced dissociation (CID) was chosen for all compounds. A multi-segment acquisition method was adopted to optimize the excitation storage level and amplitude of excitation voltage for each compound. The value of excitation storage level used was achieved by the Varian software tool "q-calculator" that provides a theoretical optimum RF value for a specific precursor ion mass. The CID Voltage was firstly optimized using the automated method development (AMD) feature. That permits to increase the excitation voltage through a range by 0.1V steps.

Instrumental parameters used in EI mode and in CI mode are shown in table 3.3.1 and table 3.3.2, respectively.

Compound	Retention times (min)	Time range (min)	Precursor ion (*m/z*)	Excitation storage level (*m/z*)	Excitation amplitude (V)	Product ion-quantifier (*m/z*)
DMP	8,47	7,00–9,00	163	65,0	0,46	133, 135, 77
DEP	9,60	9,00–11,00	149	60,0	0,44	121, 93, 65
DBP	12,12	11,00–13,00	149	60,0	0,50	121, 93, 65
BBP	15,45	13,00–16,50	149	60,0	0,50	121, 93, 65
DEHP	17,90	16,50–20,00	149	60,0	0,44	121, 93, 65
DEHP-d_4 (I.S.)	17,85	16,50–20,00	153	60,0	0,45	125, 97, 69
DOP	22,42	20,00–24,00	149	60,0	0,46	121, 93, 65

Table 3.3.1 Electron ionization tandem mass spectrometry (EI-MS/MS) parameters.

Compound	Precursor ion (*m/z*)	Excitation storage level (*m/z*)	Excitation amplitude (V)	Product ion-quantifier (*m/z*)
DMP	163	65.0	0.54	133, 135
DEP	177	72.0	0.95	149
DBP	149	60.0	0.97	121, 81
BBP	149	60.0	0.70	121, 65
DEHP	149	60.0	0.42	121, 65
DEHP-d_4 (I.S.)	153	60.0	0.46	125, 69
DOP	149	60.0	0.40	121, 65

Table 3.3.2 Chemical ionization tandem mass spectrometry (CI-MS/MS) parameters.

All the MS/MS spectra have been obtained by selecting the base peak (*m/z* 163 for DMP and *m/z* 149 for all the other analytes) as precursor ion. EI-MS/MS spectra of all PAEs except DMP (Figure 3.3.2 B) exhibit three product ions (*m/z* 121, 93 and 65) corresponding, probably, to the consecutive eliminations of formal CO groups, whereas spectrum of DMP shows an abundant ion *m/z* 133 that could correspond to the loss of formal units of formaldehyde (Figure 3.3.2 A).

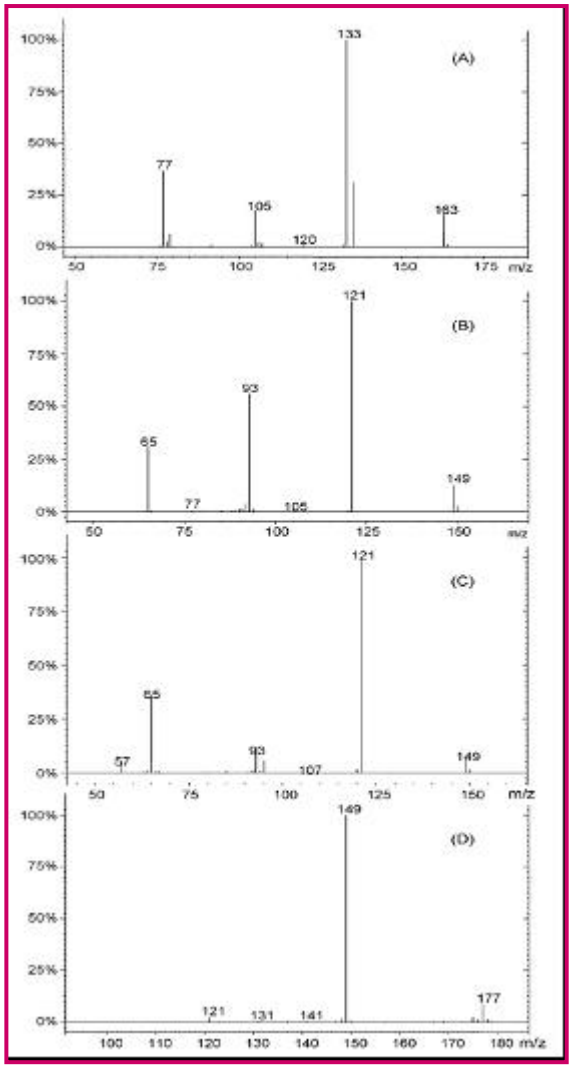


Figure 3.3.2. (A) EI-MS/MS and CI-MS/MS spectra of DMP; (B) EI-MS/MS spectra of DEP, DBP, BBP, DEHP, DOP; (C) CI-MS/MS spectra of DBP, BBP, DEHP, DOP; (D) CI-MS/MS spectrum of DEP.

Similar spectra have been obtained in CI mode except for DEP (Figure 3.3.2 C). For this phthalate, *m/z* 177 was chosen as precursor ion and the corresponding spectrum exhibit an abundant ion *m/z* 149 (Figure 3.3.2 D).

Since EI-MS/MS and CI-MS/MS spectra are similar, it was not possible to choice a priori the best ionization method for achieving the best analytical performances. In order to compare the two methodologies, the analytical parameters were therefore evaluated in both ionization modes.

A comparison between calibration standards prepared in pure solvent and spiked matrix standards was carried out. The relative response for solvent standards compared to matrix standards was found to be significantly higher for BBP due to the presence of fat traces in the final extract that co-elute with BBP. Internal standard is not subjected to the same suppression of signal and this effect would lead to quantitation bias when it was made against calibration standards prepared in pure solvent. Thus, calibration curves were constructed using spiked matrix standards for the two ionization modes.

Internal standard calibrations were performed to obtain the calibration curves by measuring the peak areas relative to that of the internal standard. A good linearity was achieved up to 0.1–2 mg $kg^{\bullet 1}$ for EI-MS/MS as it is shown in table 3.3.3. A range of 0.3–2 mg $kg^{\bullet 1}$ was only considered for DEHP. Linearity obtained in CI mode is comparable to calibration achieved in EI only for DMP, DEP and DBP, whereas R^2 values for BBP and DEHP have shown the calibration curves to be unsatisfactory. Finally, correlation Coefficient for DOP resulted satisfactory only in a more limited linear range.

Compound	EI-MS/MS			CI-MS/MS		
	Calibration range (mg kg^{-1})[a]	Curve	R^2	Calibration range (mg kg^{-1})	Curve	R^2
DMP	0.1–2	$y = 0.7642x - 0.0051$	0.99964	0.1–2	$y = 9.5330x - 0.6723$	0.99407
DEP	0.1–2	$y = 0.9177x + 0.0656$	0.99684	0.1–2	$y = 8.0579x + 0.7026$	0.99498
DBP	0.1–2	$y = 0.1638x + 0.0197$	0.99676	0.1–2	$y = 0.0361x + 0.0549$	0.99256
BBP	0.1–2	$y = 0.7603x + 0.0261$	0.99799	0.1–2	$y = 1.4619x + 0.0596$	0.98373
DEHP	0.3–2	$y = 1.4261x + 0.2301$	0.98910	0.3–2	$y = 1.8235x + 0.1594$	0.96039
DOP	0.1–2	$y = 1.6153x - 0.0171$	0.99833	0.1–0.8	$y = 1.4152x - 0.0639$	0.99990

Table 3.3.3 Calibration parameters, [a] Internal standard at 0.5 $mgkg^{\bullet 1}$.

Accuracy and precision have been evaluated at two concentration values (0.5 and 1.7mg $kg^{\bullet 1}$) in the acquisition modes previously described (Table 3.3.4). Analytical data were obtained by subjecting spiked sample to clean-up process three times and by injecting the resulting solution three times.

Compound	EI-MS/MS				CI-MS/MS			
	0.5 mg kg^{-1}	1.7 mg kg^{-1}	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	0.5 mg kg^{-1}	1.7 mg kg^{-1}	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
DMP	86.3 (9.7)	91.8 (3.1)	0.1	0.2	83.0 (11.2)	86.6 (10.9)	0.1	0.4
DEP	98.2 (2.7)	112.2 (5.8)	3.4	6.0	99.2 (6.8)	96.7 (8.8)	2.8	5.7
DBP	101.1 (9.5)	110.7 (4.7)	33	46	108.5 (64.4)	124.8 (12.4)	2.9	5.4
BBP	100.0 (3.0)	112.0 (6.8)	22	37	89.9 (30.0)	130.1 (22.3)	0.5	1.5
DEHP	71.7 (6.7)	94.8 (5.6)	148	182	92.5 (56.7)	96.4 (14.7)	156	195
DOP	84.4 (8.8)	111.9 (3.0)	0.6	1.4	65.8 (4.8)	117.5 (3.2)	4.7	13.1

Table 3.3.4 Summary of mean accuracies (%), relative standard deviations (RSD (n = 9), %, in parentheses) and limits of detection (LODs) and limits of quantitation (LOQs)

As can be seen, comparison between EI-MS/MS and CI-MS/MS acquisition shows clearly that EI-MS/MS parameters are considerably better than those obtained in CI-MS/MS for all the analytes.

Accuracy values in EI mode were generally in the range 86–112% except for DEHP at 0.5% $mgkg^{\bullet 1}$ (71.7%). The relative standard deviations obtained were very good: the RSD values were between 2.7% and 9.7%, whereas CI acquisition gave very unsatisfactory values for DBP, BBP and DEHP.

The limit of detection (LOD) and the limit of quantitation (LOQ) for the olive oil were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry, that is, as follows:

$$S_{LOD} = S_{RB} + 3\bullet_{RB}$$

$$S_{LOQ} = S_{RB} + 10\bullet_{RB}$$

where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of the reagent blank, and • RB is the standard deviation for the reagent blank. The concentrations were calculated by the standard curve. Comparing LOD and LOQ values in EI mode with those obtained in CI mode, it can be said that the

proposed method shows comparable detection limit. Only for DBP and BBP it was achieved lower values in CI mode.

An overall evaluation of all analytical parameters shows that acquisition in EI-MS/MS mode provides satisfactory data and is to prefer rather than the acquisition in chemical ionization for all the analytes of interest.

3.3.3 Comparison of MS/MS acquisition with SIM mode

Most of the studies regarding assay of PAEs in several food have been carried out by analyzing phthalates in single ion monitoring (SIM). In order to compare results obtained in tandem mass spectrometry with the more common methodology, solutions resulting from clean-up GPC step were subjected to analysis EI-SIM. Quantification ions and analytical parameters are summed in table 3.3.5.

Compound	Calibration range[a]	Quantification ions	Curve	R^2	0.5 mg kg^{-1}	1.7 mg kg^{-1}	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
DMP	0.1–2	163	$y = 0.5271x + 0.0226$	0.99461	134.9 (1.6)	89.3 (33.3)	68	195
DEP	0.1–2	149, 177	$y = 0.8065x + 0.2735$	0.99825	101.6 (2.2)	112.1 (0.7)	148	259
DBP	0.1–2	149	$y = 0.4814x + 0.0773$	0.98648	110.4 (2.9)	111.2 (6.2)	149	333
BBP	0.1–2	149	$y = 0.5699x + 0.0574$	0.99937	98.0 (0.5)	119.3 (15.0)	48	74
DEHP	0.3–2	149	$y = 1.4824x + 0.3927$	0.97852	137.7 (19.0)	94.6 (0.2)	168	201
DEHP-d_4 (I.S.)		153, 171						
DOP	0.1–2	149	$y = 1.2580x + 0.0198$	0.99874	62.2 (2.1)	54.9 (45.8)	4.6	9.8

Table 3.3.5 Summary of calibration parameters, mean accuracies (%), relative standard deviations (RSD (n = 9), %, in parentheses) and limits of detection (LODs) and limits of quantitation (LOQs) in electron ionization single ion monitoring (EI-SIM) mode

Comparison between the chromatograms of the same spiked extract obtained in SIM and in MS/MS acquisition mode (Figure 3.3.3) confirms that tandem mass spectrometry technique minimizes matrix interference and improve the signal/noise ratio. This feature allows us to obtain reconstructed chromatograms with well-defined chromatographic peaks and then more easily to integrate.

Moreover, an examination of MS/MS analytical data compared to those obtained in SIM mode underlines that better results were achieved by MS/MS acquisition. In particular, calibration parameters result better than those achieved in SIM for DBP and DEHP and similar for DMP, DEP, BBP and DOP. Comparison between accuracy

and precision values in SIM and MS/MS mode shows clearly that EI-MS/MS parameters are significantly better than those obtained in SIM for all the analytes except for DEP.

Finally, as we expected, LOD and LOQ values result lower than those achieved in SIM acquisition.

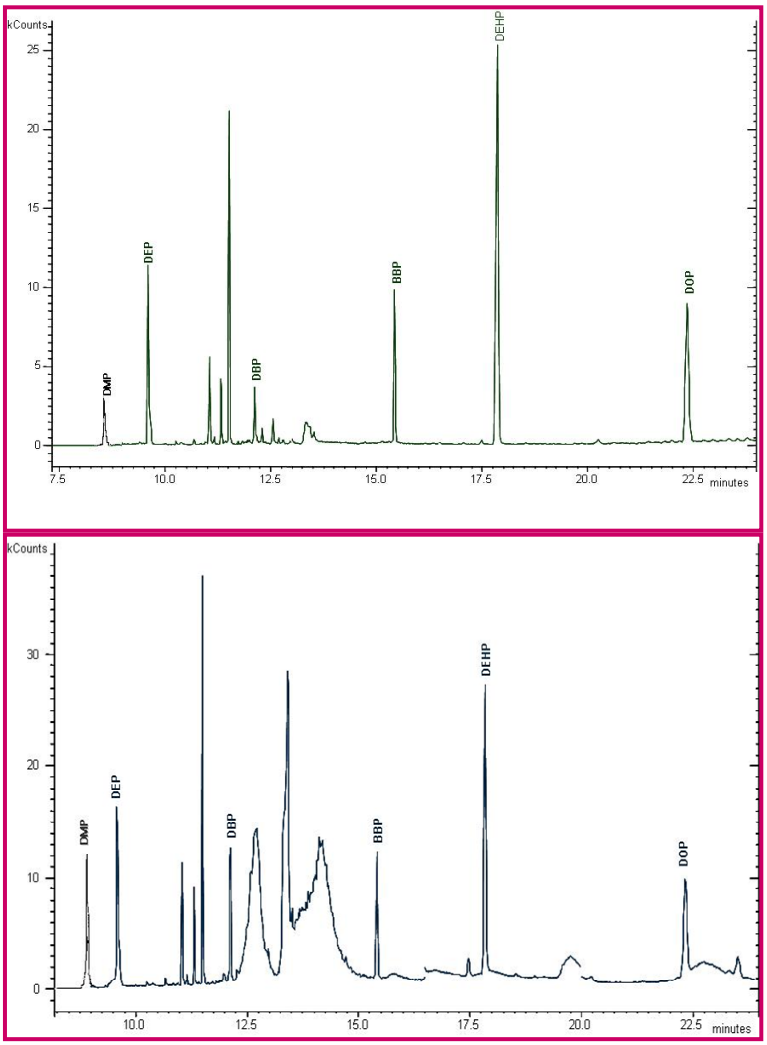


Figure 3.3.3 Gas chromatograms for a sample of olive oil spiked with a 0.5 mg kg^{-1} concentration of analyzed phthalates in EI-MS/MS mode (A) and in EI-SIM mode (B).

The developed method using GC–EI-MS/MS was applied to the analysis of 16 samples of commercial olive oil (6 of extra virgin olive oil (EOO), 6 of olive oil-composed of refined olive oils and virgin olive oils (OO) and 4 of olive-pomace oil (OPO)). This permitted us to compare the PAEs present in samples depending on the manufacturing procedure used. Each sample was analyzed in triplicate as the procedure described in Section 2.5. As can be observed from table 3.3.6, DBP, BBP and DEHP are the only phthalates present in the samples.

In general, higher concentration of PAEs was detected in olive pomace oils and lower levels in extra virgin olive oils (this can be a consequence of high accuracy paid by the producers in avoiding any unsecured use of plastic materials during all the steps going from the malaxation to the separation and bottling of extra virgin olive oil). It is important to note the remarkable concentration of DEHP in olive-pomace oils (with an average concentration of 2.84 mg $kg^{\bullet 1}$), whereas only one sample (OO-5) was not found to contain any phthalates. Finally, DEHP was present in extra virgin olive oil and in olive oil with an average concentrations of 0.85 mg $kg^{\bullet 1}$ and 1.45 mg $kg^{\bullet 1}$, respectively.

Compound	EOO-1	EOO-2	EOO-3	EOO-4	EOO-5	EOO-6	OO-1	OO-2
DMP	–[a]	–	–	–	–	–	–	–
DEP	–	–	–	–	–	–	–	–
DBP	0.368 ± 0.025	0.153 ± 0.011	–	–	–	–	0.295 ± 0.024	0.394 ± 0.107
BBP	0.366 ± 0.022	1.75 ± 0.15	0.085 ± 0.009	–	0.451 ± 0.070	0.999 ± 0.037	1.01 ± 0.05	0.285 ± 0.043
DEHP	1.66 ± 0.069	0.439 ± 0.031	0.543 ± 0.011	0.630 ± 0.005	0.483 ± 0.064	1.34 ± 0.02	2.66 ± 0.01	2.17 ± 0.22
DOP	–	–	–	–	–	–	–	–

Compound	OO-3	OO-4	OO-5	OO-6	OPO-1	OPO-2	OPO-3	OPO-4
DMP	–	–	–	–	–	–	–	–
DEP	–	–	–	–	–	–	–	–
DBP	0.096 ± 0.018	–	–	–	–	0.365 ± 0.030	0.490 ± 0.030	0.163 ± 0.003
BBP	0.029 ± 0.009	0.188 ± 0.039	–	0.401 ± 0.020	0.341 ± 0.029	0.536 ± 0.089	0.649 ± 0.068	0.171 ± 0.011
DEHP	0.386 ± 0.074	1.27 ± 0.10	–	2.11 ± 0.08	2.86 ± 0.23	4.70 ± 0.95	2.19 ± 0.22	1.62 ± 0.11
DOP	–	–	–	–	–	–	–	–

Table 3.3.6 Results (mgkg$^{\bullet 1}$) obtained in the analysis of different olive oil by GPC-GC–EI-MS/MS (EOO: extra virgin olive oil, OO: olive oil, OPO: olive-pomace oil). [a] <limit of detection (LOD)

The goal of the investigation thoroughly discussed, i.e. the exploitation of high technological methods for residue monitoring in food safety assessment, has been fulfilled, at least in the particular field of producing and stocking virgin olive oil.

A method for the determination of phthalates in olive oil was developed by GC–MS analysis following a preliminary GPC cleanup step. Three acquisition modes (EI-MS/MS, CI-MS/MS and EI-SIM) have been tested. An overall evaluation of all

analytical parameters shows that acquisition in EI-MS/MS mode provides satisfactory data for all the analytes of interest. In particular, comparison with the more common SIM methodology has proven the capability of MS/MS to increase sensitivity and selectivity of the detection and quantification. The high specificity and signal to noise ratio, accuracy, the LOD and LOQ values, better than those published, obtained by matching GPC technique with MS/MS methodology, allow to exclude any preliminary liquid–liquid extraction.

The use of spiked matrix standards has been shown to be essential for improving calibration data and accuracy. The performance of method in terms of accuracy and precision was very satisfactory for all the analytes. The application of the proposed method to commercial samples of different olive oil (extra virgin olive oil, olive oil and refined olive oil) revealed the presence of DBP, BBP and DEHP at higher concentration in refined oils and lower levels in extra virgin oils.

3.4 Screening of dimethoate in food by isotope dilution and electrospray ionization tandem mass spectrometry

Application of agrochemicals like pesticides at various stages of cultivation and during post-harvest storage has become a usual practice in modern agriculture. These chemicals help to control a wide range of pests and plant diseases, and consequently increase in the productivity thereby playing an important role in food production and quality preservation.

The toxic chemicals taken up by plants during cultivation or contaminated during preservation are passed on in the food chain causing serious health effects in human beings. The toxicity of these compounds necessitated the monitoring of pesticide residues in food products in order to assess the human exposure to pesticides through foods. European Union Commission (EU) has set the maximum residue limit (MRLs) in foodstuffs to guarantee consumer safety and to regulate international trade (Commission Regulation (EC), 1990).

Dimethoate (dimethyl S-(N-methylcarbamoylmethyl)phosphorothiolothionate) (Figure 3.4.1) is one of the most used organophosphorus (OP) pesticides, that has application in large- and small-scale agriculture; it acts against a wide range of insects, including aphids and thrips, that attack ornamental plants, vegetables, cotton, and fruit crops.[80] The toxicity of dimethoate and, in general, of OP pesticides, is due to the inhibition of acetyl cholinesterase, the enzyme responsible for catalyzing the breakdown of the neurotransmitter acetylcholine.[81,82] The accumulation of acetylcholine, caused by the inhibition of the enzyme, leads to symptoms related to the autonomous nervous system (abdominal cramps, nausea, diarrhoea and salivation) and to the central nervous system (dizziness, tremor, anxiety and confusion).[83] Epidemiological studies have suggested that exposure to OP pesticides can induce other chronic effects on the central peripheral nervous system, either after acute intoxication or as a result of lower level long-term exposure.[84,85] The use of dimethoate is still allowed in many crops, including olives, which once was based in the Mediterranean area but now is expanding rapidly throughout the world.

Dimethoate is prevalently used in olive crops because it is believed that its metabolites are eliminated in the waste water formed during olive oil production.

European and US regulatory authorities, such as the US EPA, are constantly assessing the risks of exposure to the organophosphate (OP) class of pesticides and, among these, specifically dimethoate.

The EU Directive set a dimethoate threshold limit of around 0.02 ppm for many agrifoods.[86]

Therefore, it is important to develop an analytical method whose specificity and sensitivity match the requirements needed for healthy food production.

Thus the analytical methodologies employed must be capable of residue measurement at very low levels and must also provide clear-cut evidence to confirm both identity and quantity of any residues detected.

The most frequently used methods rely on gas chromatographic (GC) separation and detection with selective and sensitive detectors such as electron-capture detection (ECD), nitrogen–phosphorus detection (NPD) and mass spectrometry (MS), because most of the pesticides are volatile and thermally stable.[87-89] In the last few years a tendency towards the use of more polar pesticides than non-polar compounds is observed due to their less persistence and higher toxicity.

Analysis of polar compounds using gas chromatography is less suitable hence use of alternative technique gained importance.[90] Liquid chromatography (LC) coupled to mass spectrometry (MS) is the most powerful technique and preferred approach for analysis of compounds that are of low volatility, high polarity and thermal liability in nature.

LC is very effective in separating analytes, while MS allows their identification and/or confirmation at trace-levels. In recent years, application of LC–MS has been widely used for the analysis of pesticide residues in fruits, vegetable and other food samples like honey.[91-95]

Moreover, the Quality and Safety (QS) system, recently introduced in Germany for the detection of multiresidues in fresh fruits, vegetables and potatoes, requires, among other things, the accreditation of the analytical laboratories to carry out analysis with the LC/MS/MS methodology.[96] A further advance in this field is represented by the use of

the isotope dilution method in conjunction with MS/MS, thus permitting contaminant residues to be assayed at the sub-picomole level.[97-99]

This work describes an isotope dilution dimethoate assay based on the application of electrospray ionization tandem mass spectrometry (ESI-MS/MS) by means of a deuterium-labeled internal standard.

Sample preparation, as one might expect, is an important step and most of the reported extraction methods are based on solvent extraction,[100,101] solid-phase extraction (SPE),[102] XAD resins,[103] ultrasonic assisted matrix solid-phase dispersion (UA-MSPD)[104] and, recently, use of the QuEChERS method.[105]

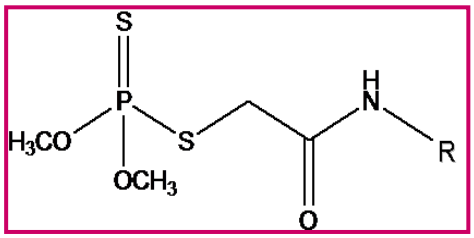


Figure 3.4.1. Structure of dimethoate; R=CH_3 Dimethoate, R=CD_3 Dimethoate-d_3

The absolute method here proposed for the assay of dimethoate in food is based on LC/ESI-MS/MS, under multiple reaction monitoring (MRM) mode, and with isotope dilution. The labeled internal standard used is the trideuteromethyl homologue of dimethoate (Figure 3.4.1).

3.4.1 Synthesis of d_3-labeled dimethoate

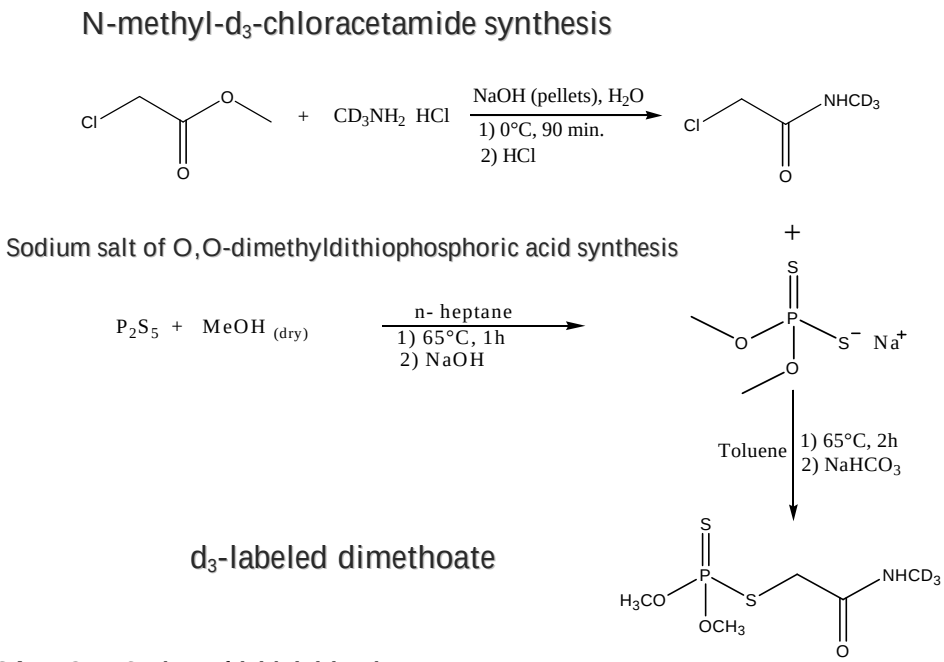
Dimethoate-d_3 was obtained by coupling N-methyl-d_3-chloracetamide with O,O-dimethyldithiophosphoric acid sodium salt (Scheme 3.4.1).[106] N-Methyl-d_3-chloracetamide was obtained by reaction between methyl-d_3-amine hydrochloride and methyl chloroacetate, adding NaOH in pellets dissolved in water and methanol.

The solvent was removed *in vacuo* and a solution of aqueous hydrochloric acid was added until the pH=5. The mixture was extracted with chloroform (3x5 mL), and the organic extract was dried over anhydrous sodium sulphate and evaporated to dryness to give a crystalline solid with 70% yield.

The O,O-dimethyldithiophosphoric acid sodium salt was prepared by reacting P_2S_5 and dry methanol in n-heptane at 65°C for 1 h. A concentrated solution of NaOH was added until the pH=6 and the mixture was partitioned between diethyl ether and water.

The aqueous phase was used for the next step. N-Methyl-d_3-chloracetamide was dissolved in toluene and heated at 65°C. The aqueous solution of O,O-dimethyldithiophosphoric acid sodium salt was added dropwise over 20 min and the reaction mixture thus formed was kept at 65°C for 2 h. A saturated solution of sodium bicarbonate was added with stirring until pH=7 was reached. The partitioned organic phase was dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure, affording an oil residue.

Dimethoate-d_3 was obtained in 55% yield as colorless crystals by adding petroleum ether to a hot chloroform solution of the oily residue.



Scheme 3.4.1. Synthesis of d_3-labeled dimethoate.

The isotopic purity of the internal standard has been verified by high resolution mass spectrometry giving the following isotopic distribution: d_2 = 4%, d_3 = 96% (Figure 3.4.2).

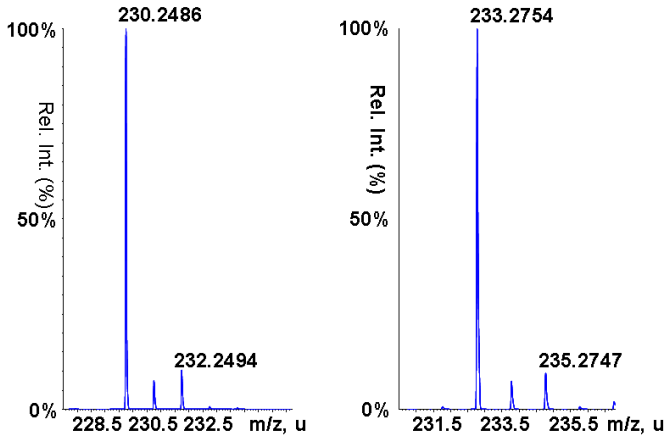


Figure 3.4.2. Mass spectra obtained by high resolution mass spectrometry.

3.4.2 Sample preparation

The extraction procedure presented here is very simple and does not need the analyte to undergo any further purification steps.

In the first part of this work we optimized the preparation sample step for the olive oil matrix, probably the most complex matrix of all.

In order to choose the better methods two different extraction methodologies were tested:

1. Gel permeation chromatography (GPC)[107,108]

   The extraction was performed using a poly(styrene-co-divinylbenzene) resin to separate the high molecular mass species present in oil from the analyte. 0,5 g of oil mixed with 0,2 ppm d_3-labeled dimethoate were placed in the GPC column and for the chromatographic separation different Mobile phase were tested. The eluent phase that gave the better result was cyclohexane:dichloromethane.

2. Solid phase extraction (SPE)

   The preparation was performed placed 0,5 gr of oil mixed with 0,1 ppm d_3-labeled dimethoate on C18 cartridges. The extraction was carried out with acetonitrile as solvent.

In both cases samples were evaporated by rotavapor and recovered with 1 mL of H_2O/CH_3CN (50:50) solution.[109]

SPE was the chosen method because although its matrix effect was higher than GPC for the LC-MS/MS experiment it hasn't show any significant problems and in addition it's less expensive and more fast than GPC method.

For the other vegetable matrices, apple, potato and spinach, the samples were crushed and homogenized using a mixer apparatus. 0,1 ppm d_3-labeled dimethoate were added to 2 g of crushed sample. Extraction was carried out with acetonitrile and the extract was filtered using a Buchner funnel.

The organic extract was evaporated to dryness and dissolved in 1 mL of H_2O/CH_3CN (50:50) solution. The crude extract is then injected directly into the instrument without further purification.

Typical mass spectra obtained using the experimental conditions described in section 4.4.3 are shown in figure 3.4.3.

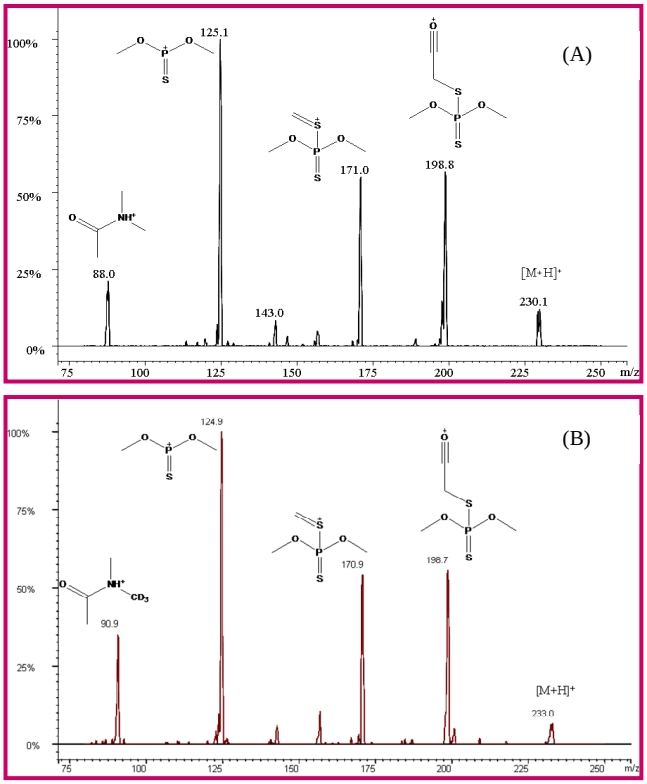


Figure 3.4.3. ESI-MS/MS spectra of $[M+H]^+$ ions of dimethoate (A) and labeled dimethoate (B).

3.4.3 Analytical parameters

The limit of detection (LOD) and the limit of quantitation (LOQ) for each foodstuff were calculated by applying Eqns. (1) and (2), following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry.

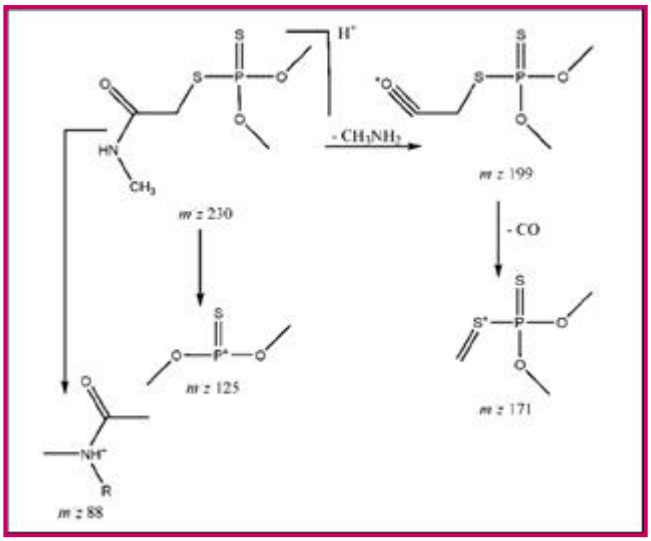
$$S_{LOD} = S_{RB} + 3\bullet_{RB}$$
$$S_{LOQ} = S_{RB} + 10\bullet_{RB}$$

where S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of the uncontaminated food matrices (organic), and S_{RB} is the standard deviation for uncontaminated matrices calculated on seven measurements. The concentrations were calculated by using the standard curve. The recovery for each foodstuff was calculated from the area of the signal obtained by analyzing an uncontaminated product added to a known amount of labeled dimethoate; the concentration was estimated by using an external calibration curve built from six standard solutions at 12.5, 25, 50, 100, 200, and 400 ppm (R^2=0.994).

The isotope dilution method (IDM), based on the combined use of MS/MS and labeled internal standards, is a powerful tool for the assay of analytes in complex natural matrices. The method has been successfully applied to many important problems related to many food safety and quality investigations.[98,99] The great improvement in both precision and accuracy of the measurements is one of the many advantages achieved by applying the method, which minimizes problems arising from calibration procedure, sample preparation, and matrix effects. Moreover, tandem mass spectrometry (MS/MS) helps in the identification, structural elucidation and quantitation of organic compounds and improves the specificity and speed of analysis. The MS/MS spectrum of dimethoate is characterized by a few easily recognizable product ions, whereas that of the d_3-labeled analogue displays, in addition to the few common product ions, other ions whose m/z values are increased by 3 m/z units with respect to those formed by the first (Figure 3.4.3 (A) and 3.4.3(B)).

The product ion at m/z 199 originates from the precursor $[M+H]^+$ ion by loss of methylamine; the subsequent loss of CO from the formed product ion generates the ion

at m/z 171. The ion at m/z 125 corresponds to the O,O-dimethyl phosphonothioate ion while the elemental composition of the ion at m/z 88 (m/z 91 for the labeled species) is in agreement with a formula of $C_4H_{10}NO^+$ (Scheme 3.4.2).



Scheme 3.4.2. Fragmentation pathways of dimethoate.

This latter product ion is presumed to be formed from a complex mechanism involving the migration of the methyl group from the phosphate ester moiety. The behavior of the labeled isomer shows that the deuterium isotopes are not involved in any breakdown mechanism of the original protonated species. Moreover, the use of the labeled compound helps to identify the product ions being formed.

The isotopic purity of the internal standard has been verified by QqToF-MS giving the following isotopic distribution: d_2=4%, d_3=96% (figure 3.4.2). The assay was performed under MRM conditions and two transitions were monitored to assure the greatest specificity. The transitions chosen were m/z 230• 199 and m/z 230• 125 for unlabeled dimethoate and m/z 233• 199 and m/z 233• 125 for the labeled internal standard (IS). For quantitative purposes the most abundant transition has been used (Figure 3.4.4).

The calibration curve was constructed using triplicate samples of six standard solutions at different concentrations with the same 0.1 ppm concentration of the IS, showing good linearity in the range from 12.5 to 400 ppb (R^2=0.9972, y=1.2419x+0.0945).

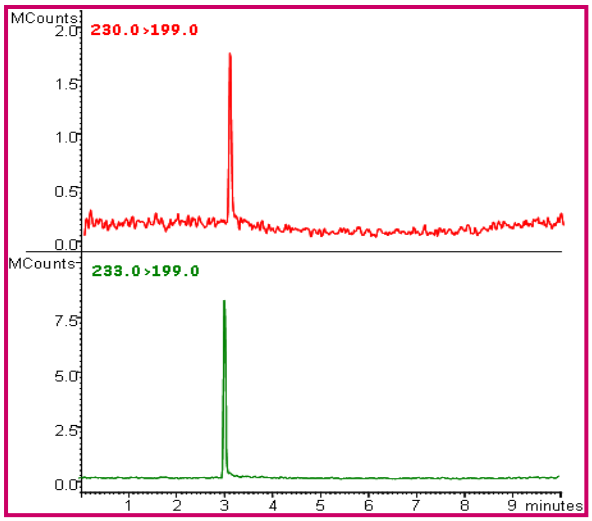


Figure 3.4.4 Following transitions obtained in MRM mode for quantitative analysis for labeled and unlabelled dimethoate.

The use of MS/MS allows us to reach very low LOQ values when interfaced with a HPLC system, even in the presence of crude extracts containing the analyte. The developed methodology has been applied to spiked samples of different matrices. The maximum value of dimethoate detected in foodstuffs ranged from 0.1 to 0.2 ppm depending on the matrix used.[86] This new method is very sensitive and allows the identification and quantification of the pesticide with high specificity at very low concentration (Table 3.4.1). The relative standard deviation (RSD) values (in all cases below 6%) and the accuracy values, obtained in the higher and lower side of the calibration curve, confirm the reliability of the developed methodology.

The calculated analytical parameters confirm the merits of this proposed approach. The values of LOQ and LOD ranged from 3.0 to 6.0 ppb, suggesting that the proposed technique is suitable for evaluating very low amounts of pesticide in different food matrices. The recovery values ranged from 60% for spinach to 92% for apple, confirming the efficacy of the extraction procedures; the reproducibility is also in all cases below 12%.

Furthermore, as evidence of the very low values of LOQ obtained, a spiked sample containing dimethoate at a concentration of 5 ppb was prepared and analyzed; in

general, accuracy values of greater than 94% were reached for all matrices (Table 3.4.2).

ppb	Calculated concentration	RSD % (avarage)	Accuracy % (avarage)
Olive oil			
20	19.0±0.3	1.7	95
100	100±3	2.7	99.6
300	315±16	5.1	103
Apple			
20	19.5±0.3	1.6	97.6
100	96±3	3.5	95.8
300	293±5	1.7	97.7
Potato			
20	20.5±0.7	3.4	106
100	98±4	4.3	97.9
300	293±9	3	97.5
Spinach			
20	19.8±0.8	4.1	99
100	97±6	5.7	96.7
300	290±10	3.5	96.6

Table 3.4.1. Analytical parameters of precision and accuracy of the method.

				Reproducibility (RSD %)		
Food matrix	LOD (ppb)	LOQ (ppb)	Recovery (%)	20 ppb	100 ppb	300 ppb
Olive oil	5.5	5.7	73.8	7.2	5.4	11.2
Apple	3.6	3.8	92.9	11.2	5.6	9.8
Potato	3.4	3.6	61.6	10.2	9.3	7.5
Spinach	2.9	3.9	69.9	9.9	4.4	1.5

Table 3.4.2. Reproducibility of the method; recovery, LOQ and LOD measurements from foodstuffs matrices.

The identification and assay of dimethoate residues in vegetable crops can be conveniently carried out with this new approach which utilizes the potential of both the isotope dilution method and tandem mass spectrometry. Satisfactory LOQ and LOD values have been obtained. The establishment of this new method for the assay of dimethoate, an extremely toxic OP pesticide, was needed because of its use in olive culture from which different foodstuffs are produced and consumed throughout the world.

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Chapter 4

Experimental

4.1 A metabolomic approach to the evaluation of the origin of extra virgin olive oil: A convenient statistical treatment of mass spectrometric analytical data

4.1.1 Materials. The survey was carried out on four samples from each Calabrian areas, i.e., Catanzaro, Corigliano, Rende, and Nocera Terinese, and on 12, 10, and 11 samples from Enfidha, Sidi, Bouzid, and Taous (Tunisian areas), respectively, with similar Jaen indexes. For each sample, 10 kg of olive was picked from three to five trees and then milled in a laboratory scale hammer mill. After 20 min of malaxation, the oil was separated by centrifugation.

5 g of drupes of Olea Europaea L. (Carolea, Coratina, Nocellara del Belice and Leccino cultivars) were used.

4.1.2 Experimental Procedure. The most suitable solid-phase microextraction (SPME) conditions for quantitative assay of the five selected compounds, hexanal (**1**), (*E*)-2-hexenal (**2**), (*E*)-2-hexen-1-ol (**3**), 1-hexanol (**4**), and (*Z*)-3-hexen-1-yl acetate (**5**), were described in a previous study. Briefly, SPME was performed with a 65 •m Carbowax/divinylbenzene (DVB) fiber (Supelco, Bellefonte, PA). Two grams of sample was placed in each septum-closed vial, and the extraction was performed in the headspace volume (~8 mL) at 40 °C for 20 min. The adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 250 °C for 3 min.

The quantitative assay was performed in chemical ionization (CI) mode, using isobutane as the reagent gas and ethyl isobutanoate as the internal standard (IS) at the concentration ranges 0.2-2 and 5-100 mg/kg, using 1 and 40 mg/kg of IS, respectively.

4.1.3 Preparation of Samples. A mother solution was prepared by dissolving 0.020 g (1.72×10^{-4} mol) of IS in 2.000 g of each sample. The samples were homogenized and the GC-MS analysis were performed after 20 min. of thermic stabilization at 40 °C on heater plate of the autosampler. Concentrations of 1 and 40 mg/kg, for the two calibration curves, respectively, were achieved through serial dilutions.

4.1.4 Instrumentation. Sample analyses were performed using a Varian (Walnut Creek, CA) Saturn 2000 GC-MS ion trap (ITD) system in positive CI modes, with isobutane as the reagent gas, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector.

The ion trap temperature was set at 210 °C with an ionization time of 2 ms, a reaction time at 50 ms, and a scan rate at 1000 ms. The transfer line temperature was set at 230 °C. The column was a 30 m Chrompack CP-Sil 8 CB low-bleed/MS (0.25 mm i.d., 0.25 *ím* film thickness). The gas chromatography (GC) oven temperature was initially held at 40 °C for 3 min, then increased at 1 °C/min to 70 °C, increased again at 20 °C/min to 250 °C, and held for 8 min. The carrier gas was helium at 1 mL/min. Analyses were performed in splitless mode. For SPME analyses, a narrow-bore Supelco 0.8 mm i.d. GC inlet liner was used.

The isobutane pressure was adjusted to produce a ratio of *m*/*z* 43-57 of approximately 1/1.2. The selective ejection chemical ionization (SECI) scan mode parameters were as follows: CI storage level, *m*/*z* 19; ejection amplitude, *m*/*z* 15; and background mass, *m*/*z* 65.

4.1.5 Statistical Analysis. The quantitative data of Calabrian olive oil samples were subjected to linear discriminant analysis (LDA) to classify samples with a priori hypothesis, which is the number of groups (areas of production), and to find the variables with the highest discriminant power. On the contrary, the Tunisian data were subjected to another two statistical tools: the Kruskal-Wallis test and the Wald-Wolfowitz test. These are the analogous nonparametric methods of one-way between-groups of variance (analysis of variance, ANOVA) and *t*-test, respectively. Nonparametric techniques are also called “distribution-free methods”, since they are not dependent on a given distribution (such as in the case of ANOVA) but generally work for a broad range of different distributions. Statistical treatment of data was performed by Statistica 7.1 (StatSoft 2005 edition).

4.2 Geographical characterization of tomato through analysis of volatile components by SPME-GC/Ion Trap Mass Spectrometry

4.2.1 Materials. The survey was carried out on ten samples of fresh tomato from each of the five areas of production (Calabria - Isola Capo Rizzuto and Crotone, Basilicata, Puglia and Emilia Romagna) and ten samples of concentrated tomato from three of the five areas of production (Calabria - Isola Capo Rizzuto and Crotone, Emilia Romagna).
Tomatoes were harvested at full maturity assessed by a colorimeter and determining the sugar level.
Fibers and the syringe used for the adsorption / desorption of the sample are commercial and purchased from Supelco.

4.2.2 Experimental Procedure. SPME was performed using Carboxen/PDMS, 85 • m fiber (Supelco, Bellefonte, PA). The tomato sample still frozen was homogenized using a kitchen blender for about one minute. From the mass thus obtained 2 g was immediately taken and placed in a 10 ml septum-closed vial, with CaCl2 (CaCl2 volume (ml) = weight of sample (g)) and appropriate amount of internal standard solution as specified below in detail.
Samples of concentrate were prepared by taking 3 g of the product and placed in a septum-closed vial, to which were added CaCl2 and a suitable amount of internal standard.
Analysis of volatile components were performed adsorbing the analytes on a Carboxen/PDMS fiber for 60 minutes at 35 °C in the headspace volume. The analytes were desorbed into the GC injector for 15 minutes at 300 °C.

4.2.3 Preparation of Samples. A mother solution of internal standard 5 mg/l was prepared. 80 • l of this solution are diluted in 2 g of fresh tomato sample and 120 • l in 3 g of paste tomato sample to obtain a concentration of 2 mg/l.

4.2.4 Instrumentation. Sample analyses were performed using a Varian (Walnut Creek, CA) Saturn 2000 GC-MS ion trap (ITD) system in electron impact (EI) mode, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector.
The ion trap temperature was set at 210 °C with an ionization time of 2 ms, a reaction time at 50 ms, and a scan rate at 1000 ms. The transfer line temperature was set at 230 °C. The column was a 30 m Chrompack CP-Sil 8 CB low-bleed/MS (0.25 mm i.d., 0.25 • m film thickness). The gas chromatography (GC) oven temperature was initially held at 35 °C for 8 min, then increased at 1,5 °C/min to 45 °C, increased again at 3 °C/min to 150 °C, increased at 2,5 °C/min to 250 °C and held for 6 min. The carrier gas was helium at 1 mL/min. Analyses were performed in splitless mode with a injector temperature of 300 °C. For SPME analyses, a narrow-bore Supelco 0.8 mm i.d. GC inlet liner was used.
The analysis was in full scan, in the range m / z 40 and m / z 400, in a time range between 0 and 60 min.

4.2.5 Statistical Analysis. The quantitative data for each sample have been correlated with different areas of production by stepwise Linear Discriminant Analysis (LDA), by LDA applied on the principal components and by Soft Indipendent Modelling of Class Analogy (SIMCA). Statistical treatment of data (LDA) was performed by Statistica 8.1 (StatSoft STATISTICA 8.0). SIMCA analysis was performed using software PARVUS 2004.

4.3 Tandem mass spectrometry in food safety assessment: The determination of phthalates in olive oil.

4.3.1 Materials

Cyclohexane, dichloromethane and ethyl acetate were purchased from Aldrich (Milan, Italy) as residue analysis grade. The phthalates mix (dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), butylbenzyl phthalate (BBP), di-2-ethylhexyl phthalate (DEHP) and di-*n*-octyl phthalate (DOP)) at 2000• $gml^{\bullet 1}$ and DEHP-d_4 (internal standard) were also obtained from Aldrich. Lowbleed septa utilized in GC injector were supplied by Phenomenex (Bologna, Italy) and subjected to further purification procedure.

Septa were put into a 50 ml Erlenmeyer flask and then cover with 30 ml of *n*-hexane for 12 h; solvent was then discharged, septa were washed with 30 ml of *n*-hexane and solvent was again eliminated. Finally, septa were put into an oven held at 80 °C for 3 h and at 200 °C for 2 h.

Analytes solution (20 $mgl^{\bullet 1}$) were prepared by diluting mix solution in *n*-hexane and storing it in a glass-stopped bottle that was placed in a freezer at • 20 °C in the dark. Internal standard solution (25 $mgl^{\bullet 1}$)was prepared by dissolving 20 mg of pure DEHP-d_4 in 50 ml of *n*-hexane and by diluting this solution 16 times.

To minimize the risk of secondary contamination, all solvents (including re-distilled acetone used for washing all laboratory glassware) were checked for the presence of phthalates.

4.3.2 Instrumentation and apparatus.

Extraction and clean-up. The GPC system comprised an Agilent 1200 Series isocratic pump and a GPC clean-up column (25mm×450mm) (LabService Analytica, Bologna, Italy) packed with Bio-Beads S-X3 styrene–divinylbenzene copolymer of 200–400 mesh so as to reach a backpressure of 6 psi. The flow-rate was set at 5.0 ml $min^{\bullet 1}$ and the mobile phase was cyclohexane: dichloromethane (7:3). An Agilent 1100 Series UV–vis detector was used to measure the absorbance at 220 and 254 nm of the eluate from GPC column.

GC–MS analysis. GC–MS analyses were performed using a Varian (Walnut Creek, CA, USA) Saturn 2000 GC–MS ion-trap system in electron ionization and positive chemical ionization mode, with isobutane as reagent gas, coupled to a Varian 3400 gas chromatograph (GC) equipped with a Varian 8200 autoinjector.

The ion trap temperature was set at 210 °C with an ionization time of 25 ms, emission current at 10 • A and scan rate at 1000 ms. The injector and the transfer line temperatures were set at 250 °C and 230 °C, respectively. The capillary column was a 30m×0.25mm i.d., 0.25 • m film thickness Varian Factor Four 5-ms (95% polydimethylsiloxane, 5% polydiphenylsiloxane).

The GC oven temperature was initially held at 100 °C for 3 min, then ramped at 16°Cmin$^{\bullet 1}$ to 250°C and held for 28 min. The carrier gas was helium at 1 mlmin$^{\bullet 1}$ of purity 99.999%. Analyses were performed in splitless mode (injected volume 2 • l). For the CI analysis the isobutane pressure was adjusted to produce a ratio of m/z 43–57 of approximately 1–1.2, ionization time was set at 2 ms and reaction time at 50 ms.

The selective ejection chemical ionization (SECI) scan mode parameters were as follows: CI storage level, *m*/*z* 19; ejection amplitude, *m*/*z* 15; background mass, *m*/*z* 65.

Analyses were acquired in SIM and MS/MS modes. The MS/MS process was carried out by collision-induced dissociation (CID) with resonant excitation for all analytes.

4.3.3 Calibration procedure

Five-point calibration curves were obtained by spiking olive oil, free from any PAEs, provided by CRA-OLI, Rende, with known amounts of analytes and internal standard to cover a concentration range of 0.1–2 mgkg$^{\bullet 1}$ with 0.5 mgkg$^{\bullet 1}$ of DEHP-d_4 as internal standard. Each experimental value corresponds to the average of three independent measurements.

4.3.4 Samples

Commercial olive oil samples were supplied by a local supermarket in Cosenza (Italy).

One gram of olive oil was weighted into a 10 ml all-glass measuring flask. After adding 200 • l of internal standard solution at 25 mgl$^{\bullet 1}$, the flask was filled to the mark with GPC mobile phase and 5 ml of this diluted sample were manually injected into the GPC

column. The eluate was collected between 18 and 27 min in a pearshaped flask and the volume of fraction was reduced by placing it in a rotary evaporator at low pressure and at a temperature below 50 °C. Finally, the extract was diluted to 1ml with mobile phase. The spiked matrix standards were prepared by spiking them previously with an appropriate amount of phthalates standard solution.

4.4 Screening of dimethoate in food by isotope dilution and electrospray ionization tandem mass spectrometry.

4.4.1 Chemicals

All chemicals and solvents were obtained from Sigma- Aldrich (St. Louis, MO, USA).

4.4.2 Synthesis of d_3-labeled dimethoate

Dimethoate-d_3 was obtained by coupling N-methyl-d_3-chloracetamide with O,O-dimethyldithiophosphoric acid sodium salt. N-Methyl-d_3-chloracetamide was obtained by reaction between methyl-d_3-amine hydrochloride (1 g, 14.2 mmol) and methyl chloroacetate (1.25 mL, 14.2 mmol), adding 0.59 g (14.8 mmol) of NaOH in pellets dissolved in 1.5 mL of water and 5 mL of methanol, at 0°C for 90 min.

The solvent was removed *in vacuo* and a solution of aqueous hydrochloric acid was added until the pH=5. The mixture was extracted with chloroform (3x5 mL), and the organic extract was dried over anhydrous sodium sulphate and evaporated to dryness to give a crystalline solid with 70% yield.

The O,O-dimethyldithiophosphoric acid sodium salt was prepared by reacting P_2S_5 (11.12 g, 50 mmol) and dry methanol (3.04 mL, 75 mmol) in 15 mL of n-heptane at 65°C for 1 h. A concentrated solution of NaOH was added until the pH=6 and the mixture was partitioned between diethyl ether (15 mL) and water (30 mL).

The aqueous phase was used for the next step.

N-Methyl-d_3-chloracetamide (1.21 g, 10.96 mmol) was dissolved in 10 mL of toluene and heated at 65°C. The aqueous solution of O,O-dimethyldithiophosphoric acid sodium salt was added dropwise over 20 min and the reaction mixture thus formed was kept at 65°C for 2 h. A saturated solution of sodium bicarbonate was added with stirring until pH=7 was reached. The partitioned organic phase was dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure, affording an oil residue.

Dimethoate-d_3 was obtained in 55% yield as colorless crystals by adding 20 mL of petroleum ether to a hot chloroform (8 mL) solution of the oily residue.

4.4.3 Sample preparation

Olive oil samples. A stock solution of the internal standard (25 mL, 2 ppm) was added to 0.5 g of olive oils. The oil with the internal standard was homogenized with 1mL of acetonitrile and loaded into the SPE cartridge (C18, 1 g, 6 mL; J. T. Baker, Phillipsburg, NJ, USA), previously activated with acetonitrile (2x5 mL); the analyte was then eluted with 15 mL of acetonitrile. The eluted solution was evaporated to dryness and dissolved in 0.5 mL of H_2O/CH_3CN (50:50) solution.

Apple, potato and spinach samples. The samples were crushed and homogenized using a mixer apparatus. A stock solution (25 mL, 4 ppm) of labeled internal standard was added to 2 g of crushed sample and homogenized. The final mixture was extracted twice with 15 mL of acetonitrile and filtered on paper using a Buchner funnel. The organic extract was evaporated to dryness and dissolved in 1 mL of H_2O/CH_3CN (50:50) solution.

4.4.4 Instrumentation

The LC/MS analysis was carried out with a LC 320 triple quadrupole mass spectrometer (Varian Inc., Palo Alto, CA, USA), equipped with an ESI source interfaced with an HPLC Prostar 210 (Varian Inc.) equipped with a binary pump, online degasser and autosampler.

HPLC analysis. A reversed-phase C18 column (Pursuit, 50x2.0mm, 5 mm particle size; Varian Inc.), with a pre-column of the same material, was used for the stationary phase. The sample solutions (20 mL) were injected and the analytes were eluted using a linear gradient: A (H_2O, 0.1% formic acid), solvent B (CH_3CN); from 5% B to 95% B in 5 min; 3 min at 95% B isocratic; from 95% B to 5% B in 2 min; 2 min at 5% B isocratic at flow rate fixed at 0.25 $mLmin^{-1}$.

Mass spectrometry conditions. The needle voltage was fixed at 5.8 kV, the capillary voltage at 45 V, the drying gas (N_2) pressure at 20 psi and temperature at 200 °C, the nebulizing gas (N_2) pressure at 45 psi, the housing temperature at 50 °C, and the electron multiplier at 1350 V. The dwell time was 0.200 s/scan, and the mass windows of the quadrupole were set to 0.9 m/z units. The collision gas (Ar) pressure was fixed at 2 mTorr, and the collision energy was optimized to obtain the maximum response, and set to 20 eV for the transitions m/z 230 $[M_H+H]^+$ • m/z 125 and m/z 233 $[M_D+H]^+$ •

m/z 125, while for the transitions m/z 230 $[M_H+H]^+$ • m/z 199 and m/z 233 $[M_D+H]^+$ • m/z 199 the collision energy was set to 8 eV.

The instrument was tuned and calibrated with a standard solution of poly(propylene glycol).

The quadrupole time-of-flight (QqToF) experiments were carried out using a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystems, Toronto, Canada) mass spectrometer equipped with an ionspray ionization source. Samples were introduced by direct infusion (flow rate of 3 mL/min) of the sample containing the analyte (5 ppm), dissolved in a solution of 0.1% acetic acid, H_2O/CH_3CN (50:50) at the optimum electrospray (ES) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) were set at pressures of 20 and 25 psi, respectively, and the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10V relative to ground, respectively; the mass resolution of the instrument was kept at 8000 FWHM.

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A Metabolomic Approach to the Evaluation of the Origin of Extra Virgin Olive Oil: A Convenient Statistical Treatment of Mass Spectrometric Analytical Data

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The selection of suitable markers from the secondary metabolism of lipoxygenase, in experimental olive oils produced from drupes harvested in different areas of the Italian Calabria region and of Tunisia, allows an easy discrimination between each cluster of samples. The origin of the foodstuff can be ascertained even when the distances between the production zones are very close to each other as in Calabria. Olive oils produced from irrigated and nonirrigated farms in Tunisia were also clearly distinguishable. The markers were detected by chemical ionization mass spectrometry with an ion trap gas chromatography−mass spectrometry apparatus. The quantitative data of Calabrian olive oil samples were subjected to linear discriminant analysis, whereas the Tunisian data were treated by means of other two statistical tools, i.e., the Kruskal−Wallis test and the Wald−Wolfowitz test.

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Tandem mass spectrometry in food safety assessment: The determination of phthalates in olive oil

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ABSTRACT

A gas chromatography–tandem mass spectrometry (GC–MS/MS) method for the detection of six phthalates in olive oil was developed. A gel permeation chromatography (GPC) clean-up step with cyclohexane:dichoromethane 7:3 as mobile phase was employed to remove the high-molecular mass species present in oil. Two ionization methodologies, i.e. electron (EI) and isobutane-chemical ionization (CI), were compared, in MS/MS mode, to achieve better analytical performances. An overall evaluation of all analytical parameters shows that the EI-MS/MS approach provides satisfactory results and is to be preferred to CI-MS/MS, at least in the case of the examined analytes. The observed accuracies, ranging from 71.7% to 112.2%, and the RSD values less than 9.7%, confirm the effectiveness of the proposed method in the assay of phthalate content in such a complex matrix as olive oil. The proposed protocol for the identification and assay of phthalates in olive oil might be of interest for the implementation of the QS (quality assurance scheme) for residue monitoring in food safety assessment.

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RCM

Screening of dimethoate in food by isotope dilution and electrospray ionization tandem mass spectrometry

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Crop control is an important issue in both developed and developing countries. An environmentally friendly approach is represented by the so-called Integrated Pest Management (IPM), whereby synthetic pesticides are only applied as a last resort, under the strict control of suitable experts. European and US regulatory authorities, such as the US EPA, are constantly assessing the risks of exposure to the organophosphate (OP) class of pesticides and, among these, specifically dimethoate. The use of dimethoate is still allowed in many crops, including olives, which once was based in the Mediterranean area but now is expanding rapidly throughout the world. An important aspect of IPM protocols is represented by the availability of reliable and sensitive methods to detect pesticides residues. This paper describes an isotope dilution dimethoate assay based on the application of electrospray ionization tandem mass spectrometry (ESI-MS/MS) by means of a deuterium-labeled internal standard.