# Chapter 1
# Introduction

## 1.1 Edible Oils

### *1.1.1 Introduction*

Edible oils are mainly vegetable oils which have been subjected to several processes to removed undesirable constituents. In order to make them suitable for human consumption, most edible oils are subjected to refining processes, such as neutralization, bleaching and deodorization. Among edible oils, only virgin olive oil (a natural juice obtained by olive pressing), can be consumed without refining.

Edible oils are characterized by a wide range of physical and chemical properties, since their composition depend on the type of oil (Rossell 1991).

Oil suitability for most uses depends on its quality and chemical composition. Several tests can be used to determine oil purity, such as the determination of triacylglycerols, Ts, sterols and other constituents of the unsaponifiable fraction of oil.

### *1.1.2 Constituents of Edible Oils*

The constituents of edible oils can be grouped into the saponifiable (triacylglycerols, free fatty acids, phosphatides) and the unsaponifiable (hydrocarbons, fatty alcohols, etc.) fractions. The unsaponifiable fraction accounts, in general, 0.5–1.5 % of the oils.

#### 1.1.2.1 Saponifiable Fraction

The saponifiable fraction accounts for 98.5–99.5 % of oils. The major part of this fraction are triacylglycerols and free fatty acids, although other fatty acid

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derivatives such as mono- and diacylglycerols, phospholipids, waxes and sterol esters are also found.

*Triacylglycerols*. These compounds comprise 98–99 % of the oils. They are esters derived from the union of glycerol (1,2,3-propanetriol) and fatty acids. Generally, the fatty acids at the central position of the glycerol molecule are generally unsaturated, although saturated acids can be found at this position when the total concentration of saturated fatty acids in the oil is very high. The most abundant triacylglycerols found in olive oil are OOO (43.5 %), POO (18.4 %), OOL (6.8 %), POL (5.9 %) and SOO (5.1 %) (being O = oleic acid; P = palmitic acid; S = stearic acid and L = linoleic acid) (Fedeli 1977).

*Mono- and diacylglycerols*. Jointly with triacylglycerols, edible oils also contain partial glycerols such as mono- and diacylglycerols, comprising 0.2 and 1.3 % of total fatty acids, respectively. Their present in an olive oil is an index of low quality (Mariani and Fedeli 1985). For this reason, their determination is often used as an oil quality marker.

*Free fatty acids*. Their proportion in the oil depends on the hydrolysis degree of triacylglycerols, being their composition variable according to the botanical variety of oil, or, in the case of olive oil, according to the genetic variety, climatic conditions, fruit maturity and geographical origin of olives (Aparicio et al 1994; Boskou 2002; D'Imperio et al. 2007; Stefanoudaki Kotsifaki and Koutsaftakis 1999; Torres and Maestri 2006). Major fatty acids in olive oils are oleic (55–85 %), palmitic (7.5–20 %), linoleic (7.5–20 %), stearic (0.5–5 %), palmitoleic (0.3–3.5 %) and linolenic (0.0–1.5 %) acids, although traces of myristic, arachidic and margaric acids could be also found.

*Phospholipids*. Phospholipids are found in small quantities in freshly produced olive oils (40–135 mg/kg) (Tiscornia et al. 1982), being their concentration lower with oil aging. The most important phospholipids in olive oil are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine (Alter and Gutfinger 1982; Boskou 2002).

*Waxes*. These compounds are esters of fatty alcohols with fatty acids. The main waxes detected in olive oils have a high and even carbon number, in particular, $C_{36}$–$C_{46}$ esters. Their amount is very low, not exceeding 35 mg/100 g (Boskou 2002).

*Sterol esters*. These compounds are combinations of fatty acids with different types of sterols (sterols will be discussed below).

#### 1.1.2.2 Unsaponifiable Fraction

The unsaponifiable fraction of edible oils contain different compounds which are not chemically related to fatty acids, such as hydrocarbons, fatty alcohols, free sterols (common sterols, 4-α-methylsterols, 4,4-dimethylsterols or triterpene alcohols and triterpene dialcohols), Ts and $T_3$s, pigments, different volatile compounds and aromatic hydrocarbons, phenolic compounds and proteins.

*Hydrocarbons*. The most important hydrocarbon found in both virgin and refined olive oils is squalene. Squalene contents comprised between 2,500 and

9,250 μg/g has been found in olive oils, which are widely larger in comparison to those found in other edible oils, which ranged from 16 to 370 μg/g, according to Gutfinger and Letan 1974. Other hydrocarbons also present in olive oil are $C_{14}$–$C_{30}$ *n*-alkanes, some *n*-alkenes and terpene hydrocarbons such as α-farnesene. The concentration of these hydrocarbons is approximately 150–200 μg/g (Lanzon et al. 1994). β-carotene, which could be included in the family of terpene hydrocarbons due to its chemical structure, will be discussed in the pigment section.

*Fatty alcohols*. These minor compounds are also important constituents of edible oils, and, in the case of olive oil, they can be used to distinguish different olive oil types (Regulation (EEC) N° 2568/91). Fatty alcohols can be linear (aliphatic) o triterpene (see sterol section). Other alcohols, such as diterpene alcohols or acyclic diterpene alcohols are also found in olive oils.

Aliphatic alcohols are compounds of linear structure. On the other hand, they are precursors of the formation of waxes. The main linear alcohols present in olive oil, whose concentrations are usually lower than 35 mg/g, are docosanol (C22), tetracosanol (C24), hexacosanol (C26) and octacosanol (C28). Other alcohols with an odd atom number, present at trace levels, are tricosanol (C23), pentacosanol (C25) and heptacosanol (C27).

Olive oil also contains two important diterpene alcohols: phytol (found at concentrations ranging from 120 to 180 mg/kg) and geranylgeraniol (Paganuzzi 1979).

*Sterols*. There different types of sterols in vegetable oils: common sterols or 4-α-desmethylsterols, 4-α-methylsterols, 4,4-dimethyl sterols or triterpene alcohols and triterpene dialcohols.

4-α-desmethylsterols are the most abundant sterols in oils, and its content in olive oil ranged from 100 to 200 mg/g. The main sterols in olive oil are β-sitosterol (75–90 %), $\Delta^5$-avenasterol (5–36 %) and campesterol (which accounts approximately 3 % of the total sterol fraction). However, other sterols such as cholesterol, campestanol, stigmasterol, $\Delta^7$-campesterol, chlerosterol, sitostanol, $\Delta^{5,24}$- stigmastadienol, $\Delta^7$- stigmasterol and $\Delta^7$-avenasterol have been also found at trace levels (Boskou 2002).

4-α-methylsterols are found in low concentrations, which normally ranged between 20 and 70 mg/g (Boskou 1996) as a consequence of sterol biosynthesis. The most abundant 4-α-methylsterols found in olive oil are obtusifoliol, cycloeucalenol, gramisterol and citrostadienol.

On the other hand, major triterpene alcohols present in olive oil, whose content ranged between 100 and 150 mg/100 g oil (Kiosseoglou et al. 1987), are α- and β-amyrin, cycloartenol, butyrospermol, 24-methylenecycloartanol, taraxerol, dammaradienol and 24-methylene-24-dihydroparkeol (Boskou 2002; Kiritsakis et al. 2003; Paganuzzi 1982).

The main triterpene dialcohols are erythrodiol and uvaol. Their total content in olive oil ranged from 1 to 20 mg/g, although a content up to 280 mg/g could be found in β-residual oils (Boskou 2002; Mariani et al. 1987).

*Ts and $T_3$s*. Ts and $T_3$s are important fat-soluble vitamins. Both series of compounds contribute to the stability of oils protecting them from oxidation (Blekas et al. 1995; Manzi et al. 1998; Psomiadou and Tsimidou 1998) preventing

lipid peroxidation in biological membranes (Panfili et al. 2003; Solomon 1998), having also a beneficial biological role as antioxidants (Mateos et al. 2005). While Ts are found in all oils, $T_3$s are mainly found in palm oil (Choo et al. 1996) and in oils obtained from cereals. The relative concentrations of Ts and $T_3$s vary with the type of oil, being α-T the most abundant in olive oil, representing 95 % of Ts (Gimeno et al. 2000; Tasioula-Margari and Okogeri 2001). The other 5 % are mainly β- and γ-Ts.

*Pigments*. The main pigments present in edible oils are carotenoids (Serani and Piacenti 1992). The main carotenoids present in olive oil are β-carotene and lutein, although small quantities of xanthophylls such as violaxanthin and/or neoxanthin have been also reported (Boskou 2002). The total pigment content ranged from 1 to 20 mg/kg, although in most cases its quantity does not exceed 10 mg/kg. Other pigments also found in olive oils (10–30 mg/kg) are chlorophylls. Olive oil green colour is a consequence of the presence of these pigments. Pheophytin α is the most abundant chlorophyll found in packed oils, although the occurrence of other chlorophylls such as chlorophyll α, chlorophyll β, pheophytin α and pheophytin β have been also reported in fresh oils (Miguez-Mosquera et al. 1990).

*Volatile and aromatic compounds.* They are responsible of the aroma and flavor of virgin olive oils. There are more than one hundred components directly related to the aroma and flavor, such as hydrocarbons, alcohols, aldehydes, esters, phenols, terpenes and furan derivates (Boskou 1996; Morales and Aparicio 1999 and 2003; Reiners and Grosch 1998). The most important constituents of olive oil aroma are 6-carbon aldehydes and the alcohols formed in the fruit from polyunsaturated fatty acids.

*Phenolic compounds.* These compounds, more commonly known as polyphenols, are minor constituents of olive oil. Their antioxidant potential has attracted great interest, since they are supposed to have chemoprotective properties in human beings (Bendini et al. 2007; Caponio et al. 1999; Vissers et al. 2001) and also for being one of the most important olive oil antioxidants (Caponio 1999; Tsimidou 1998; Tura 2007; Velasco 2002). Their antioxidant activity has been related to the protection against chronic and degenerative diseases such as heart disease, diseases of neuro-degenerative aging and tumors located in various parts of the human body (Franceschi 1999; Hodge 2004). In addition, polyphenols also contribute to the sensorial properties of virgin olive oils (Servili 2002) by conferring bitterness, pungency, and astringency (Gutiérrez-Rosales 1992 and 2003; Tsimidou 1998).

Phenolic compounds can be mainly grouped into the following categories (Harborne 1989):

- Simple phenols, such as TY, the HYTY, p-hydroxyphenylacetic acid and homovanillic acid.
- Phenolic acids, with the basic structure C6–C1 (benzoic acids), such as gallic, gentisic, benzoic, vanillic, protocatechuic, *p*-hidroxybenzoic and syringic acid, or with the basic structure C6–C3 (cinnamic acids), such as caffeic, *p*-coumaric, *o*-coumaric, ferulic, cinnamic and sinapic acids.

- Phenolic alcohols, such as 3,4-DHPEA and 2-(4-hydroxyphenyl)ethyl acetate.
- Secoiridoids such as oleuropein, ligstroside, OA, LAg, deacetoxy oleuropein aglycon, deacetoxy ligstroside aglycon, dialdehydic form of oleuropein and dialdehydic form of ligstroside.
- Flavonoids, such as API, LUT and taxifolin.
- Lignans, such as AcPIN, pinoresinol and 1-hydroxypinoresinol.

*Proteins*. Finally, the presence of proteins in vegetable oils has been described (Hidalgo et al. 2001a, b, 2002). The total protein content varies widely depending on the type of oil and its extraction method (Hidalgo 2006). On the other hand, it has been shown that there are significant differences in protein content in olive oils depending on the crop and the maturity of the fruit (Zamora 2001).

## *1.1.3 Methods of Analysis of Main Edible Oil Constituents*

### 1.1.3.1 Determination of Triacylglycerols

Different LC techniques have been used for the analysis of triacylglycerols in vegetable oils, such as TLC (Christie 1992), RP-HPLC (Carelli 1993; Cunha and Oliveira 2006a; Holčapek 2005; Parcerisa 1995) and high-temperature-capillary GC (Aparicio 2000; Carelli 1993). Columns packed with silver ions have been the most commonly used in RP-HPLC, since silver presence in the stationary phase promotes the selective retention of unsaturated compounds (Macher 2001). On the other hand, comprehensive two-dimensional chromatography using both a C18 column with a second column load with silver ions have been also employed to achieve selectivity and high peak capacities (Dugo 2006; Robison 1985; van der Klift 2008). Among these techniques, the most widely employed methodology for triacylglycerol analysis has been RP-HPLC. The official method of analysis involves the use of an HPLC coupled with a refractive index detector (Parcerisa 1995), but, since this detector is not compatible with the use of gradient elution (desirable to reduce analysis times and to improve chromatographic resolution), other detectors have been also used, such as UV at low wavelengths (Carelli 1993; Holčapek 2005; Van der Klift 2008), ELSD (Holčapek 2005; Macher 2001; Perona 2001; Van der Klift 2008) or MS (Holčapek 2005; Van der Klift 2008).

### 1.1.3.2 Determination of Fatty Acids

Analysis of free fatty acids has been usually carried out by GC-FID, being this technique that proposed by the official method of analysis (Hajimahmoodia 2005; Regulation (EEC) N° 2568/91, annex X; Sakouhia 2008). However, other analytical methods, such as HPLC (Kotani 2002), CEC (Dermaux 1999) or NMR (Sacchi 1997) have been also developed for this purpose.

#### 1.1.3.3 Determination of Alcohols

Aliphatic and triterpene alcohols present in vegetable oils have been usually determined by GC-FID (Abou Hadeed 1990; Azadmard-Damirchi 2005; Benitez-Sánchez 2003; Lazzez 2008; Ntsourankoua 1994; Ranalli 2002; Rivera del Álamo et al. 2004; Sindhu-Kanya 2007) o GC–MS (Abou Hadeed 1990; Azadmard-Damirchi 2005; Cunha et al. 2006b; Ntsourankoua 1994; Sindhu-Kanya 2007). Only some studies have described the use of HPLC after derivatization of alcohols with 3,5-dinitrobenzoyl chloride (Cortesi 1987).

#### 1.1.3.4 Determination of Sterols

The analysis of sterols have been usually carried out by GC-FID (Cercaci 2007; Galeano 2005; Parcerisa 2000; Ranalli 2002; Rivera del Álamo et al. 2004) or GC–MS (Cercaci 2007; Cunha et al. 2006b; Parcerisa 2000; Thanh 2006; Medvedovici 1997), after extraction of the sterol fraction by TLC followed by derivatization, as indicated by the official method (Regulation (EEC) N° 2568/91, annex V). The major disadvantage of GC is the requirement of thermally stable columns and the need of chemical derivatization prior to analysis. For this reason, alternative methods have been described based on the use of HPLC–MS (Cañabate-Díaz 2007; Martínez-Vidal 2007; Segura-Carretero 2008) and CEC (Abidi 2004).

#### 1.1.3.5 Determination of Ts and $T_3$s

Ts and $T_3$s analysis has been mainly carried out by GC (Melchert 2002) and HPLC using several detectors (Abidi 2000; Cunha et al. 2006c; Gruszka and Kruk 2007). Both, NP-HPLC, which is capable of separating $\beta$ and $\gamma$ isomers (Abidi 2000), and RP-HPLC, which shows higher column stability, better reproducibility, and shorter analysis times, have been used (Abidi 2000; Gimeno et al. 2000b). On the other hand, Ts have also been determined by FTIR spectroscopy (Silva et al. 2009), synchronous fluorescence spectroscopy (Sikorska et al. 2005) and by CEC using packed columns (Aturki et al. 2005).

#### 1.1.3.6 Determination of Volatile Compounds

Many efforts have been made to develop instrumental methods capable of determining the components responsible for flavor and aroma of olive oils, and to remove the subjectivity and other disadvantages coming from the sensory evaluation by tasting panels. Traditionally, volatile compounds were determined by GC–MS (Baccouri et al. 2008a, b; Guth and Grosch 1993; Tateo et al. 1993). Later, another approach was proposed for the determination of volatiles, based on the use of sensor arrays (electronic nose), which is able to assess the basic

perceptions produced by the oil (Aparicio 1995; Tena 2007). In some cases, the results obtained by the electronic nose have been compared with those provided by a tasting panel (Camurati 2006). On the other hand, GC has been also used in combination of sensor arrays (Cimato et al. 2006; García-Gónzalez 2010; López-Feria 2008; Morales 1994; Tena 2007).

#### 1.1.3.7 Determination of Phenolic Compounds

Several methods have been described for the analysis of phenolic compounds in olive oil. The most used technique has been LC, coupled to UV–Vis (Allalout 2009; Baccouri et al. 2008a, b; Bendini 2003; Bonoli 2004; Cerretani 2006; Gutierrez-Rosales 2003; Ocakoglu 2009), electrochemical (Brenes 2000), fluorimetric (Cartoni 2000; García 2003) or MS detectors (Baccouri 2008b; Bendini 2003; Bonoli 2004; Carrasco-Pancorbo 2007a; Gutierrez-Rosales 2003; Suárez 2008). Other techniques, such as GC (Carrasco-Pancorbo 2005; Liberatore 2001; Ríos 2005; Saitta 2009) and CE (Bendini 2003; Bonoli 2004; Carrasco-Pancorbo 2004, 2006,2007; Gómez-Caravaca 2005) coupled to different detectors, have been also widely used. In addition, and more recently, voltammetric sensors (Rodríguez-Méndez 2008) and high-resolution NMR (Christophoridou 2009) have also been used for this purpose.

#### 1.1.3.8 Determination of Proteins

Due to the recent description of proteins as trace components of vegetable oils, only one method for their determination has been described (Hidalgo 2001). This method involves, in first place, protein hydrolysis followed by derivatization of the amino acids obtained with diethyl ethoxymethyl malonate, and subsequent analysis of the derivatives by HPLC–UV-Vis.

### *1.1.4 Detection of Adulteration*

EVOO is often illegally adulterated with cheaper vegetable oils such as corn, peanut, sunflower and soybean oils (Kiritsakis 1998), although the most common adulteration is performed with hazelnut oil, due to the difficulty of its detection by the great similarity between hazelnut and olive oil chemical compositions. EVOO is also adulterated with other olive oils of lower quality, such as olive pomace (Kiritsakis 1998).

To analyze and detect adulteration different physical and chemical tests (Comission of Codex Alimentarius 1993; Fedeli 1977; Kiritsakis 1991), and chromatographic (Fasciotti 2010; Marcos Lorenzo et al. 2002; Maryam 2009; Saba 2005), spectroscopic (Agiomyrgianaki 2010; Fragaki et al. 2005; Fronimaki et al. 2002; Maryam et al. 2009; Poulli et al. 2006; Vlachos et al. 2006) and termic

(Chiavaro et al. 2008; Maryam et al. 2009) methods, among others, have been applied.

On the other hand, the analysis of the unsaponifiable fraction is an alternative and powerful tool to detect adulteration and to increase reliability to differentiate between vegetable oils from different botanical origins, or between olive oils of different quality. Among the constituents of the unsaponifiable fraction, the sterol family, which composition in EVOOs is very characteristic, provides excellent adulteration tracers, both to distinguish between vegetable oils from different botanical origin (Ballesteros et al. 1995; Cañabate-Díaz et al. 2007; Cercaci et al. 2003; Mariani et al. 2006) or to differentiate olive oils of different quality (Martínez-Vidal et al. 2007; Philips et al. 2002), which explains why this family of compounds has been widely used in oil authentication.

## 1.2 Olive Oil

Olive oil is an olive juice directly obtained from *Olea europaea* fruits. This oil could present excellent organoleptic properties depending on the quality of the fruits used for its elaboration (fresh fruits free of defects, adequate maturation degree, etc.).

### *1.2.1 Legal Classification of Olive Oil*

There are several quality levels in olive oil, which can be classified as virgin olive oils and olive–pomace oils. Their characteristics, within the European Union, were established in the Commission Regulation (EEC) No. 2568/91, as amended by EEC 796/2002.

#### 1.2.1.1 Virgin Olive Oil

Virgin olive oil is that obtained only by mechanical or physical methods that do not involve any alteration, particularly thermal. Virgin olive oil must have not undergone any treatment other than washing, decantation, centrifugation and filtration. However, not all virgin olive oils are characterized by an excellent quality. For this reason, virgin olive oils could be classified into several categories according to their acidity, organoleptic score and the absence of defects. These categories are:

- *Extra virgin olive oil or EVOO.* It is considered the best olive oil. Its organoleptic score (set of aromas and flavours) must be equal to or greater than 6.5, and its free acidity (percentage of oleic acid) can not exceed 0.8 g/100 g. EVOOs can present different characteristics, depending on several factors ranging from the olive genetic variety to the cultivation conditions. According to these

factors, EVOOs could be subdivided into at least three major groups: monovarietal oils, made with a single variety of olives; coupages, prepared from different olive varieties to always get the same standards of taste and aroma; and PDO oils, prepared from olives from one geographical area, which are officially recognized.

- *Virgin olive oil or VOO*. It is the virgin olive oil that can be slightly altered, either in their analytical indexes or in their sensory characteristics, but always at a small scale. These alterations, especially sensory, may be almost imperceptible, but depreciated in relation to quality EVOO. In this case, the organoleptic score should be equal to or greater than 5.5, and maximum free acidity of 2° (2 g of oleic acid per 100 g of oil).
- *Ordinary virgin olive oil.* It features sensitive alterations in their physicochemical parameters or in their sensory characteristics. Its organoleptic score must be equal or superior to 3.5, and its maximum acidity of 3.3°. It is used as a component of the so-called olive oil (defined below) but only when its organoleptic characteristics are not significantly altered, being then used to be refined.
- *Lampante virgin olive oil or LVOO*. It is not intended in any way for direct consumption, and must be necessarily subject to a refining process to make it edible. Free acidity is greater than 3.3° and its organoleptic score lessthan 3.5.
- *Refined olive oil*. It is the resulting oil when the previous two oils are subjected to a refination process. Refined olive oil must have a free acidity not higher than 0.3°, and should present almost neutral sensory characteristics (without taste or smell), so it is used as the basis for the manufacturement of other olive oils.

#### 1.2.1.2 Crude Olive–Pomace Oil

Crude olive–pomace oil is the one extracted with organic solvents from the solid waste of mills. It is necessarily subjected to refinement since it is not directly suitable for human consumption. It is commercialized, as explained below, mixed with virgin olive oil.

#### 1.2.1.3 Other Commercial Olive Oils

- *Olive oil*. It is another commercial product obtained by mixing different proportions of VOO or common virgin olive oil with refined olive oil. Its maximum acidity is 1°.
- *Olive–pomace oil or OPO*. It is that obtained by mixing VOO with refined pomace oil or ROPO.

**Table 1.1** Discriminant tests in sensory analysis

| Test | Short description |
|---|---|
| Paired | Two labeled samples. Assessor must indicate the differences between them |
| Duo–trio | Three samples, two identical and one different. One of the two identical samples is identified as reference. Assessor must recognize which sample is identical to the one used as reference |
| Triangle | Three samples, two identical and one different. The judge must indicate which sample is different |
| Classification | Assessor must order a set of samples according to the intensity of a certain attribute |
| Threshold | A series of samples with decreasing concentrations of an attribute is presented. Assessor must indicate for each sample if the attribute is detected |

## *1.2.2 Sensory Assessment of Virgin Olive Oils*

As it can be deduced from Sect. 1.2.1, sensory analysis is, jointly with the chemical analysis of olive oils, an excellent marker of oil quality.

Sensory analysis is a discipline that uses human senses to evaluate food products. With sight, product appearance, colour and shape is evaluated, while smell evaluated food aromas. Consistency and other characteristics such as fluidity and/or viscosity are evaluated by both touch and hearing. Finally, food flavour is evaluated through a combination of smell, taste and touch.

### 1.2.2.1 Methods for Evaluating Sensory Characteristics

Various sensory tests have been developed to provide the evaluation of organoleptic characteristics of foods, including oils. Some of these tests are planned to give information about the overall acceptability of a product or to determine the preference of assessors or consumers between two products or among samples of the same food (preference tests). Other tests are used to evaluate qualitative and quantitative sensory differences between two products (discriminant tests), or to identify, describe, and quantify each sensory quality perceived in an assessment (descriptive tests). Discriminatory tests (which are the ones used in this thesis) are summarized in Table 1.1.

### 1.2.2.2 Organoleptic Assessment of Virgin Olive Oil

The purpose of this international method (IOC, COI/T.20/Doc. No 15/Rev. 4 November 2011; Regulation (EC) No. 796/2002) is to determine the procedure for assessing the organoleptic characteristics of virgin olive oil and to establish the method for its classification on the basis of those characteristics. This method can be used only for grading virgin oils on the basis of fruitiness and intensity of

**Table 1.2** Specific vocabulary for olive oil described in Annex XII of the Commission Regulation (EC) No. 796/2002, Off. J. Eur. Commun. (2002)

| Positive attributes | |
|---|---|
| Fruity | Range of smells (dependent on variety) characteristic of oil from healthy fresh fruit, green or white, perceived directly or retronasally |
| Bitter | Characteristic taste of oil from green olives or olives turning colour |
| Pungent | Tingling sensation characteristic of oil made at the beginning of the season mainly from olives that are still green |
| **Negative attributes** | |
| "Atrojado" (fusty) | Characteristic flavour of oil from piled olives in advanced anaerobic fermentation |
| Mustiness/ humidity | Characteristic flavour of oil from olives in which large numbers of fungi and yeasts had developed as a result of storage for several days in humid conditions |
| Muddy sediment | Characteristic flavour of oil that has remained in contact with sediment in vats and tanks |
| Winey/vinegary | Characteristic flavour of certain oils reminiscent of wine or vinegar, due basically to formation of acetic acid, ethyl acetate and ethanol by fermentation of the olives |
| Metallic | Flavour reminiscent of metal, characteristic of oil that has been in prolonged contact with metal surfaces during crushing, mixing, pressing or storage. |
| Rancid | Flavour of oil that has become oxidised |
| Heated or burnt | Characteristic flavour caused by excessive and/or prolonged heating during production, particularly by thermo-mixing of the paste in unsuitable conditions |
| Hay/wood | Characteristic flavour of certain oils from dry olives |
| Rough | Thick and pasty mouthfeel produced by some oils |
| Greasy | Flavour reminiscent of diesel, grease or mineral oil |
| Vegetable water | Flavour acquired by oil through prolonged contact with the vegetable water |
| Brine | Flavour of oil from olives preserved in salt solution |
| Esparto | Characteristic flavour of oil from olives pressed in new esparto mats. It can vary according to whether the mats are of green or dried esparto |
| Earthy | Flavour of oil from olives collected with earth or mud on them and not washed |
| Grubby | Flavour of oil from olives heavily attacked by grubs of the olive fly (*Bactrocera oleae*) |
| Cucumber | Characteristic flavour of oil kept too long in hermetically sealed containers, notably in tins, attributed to formation of 2,6-nonadienal |

defects by a group of selected trained tasters operating as a panel. Candidates shall be selected, trained and monitored by the panel leader in accordance with their skills in distinguishing between similar samples; it should be borne in mind that their accuracy will improve with training. Tasters must act like real sensory observers, setting aside their personal tastes and solely reporting the sensations they perceive. Between 8 and 12 tasters are required for each test. The selection of a group of people can average the differences that exist in some odour thresholds depending on the person, probably related to genetic, cultural and environmental factors, thus providing a final result that represents all consumers. To solve this

problem, assessors must use the same vocabulary. One part of this vocabulary, common to all foods, is the "general vocabulary", which included terms such aspect, attribute, panel, sensitivity, taster, response, fatigue, sensory stimulation, aroma, flavour, texture, etc. The "specific vocabulary" for the analysis of virgin olive oil was developed by experts from the IOC. A summary of this vocabulary is shown in Table 1.2.

However, and as published by the IOC (COI/T.20/Doc. No 15/Rev. 4 November 2011), other measures must be taken into consideration to assess olive oil quality. Thus, the dimensions, form and colour of the tasting glass have been established. Glasses should have a narrow mouth which helps to concentrate the odours and facilitates their identification, and should be made of dark-coloured glass to prevent the taster from perceiving the colour of the oil, thus eliminating any prejudices and impeding the possible formation of biases or tendencies that might affect the objectiveness of the determination. The glass shall contain 14–16 ml of oil, or between 12.8 and 14.6 g if the samples are to be weighed, and shall be covered with a watch-glass. Moreover, the oil samples shall be kept in the glasses at 28 °C ± 2 °C throughout the test. This temperature has been chosen because it makes it easier to observe organoleptic differences than at ambient temperature and because at lower temperatures the aromatic compounds peculiar to these oils volatilise poorly while higher temperatures lead to the formation of volatile compounds peculiar to heated oils. For this purpose, a heating device (IOC, COI/T.20/Doc. No 5/Rev. 1 September 2007) shall be installed in each booth within the taster's reach.

On the other hand, when organoleptically assessing a virgin olive oil, it is recommended that four samples are the most be evaluated in each session with a maximum of three sessions per day, to avoid the contrast effect that could be produced by immediately tasting other samples.

Assessors use a profile sheet (Fig. 1.1) that contains the sensory defects normally found in virgin olive oils. Among the positive perceptions, only the attributes fruity, bitter and pungent are evaluated. Fruity perception is assessed by direct inhalation, while the rest of sensations are perceived via retronasal. It is appropriate to adopt a scale, which is easy to use both by experts and amateurs, to quantify the different stimuli and to process statistical data. It uses a linear scale of 10 cm length to measure the intensity of sensory notes defined in the vocabulary. The intensity data provided by the tasters, expressed in centimeters, are treated statistically.

### *1.2.3 Genetic Varieties*

EVOO consumption has increased considerably in the last years when compared to the consumption of olive oils of lower quality. This increase has been attributed to the excellent organoleptic and nutritional properties of EVOOs. For this reason, more monovarietal EVOOs are being produced to respond at this growing demand,

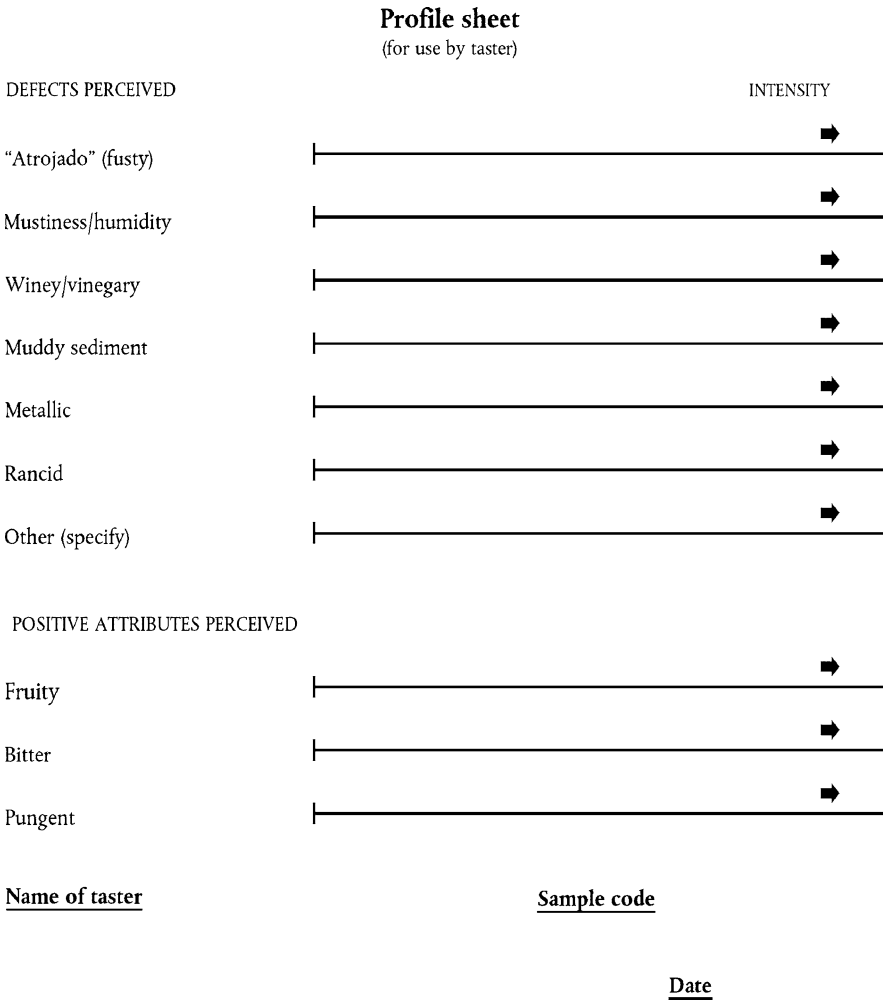



**Fig. 1.1** Oil tasting sheet of virgin olive oil adopted by Regulation (EC) N° 640/2008

since chemical composition and organoleptic properties of EVOOs vary depending on the genetic variety of the olive used in its preparation (Baccouri et al. 2008b; Tura et al. 2007).

EVOOs are produced from very different genetic varieties (Barranco et al. 2001). The most widespread varieties in Spain, which accounts 95 % of the produced olives, are Arbequina, Hojiblanca and Picual varieties, although the number of other varieties cultivated in Spain is also high and very diverse, depending on the geographic region where the crop is located. The cultivar Picual, also know as Marteña, is the most important and widespread, mainly cultivated in Andalucía, Castilla-La Mancha and Extremadura. Its production is approximately 50 % of the total Spanish olives,

and about 20 % of world production. On the other hand, Hojiblanca variety is mainly cultivated in Málaga, but it is also very abundant in Sevilla and Córdoba (Andalucía). Finally, Arbequina variety is mainly grown in Lérida, Tarragona and Córdoba. Among the other varieties cultivated in Spain, Cornicabra Lechín, Picuda and Verdial are the most important, among others.

Other genetic varieties of particular interest in the development of this thesis are those grown in the Comunidad Valenciana, Spain, including Serrana de Espadán and Borriolenca (mainly grown in the provinces of Castellón and Valencia), and the varieties Farga and Canetera (mainly grown in the province of Castellón).

### *1.2.4 Geographical Origin*

In addition to the genetic variety of olives, EVOOs may present very different characteristics depending on the geographical origin of the cultivar (Cerretani et al. 2006). Only in Spain, there are 32 PDOs, which are distributed along the country (Ministerio de Medio Ambiente y Medio Rural y Marino (ed.): Aceites de oliva virgen con denominación de origen) (see Table 1.3). Each PDO is governed by its own rules, which have been established in order to preserve olive oil quality. These rules are committed to the final product quality through rigorous quality controls, setting patterns of cultivation, harvesting, processing, bottling and labeling. As it can be observed in Table 1.3, most PDOs are located in Andalucía, which is the main olive oil producing area in the world.

### *1.2.5 Oxidation Compounds from Olive Oil*

Olive oil is characterized by a high oxidative stability compared to other edible oils, which is due to its fatty acid composition, with high concentrations of oleic acid and low in polyunsaturated fatty acids, as well as the presence of natural antioxidants as α-T and phenolic compounds (Tsimidou et al. 1992; Bendini et al. 2007). However, olive oil is susceptible to be oxidized, like other edible oils. Oxidation processes depend on several factors, being the presence of oxygen and trace metals, temperature and light (Al-Ismail et al. 1998, 1999) some of the causes of oil deterioration. The oxidative deterioration may be due to enzymatic oxidation processes (occurring when oil is still in the fruit, or during the extraction process itself), or chemical oxidation processes, such as photo-oxidation (which occurs when the oil is exposed to light) or autoxidation (produced mainly during processing or storage of oil in contact with oxygen) (Bendini et al. 2009; Frankel 1985).

Oil oxidation produces hydroperoxides, which are very unstable primary products, which could decompose or react with other molecules present in the lipid matrix, producing a wide range of secondary oxidation products that derive from fatty acids and other minor compounds, such as oxidised fatty acids (OFAs), oxidised polymers and volatile compounds among others (Choe and Min 2006).

**Table 1.3** Name of the Spanish virgin olive oil PDOs and their geographical distribution in Spain

| No. | Name of the PDO | Geographical area |
|---|---|---|
| 1 | Sierra de Cádiz | Andalucía |
| 2 | Antequera | Andalucía |
| 3 | Estepa | |
| 4 | Lucena | Andalucía |
| 5 | Priego de Córdoba | Andalucía |
| 6 | Poniente de Granada | Andalucía |
| 7 | Baena | Andalucía |
| 8 | Jaén Sierra Sur | Andalucía |
| 9 | Montes de Granada | Andalucía |
| 10 | Sierra Mágina | Andalucía |
| 11 | Sierra de Cazorla | Andalucía |
| 12 | Sierra de Segura | Andalucía |
| 13 | Campiñas de Jaén | Andalucía |
| 14 | Montoro-Adamuz | Andalucía |
| 15 | Monterrubio | Extremadura |
| 16 | Gata-Hurdes | Extremadura |
| 17 | Aceite Campo de Montiel | Castilla-La Mancha |
| 18 | Campo de Calatrava | Castilla-La Mancha |
| 19 | Aceite Montes de Alcaraz | Castilla-La Mancha |
| 20 | La alcarria | Castilla-La Mancha |
| 21 | Montes de Toledo | Castilla-La Mancha |
| 22 | Aceite de Mallorca | Mallorca |
| 23 | Baix Ebre-Montsià | Catalunya |
| 24 | Oli de Terra Alta | Catalunya |
| 25 | Siurana | Catalunya |
| 26 | Les Garrigues | Catalunya |
| 27 | Oli de l'Empordà | Catalunya |
| 28 | Aceite de La Rioja | La Rioja |
| 29 | Aceite de Navarra | Navarra |
| 30 | Aceite del Bajo Aragón | Aragón |
| 31 | Aceite de la Comunitat Valenciana | Comunitat Valenciana |
| 32 | Aceite de Madrid | Comunidad de Madrid |

All these compounds affect negatively oil flavor and aroma. Therefore, the study of the oxidative stability of oils is important in determining both the quality and shelf life (Hamilton 1994).

#### 1.2.5.1 Evaluation of Primary Oxidation Products

The analytical tests typically used to evaluate oil primary oxidation products are peroxide value, and the measurement of conjugated diene and triene absorptivity in the UV.

Peroxide value is one of the most used parameter to evaluate oil oxidative deterioration. It has been traditionally determined by iodometric titration (official method Regulation (EEC No 2568/91), although other methods that use enzymes,

HPLC and colorimetry, have been also developed. However, and despite the good estimation that peroxide index provided about the oxidative state of oils, its determination must be done jointly with the determination of secondary oxidation products, in order to have a more complete knowledge of oxidation evolution (Gordon 2001).

On the other hand, during the formation of peroxide and hydroperoxide radicals, there is a change in the position of the double bonds, which modifies its conjugation. The diene and triene test is based on conjugation changes affecting these molecular structures. The conjugated dienes show an absorption band at 232 nm and trienes at 268 nm. However, there are also other products derived from hydroperoxides, which have the same or similar conjugation, which may contribute to the absorption, making the measurement of conjugated dienes and trienes less specific than the peroxide one.

#### 1.2.5.2 Evaluation of the Secondary Oxidation Products

Several approaches have been attempted to find a reliable oxidation index that, combined with the evaluation of primary oxidation products, would provide a realistic idea about the oxidation status of the fatty matrix (Farhoosh and Pazhouhanmehr 2009). The secondary oxidation indices more widely applied to fat and vegetable oils are the p-anisidine value and thiobarbituric acid reactive substances (Frankel 1998) as well as the content of hexanal or nonanal or their ratio (Frankel 1998; Vichi et al. 2003). Among the chemical methods, the measurements of total polar compounds and polymerized triglycerides (Caponio et al. 2002; Melton et al. 1994), measurement of OSI time, and more recently the determination of OFAs by HPLC (Cortesi et al. 1991), have been also used. Among them, OSI time and OFA determination, which are the methods used in this thesis, will be discussed below.

OSI is an instrument that works under standardized conditions of air flow and temperature (90–110 °C). One advantage of this instrument is that, working under controlled conditions, thermo-oxidation was reduced, which is important for assessing the amount of volatile byproducts. In an OSI, a stream of purified air was passed through the oil sample that is heated to 90–110 °C, and the effluent air for the oil sample was then bubbled through a vessel containing deionized water. The effluent air contains especially volatile organic acids as formic acid (Jebe et al. 1993) and other volatile compounds formed during thermal oxidation of the oil, which increased the conductivity of the water. The results, expressed as induction time in hours, allow a comparative evaluation between different lipid matrices, although the measurement of OSI time can not be applied to predictive studies of oxidation, or to know a priori the oil life. In fact, heat stress conditions jointly with the air flow, accelerate the oxidation of oil promoting kinetics of reaction between oxygen and fatty acids that differ from those occurring in normal conditions of oil storage.

On the other hand, the use of HPLC, according to the method developed by Cortesi et al. (1991), allows the identification and quantification of the main OFAs (hydroxy,

keto, epoxy, and epidioxy) after a simple derivatization step with sodium benzyl oxide of the triglycerides. As these latter compounds are more stable than peroxides, OFAs seem to be a good index of oxidative changes in lipids.

## 1.3 Analytical Techniques

### *1.3.1 CEC*

CEC is a separation technique in which the flow of the mobile phase or buffer is driven through a chromatographic column by an electric field. Thus, this technique combines the high selectivity and reproducibility of HPLC and the high efficiency of CZE, which is originated from the plug-like flow profile. As in CZE, EOF in CEC is generated by an electric field, in which EOF is an interfacial phenomenon generated as a consequence of the surface charge on the interior wall of the column.

In CEC the separation mechanism is double (Rathore and Horváth 1996). There is a chromatographic mechanism, since there is a distribution of solutes between mobile and stationary phases. On the other hand, the ionic solutes are also separated by an electrophoretic mechanism, which is based on the differences in electrophoretic mobility, so the nature of the stationary phase of the column determines the EOF and influences the quality of the separation.

#### 1.3.1.1 Instrumentation in CEC

A CEC instrument (see Fig. 1.2) consists basically of a high voltage source, a system to provide solvent and/or sample to the inlet and outlet vials of the column, a capillary column, an isothermal compartment for the column and a detection system capable of recording the concentration profiles of the analytes in the eluent.

The instrumentation used in CEC is basically the same as in CE, although CE systems are frequently modified for CEC by applying pressure (2–15 bar) at the inlet and outlet vials (without providing a net pressure drop across the column). This pressure is necessary to avoid bubble formation, which could interrupt current. The formation of bubbles can be attributed to local differences in EOF rate (Rathore 1998), local differences in the electric field due to the loss of gas trapped in the pores of the stationary phase or by electrochemically formed gas (Carney et al. 1999), by heating (Knox 1988; Tsuda 1987) or in the case of packed columns, by the presence of the retaining frits that maintain the structural integrity of the stationary phase (Rebscher 1994). Pressurization could be applied to the inlet or outlet vials, but is usually performed in both vials to ensure a reproducible flow. An inert gas, usually $N_2$, is used to pressurize the vials.

The quality of a separation technique is characterized by efficiency, selectivity, sensitivity and reproducibility. To obtain reproducible results in CEC, it is

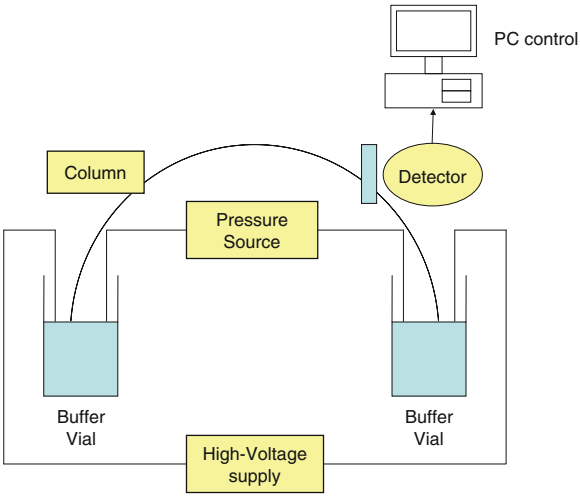



**Fig. 1.2** Scheme of a CEC instrument

necessary to control different parameters such as column temperature, applied voltage and pressure. In CE commercial equipments, these parameters are controlled automatically, leading to significant improvements in separation reproducibility.

The UV–Vis spectrophotometric detection is the most commonly used in CEC (Choudhary 2000; Rozing 2001; Devowsky 2002; Cahours 2002) with both direct and indirect modes. Other detection techniques widely used are laser-induced fluorescence (Wall 2002; Horstkötter 2002; Liu 2001), and most recently, MS (Shamsi 2004; Klampfl 2004; Barceló-Barrachina 2004).

### 1.3.1.2 Columns Used in CEC

CEC columns are usually prepared using fused silica capillaries with internal diameters between 100 and 200 μm. Based on the differences in the chromatographic supports used in their preparation, three types of columns could be distinguished: open-tubular, packed and monolithic columns.

Monolithic columns consist of a continuous porous bed which allows their use on HPLC at high flow rates, providing fast separations without incurring in an excessive pressure increase. This is one of the advantages of these columns when compared to packed ones.

In CEC, monolithic columns also represent an alternative to packed columns due to: (1) the easy preparation, (2) no need of frits, (3) the nearly total exclusion of bubble formation during operation, (4) adjustable porosity and pore size (which allows the use of long columns that could achieve highly efficient separations) and (5) the wide variety of monomers available for the synthesis of stationary phases with many different functionalities. Moreover, monolithic columns could be

prepared in situ, so that the preparation of monolithic beds is relatively simple when compared to the particle packing techniques.

Monolithic materials can be classified into two main categories, organic polymer- and silica-based monoliths. Polymeric stationary phases are usually prepared by in situ polymerization of a mixture composed of functional monomer/s, cross-linker, porogens and a radical initiator. The hydrophobicity of the resulting monolith can be controlled by selecting the nature of the monomer (Liao 1996; Palm 1997). In all cases, EOF is ensured by the addition of monomers derived from acrylic acid or sulfonic acid, or by the addition of quaternary ammonium salts in the polymerization mixture (Ericson 1999). Polymerization reaction is commonly initiated thermally, chemically or by UV irradiation.

In order to enable covalent attachment of the monolith to the wall, a surface modification of the inner wall of the fused-silica capillaries is performed. For this purpose, the wall is silanized using, in most cases, 3-(trimethoxysilyl)propyl methacrylate, also called *silane binding*.

Different types of polymers, such as acrylamide, styrene, methacrylate and acrylate esters, have been employed as stationary phases in CEC. The first monolithic columns described were prepared using acrylamide and methacrylamide (Hjertén 1989; Fujimoto 1995; Hoegger 2001). These polymers are prepared by polymerization of acrylamide or methacrylamide in the presence of methylenebisacrylamide or piperazine diacrylamide as crosslinkers. Polystyrene-based monolithic columns (Gusev 1999; Petro 1996) are obtained by polymerization of styrene or its derivatives using divinylbenzene as crosslinker. Monoliths based on methacrylate esters (Merthar 2003; Zhang 2003; Peters et al. 1998a, b) are prepared by polymerization of butylmethacrylate or other methacrylate esters using ethylene glycol dimethacrylate as crosslinker.

#### 1.3.1.3 Polymerization of Monolithic Columns Based on Methacrylate and Acrylate Esters

Polymethacrylate-based monolithic columns are the most widespread and best characterized columns, being mainly developed by Svec et al. (Peters et al. 1998a , b), who have described several applications in both HPLC and CEC. Methacrylate and acrylate polymers present several advantages such as easily adjustable polarity, fine control of pore characteristics and high stability under extreme pH conditions (2–12).

The polymerization is carried out through a radical reaction, usually initiated by a high temperature, by UV irradiation, or by chemical agents at room temperature. For thermal initiation AIBN (Peters 1998a, b; Chirica 2001), benzoyl peroxide (Xie 1997) or other peroxides (Cantó-Mirapeix 2008) are usually added to the polymerization mixture.

Svec et al. (Peters 1998a and b) have demonstrated that the chromatographic properties (efficiency, selectivity, permeability, etc.) of these materials can be altered by varying the composition of the polymerization mixture, which is

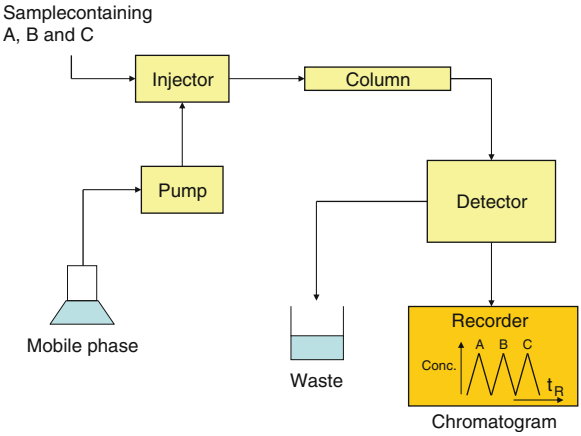



**Fig. 1.3** Block diagram of the components of a liquid chromatograph

interesting not only for the development and optimization of chromatographic separations, but also for environmental, biochemical and industrial applications.

### *1.3.2 LC*

Chromatography is a physical separation method in which the components to be separated are distributed between two phases, called stationary and mobile phases. A chromatographic system (see Fig. 1.3) comprises at least one pump system, a device for sample introduction or injector, a column, a detector and a system for data acquisition and control.

According to the different mechanisms of chromatographic retention, different column types could be found:

- Partition chromatography, in which columns with a bonded liquid phase are used. Depending on the relative polarities of the mobile and stationary phases, there are two modes: RP, where the stationary phase is apolar and the mobile phase is polar, or NP, where the stationary phase is polar and the mobile phase is apolar.
- Ion chromatography, in which the stationary phase is an ion (cationic or anionic) exchange resin.
- Size exclusion chromatography, in which the pores of a microporous solid or gel are used as stationary phase.

On the other hand, it is important to degas solvents to prevent the formation of bubbles in the system. To this end, liquid chromatographs typically include an online degasser.

The most employed detection techniques in LC are UV–Vis spectrophotometry and MS. However, other detectors, such as refractive index and different evaporative detectors are also commonly used. Amperometric and fluorimetric detectors

**Table 1.4** Representative characteristics of the different miniaturization techniques

| Parameter | Classic LC | Micro-LC | Capilar-LC | Nano-LC |
|---|---|---|---|---|
| Internal diameter | 4.6 mm | 1–2,1 mm | 0.1–1 mm | 25–100 μm |
| Length | 1.5–25 cm | 1.5–20 cm | 1.5–20 cm | 1.5–20 cm |
| Particle diameter | 1.8–5 μm | 1.8–5 μm | 1.8–5 μm | 1.8–3 μm |
| Injection volume | 5–100 μL | 1–5 μL | 0.03–0.3 μL | 0.03–0.3 μL |
| Typical flow rate | $< 1$ mL $min^{-1}$ | 10–100 μL $min^{-1}$ | 1–10 μL $min^{-1}$ | 0.1–1 μL $min^{-1}$ |

are also used for specific applications. Finally, conductivity detection is commonly used in ion chromatography.

In UV–Vis spectrophotometry, the signal is proportional to the solute molar concentration, which molar absorptivity depends on the nature of the absorbent group/s. There are two different types of UV–Vis detectors: variable wavelength detector and DAD. In the first type, a fixed wavelength is measured, while in a DAD all spectral range is measured several times per second.

In MS, the detector is an instrument that provides high-level information about analyte molecular structures, distinguishing between functional groups, chemical elements and isotopes, separating the fragments according to their *m/z* ratio. A MS detector coupled to a liquid chromatograph is able to differentiate compounds with similar retention characteristics, being also possible the identification and/or the quantification of compounds that are only partially resolved or even unresolved. More information about MS is given in Sect. 1.3.5.

More recently, different chromatographic modalities has been introduced involving miniaturization, either in the column size or the particle diameter. Miniaturization is one of the present trends in science and technology, especially in the field of analytical chemistry. The use of these miniaturized techniques offers several advantages over the classical ones, such as better separation efficiencies, increase in sensitivity, shorter analysis times, and lower sample and reagent consumption. However, there are also some disadvantages, such as major instrumental requirements (use of micro-pumps, micro/nano-nebulizers, miniaturized connections, etc.).

Considering column diameter and other related features, a distinction between micro-LC, capillary LC and nano-LC should be performed. The following table shows some of the representative characteristics of these modalities (Table 1.4).

On the other hand, there is another chromatographic method, the UPLC, which enables the use of columns packed with smaller particle sizes, which were able to deliver mobile phases at higher pressures (up to 1,000 bar) than those provided by a conventional chromatography system. With these improvements, higher speed of analysis, peak capacity, resolution and sensitivities are obtained.

### *1.3.3 Chromatographic Parameters*

To successfully carry out a chromatographic separation, analyst must determine if the analytes of interest can be accurately separated from other sample components,

and if this separation is enough to correctly detect and/or determine them. The time elapsed between the injection of the sample components into the column and their detection is known as retention time ($t_R$). $t_R$ is longer when the solute has higher affinity to the stationary phase due to its chemical nature. A non retained substance passes through the column at a time $t_0$, called void time. The retention factor or capacity factor ($k$) measures the degree of retention of an analyte:

$$k_i = \frac{t_{R,i} - t_0}{t_0} \tag{1.1}$$

where $t_{R,i}$ is the retention time of the analyte $i$. The optimum $k$ value ranged from 1 to 5, being values between 0.2 and 20 also acceptable. $k$ values below 0.2 indicate poor retention, while $k$ values above 20 indicate a high retention caused by an excessive preference of the solute for the stationary phase. This high retention provides very long analysis times and broad and low height peaks, which are difficult to detect and to accurately measure.

The ability of a chromatographic system to distinguish between two solutes is expressed by the selectivity factor $\alpha_{i,j}$, which is calculated as the ratio between the relative retention of both solutes:

$$\alpha_{i,j} = \frac{k_j}{k_i} \tag{1.2}$$

being $i$ and $j$ two adjacents peaks, and $i$ the less retained one.

The degree of separation between two solutes is measured by resolution, $R$:

$$R = \frac{t_{R,i} - t_{R,j}}{0,5(w_i + w_j)} \tag{1.3}$$

where $w_i$ and $w_j$ are the widths at the base of peaks $i$ and $j$.

On the other hand, efficiency is a measure of the capacity of the column to restrain peak dispersion and thus, provide high resolution. The higher the efficiency, the more the peak dispersion is restrained, and the better the column. Column efficiency is measured in theoretical plates ($N$):

$$N = 16 \cdot \left(\frac{t_R}{w}\right)^2 \text{ and } N = 5,54 \cdot \left(\frac{t_R}{w_{1/2}}\right)^2 \tag{1.4}$$

where $t_R$ is solute retention time and $w$ and $w_{1/2}$ are the width of the base peak and its width at half height, respectively.

The height equivalent to the theoretical plate or the variance per unit length of a column ($H$) is calculated as the ratio of the column length to the column efficiency:

$$N = \frac{L}{H} \tag{1.5}$$

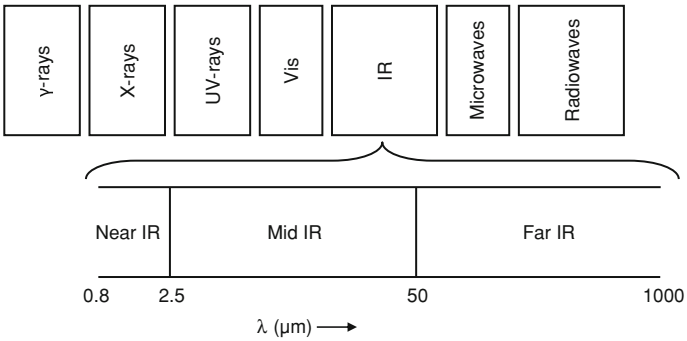



**Fig. 1.4** IR region of the electromagnetic spectrum

Van Deemter equation describes which parameters contribute to $H$, that is, indicates how the different factors of column construction and operation affect its effectiveness. In the case of microparticulate columns both in HPLC and in CEC:

$$H = A + \frac{B}{\bar{u}} + C \cdot \bar{u} \tag{1.6}$$

where $\bar{u}$ is the mean of the linear velocity of the mobile phase.

In this equation the parameters that influence the overall peak width, are expressed in three terms:

- $A$-term, also known as eddy diffusion. This diffusion is due to the different lengths of the flow paths and the different velocities of the analytes that travel along the chromatographic bed. This contribution to $H$ is a function only of the geometry of the stationary phase, that is, it does not depends on $\bar{u}$.
- $B$-term, which represents longitudinal diffusion: This diffusion is proportional to the diffusivity of the solutes and the time that sample passed into the column. As this time increases, diffusion is higher, and therefore the term $B$ becomes important only at low flow rates. This dependence with time is reflected in the inverse of this contribution with respect to $\bar{u}$.
- $C$-term, which is the resistance against mass transfer between mobile and stationary phases. This term is proportional to $\bar{u}$ due to the slow mass transfer of solute between both phases. Thus, the contribution of $C$-term increases with mobile phase velocity as the equilibration time between the two phases is lower. The slow or delay with which mass transfers are made after each mobile phase movement leads to an additional peak broadening.

### *1.3.4 IR Spectroscopy*

The IR region of the electromagnetic spectrum is that located between visible and microwave regions, as shown in Fig. 1.4.

The section of the IR region that provides more structural information at molecular level ranged between 4,000 and 650 $cm^{-1}$. This region is called mid-IR. However, the use of far-IR (between 650 and 200 $cm^{-1}$) has expanded considerably in recent decades, especially for the study of organometallic and inorganic compounds. The near-IR region, between 12,500 and 4,000 $cm^{-1}$, has been widely used for sample authenticity and classification purposes, although its used for structural purposes is limited. It could be said that the IR spectrum characterizes a molecular structure, since two different molecules, with the exception of optical isomers, should show different IR spectra. This property has been extensively used in the characterization of organic compounds. The existence of large databases of IR spectra allows the use of this technique coupled to chromatographic systems in the identification and rapid determination of components of organic mixtures. Moreover, IR spectroscopy could be applied for quantitative purposes using the Beer-Lambert law.

#### 1.3.4.1 IR Spectrophotometers

According to the measurement technique used, IR spectrophotometers could be classified in dispersive and FTIR. The dispersive IR spectrophotometer emerged in the 1940s, while FTIR were developed for commercial use in the 1960s. The main difference between both types is that a dispersive IR spectrometer measures intensity over a narrow range of wavelengths at a time, while an FTIR spectrophotometer simultaneously collects spectral data in a wide spectral range. For this reason, and also by the speed, sensitivity, resolution and signal/noise ratio obtained with the FTIR spectrophotometer when compared with the dispersive one, FTIR is nowadays the most widely spectrophotometer used.

#### 1.3.4.2 *Reflection Techniques in the IR* (© 2005–2012 PerkinElmer, Inc. All Rights Reserved. Adapted with Permission)

One of the strengths of IR spectroscopy is its ability as an analytical technique to obtain spectra from a very wide range of solids, liquids and gases. However, in many cases some form of sample preparation is required in order to obtain a good quality spectrum. Sample preparation is easier for liquid transmission studies when compared to solid transmission sampling but both suffer from inevitable reproducibility issues given the complexity of the sample preparation methods. In addition, preparation can be very messy and time consuming and is further complicated by difficulties in getting sample to matrix ratios correct and homogenous throughout the sample. The materials involved are fragile and hydroscopic and the quality of measurements can be adversely affected if handled or stored incorrectly. In these cases, reflection techniques address these issues. Among them, the ATR technique, which is used in this thesis, is discussed below.

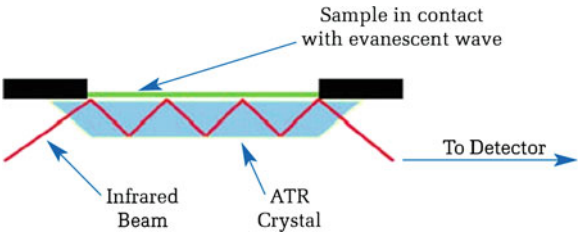



**Fig. 1.5** A multiple reflection ATR system (© 2005–2012 PerkinElmer, Inc. All rights reserved. Reprinted with permission)

An ATR accessory operates by measuring the changes that occur in a totally internally reflected infrared beam when the beam comes into contact with a sample (indicated in Fig. 1.5). An infrared beam is directed onto an optically dense crystal with a high refractive index at a certain angle. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the sample held in contact with the crystal. It can be easier to think of this evanescent wave as a bubble of infrared that sits on the surface of the crystal. This evanescent wave protrudes only a few microns (0.5–5 $\mu$) beyond the crystal surface and into the sample. Consequently, there must be good contact between the sample and the crystal surface. In regions of the IR spectrum where the sample absorbs energy, the evanescent wave will be attenuated or altered. The attenuated energy from each evanescent wave is passed back to the IR beam, which then exits the opposite end of the crystal and is passed to the detector in the IR spectrometer. The system then generates an infrared spectrum.

For the technique to be successful, the following two requirements must be met: (1) sample must be in direct contact with the ATR crystal and (2) the refractive index of the crystal must be significantly greater than that of the sample or else internal reflectance will not occur—the light will be transmitted rather than internally reflected in the crystal. Typically, ATR crystals have refractive index values between 2.38 and 4.01 at 2,000 $cm^{-1}$. It is safe to assume that the majority of solids and liquids have much lower refractive indices.

The traditional ATR design in which a thin sample was clamped against the vertical face of the crystal has been now been replaced by a horizontal design. In horizontal ATR units, the crystal is a parallel-sided plate with the upper surface exposed (Fig. 1.5). The number of reflections at each surface of the crystal is usually between five and ten, depending on the length and thickness of the crystal and the angle of incidence.

The issue of solid sample/crystal contact has been overcome to a great extent by the introduction of ATR accessories with very small crystals. The most frequently crystal is diamond because it has the best durability and chemical inertness. However, there are other materials available for ATR, such as zinc selenide (ZnSe) and germanium. ZnSe, which is the one employed in this thesis, is a relatively low cost ATR crystal material and is ideal for analyzing liquids and non-abrasive pastes and gels.

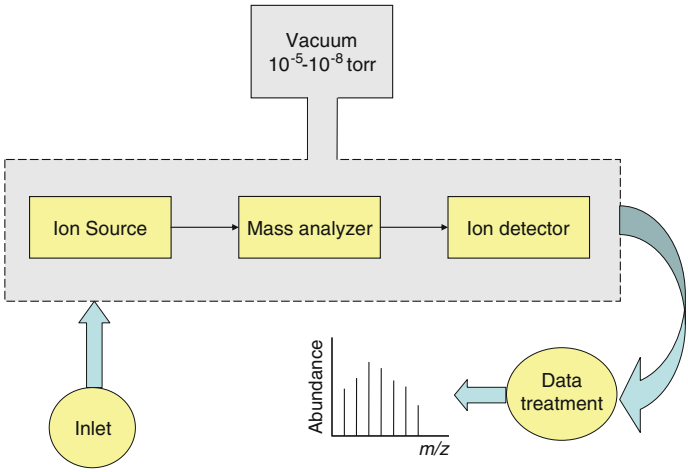



**Fig. 1.6** Components of a mass spectrometer

### *1.3.5 MS*

MS is a technique of wide application able to provide information about qualitative and quantitative composition of both organic and inorganic analytes in complex samples. Mass spectra are obtained by conversion of sample components into their gas-phase ions, which are separated according to their *m/z* ratio, being the analytical signal the abundance or intensity for each *m/z* value. The diagram showing the main components of a mass spectrometer is represented in Fig. 1.6.

As illustrated in this figure, a mass spectrometer is composed by an entry system that allows the introduction of a small amount of sample into the mass spectrometer. There are different entry systems depending on the type of sample to be analyzed (solid, liquid or gaseous). Sample can be introduced in a discrete manner using a syringe or continuously by coupling mass spectrometer with flow injection or with chromatographic or electrophoretic systems.

Jointly with the entry system, the ion source is the responsible of converting sample components into ions by bombardment with electrons, molecules, photons, etc. In many cases, such as the spectrometers used in this thesis, the entry system and the ion source are combined into a single component. Once the ions are produced, they pass into the mass analyzer, which is responsible of separating the various fragment ions produced in the ion source according to their ratio *m/z*. These fragments arrive at detector, which is responsible of converting the ion beam into an electrical signal which is amplified and recorded. These four components are usually found under a vacuum atmosphere at pressures of $10^{-7}$–$10^{-10}$ atm, to avoid collisions with background gas and other molecules, although in some cases, vacuum is

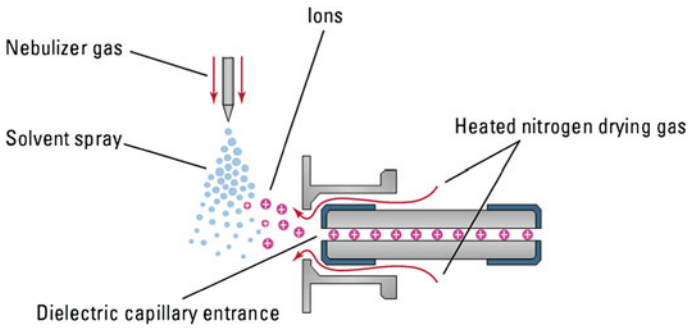



**Fig. 1.7** ESI source scheme (Copyright 2001 Agilent Technologies, Inc. reproduced with permission)

only applied on mass analyzer and detector. Finally, once the analytical signal is registered, it is processed and analyzed providing a mass spectrum.

#### 1.3.5.1 Ion Sources

The most commonly used ion sources, especially in the LC–MS coupling, are ESI, APCI and APPI, which are also used in the studies described below.

In an ESI source (see Fig. 1.7), the sample in solution was passed through a capillary nebulizer jointly with a coaxial $N_2$ flow. A high electric potential is applied at the exit of nebulizer that, jointly with $N_2$ flow, creates a fine spray of charged particles. The gas present at atmospheric pressure in the spray chamber carries the ions into capillary entrance, where the pressure is reduced to about 3 mbar by a vacuum pump.

APCI source (see Fig. 1.8) uses the heat provided by a vaporizer, which usually works at temperatures of 250–400 °C, and a coaxial $N_2$ flow, to convert the sample solution into a fine spray. With solvent evaporation, the resulting gas phase molecules are ionized by accelerated electrons which are formed by in an electrode by a corona discharge. This electrode is composed by a metal needle at which a high potential is applied.

In an APPI source (see Fig. 1.9), as in the APCI, the nebulization and ionization processes occur independently. For the correct used of this source, a certain concentration of a solvent that absorbs UV radiation (doping agent), such as a 1 % acetone, has to be added in the mobile phase. Also in this source, the heat provided by a vaporizer and a coaxial $N_2$ flow are used to convert the eluate into a fine spray, which passes through and area lit by a krypton lamp. The UV radiation produced by krypton lamp ionize dopant agent, which in turn ionizes solutes, being the ionization wavelength is short enough to not ionize common solvents such as

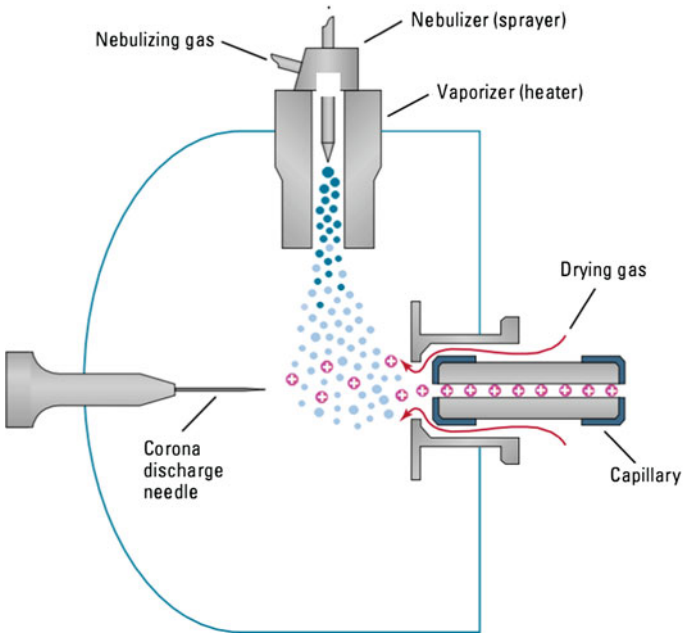



**Fig. 1.8** APCI source scheme (Copyright 2001 Agilent Technologies, Inc. reproduced with permission)

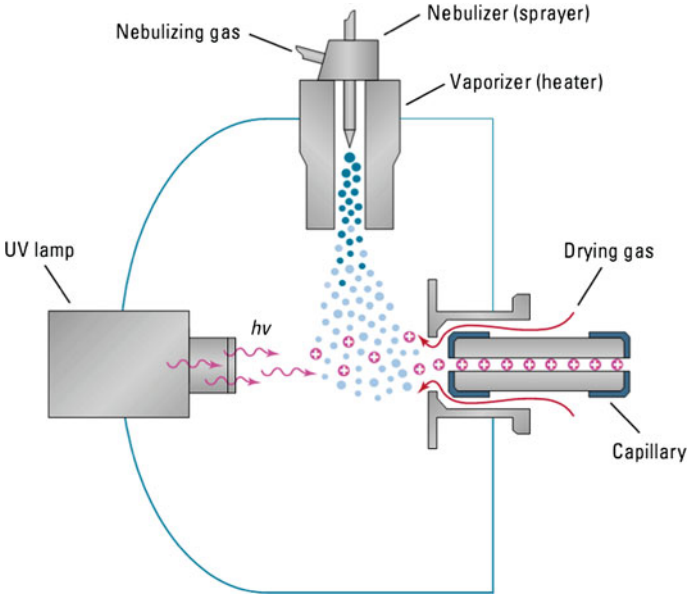



**Fig. 1.9** APPI source scheme (Copyright 2001 Agilent Technologies, Inc. reproduced with permission)

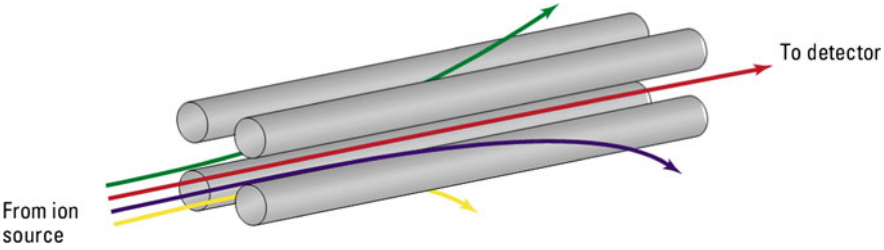



**Fig. 1.10** Scheme of a quadrupole mass analyzer (Copyright 2001 Agilent Technologies, Inc. reproduced with permission)

water, MeOH and ACN. Finally, the ions produced by the solutes are attracted to the capillary and solvents are discarded.

### 1.3.5.2 Mass Analyzers

The most widely used mass analyzers are quadrupole (single or triple), ion trap and time of flight.

A quadrupole mass analyzer or quadrupole mass filter use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency quadrupole field created between 4 parallel rods (see Fig. 1.10). Only the ions in a certain range of *m/z* ratio are passed through the system at any time, but changes to the potentials on the rods allow a wide range of *m/z* values to be swept rapidly, either continuously or in a succession of discrete hops.

A common variation of the transmission quadrupole is the triple quadrupole mass spectrometer. The "triple quad" has three consecutive quadrupole stages, the first acting as a mass filter to transmit a particular incoming ion to the second quadrupole, a collision chamber, wherein that ion can be broken into fragments. The third quadrupole also acts as a mass filter, to transmit a particular fragment ion to the detector. If a quadrupole is made to rapidly and repetitively cycle through a range of mass filter settings, full spectra can be reported. Likewise, a triple quad can be made to perform various scan types characteristic of tandem MS.

On the other hand, in an ion trap mass analyzer ions are trapped and analyzed, following the scheme described in Fig. 1.11, in a space defined by a ring electrode (usually connected to the main radio frequency potential) and between two endcap electrodes (typically connected to DC or auxiliary AC potentials).

The ions formed in the ionization source enter the analyzer, where different voltages are applied, generating a three-dimensional electric field in the cavity of the trap. During the storage phase, this field captures and focuses the ions into stable oscillation paths, depending on these potentials and the *m/z* of the ions. During the scanning or detection stage, the electrode potentials are altered to cause instability in ion trajectories and ejected them in the axial direction. The expulsion or withdrawal of the trap is made based on ion *m/z*, resulting in a mass spectrum. One of the characteristics of the quadrupole ion trap is its ability to isolate an ion

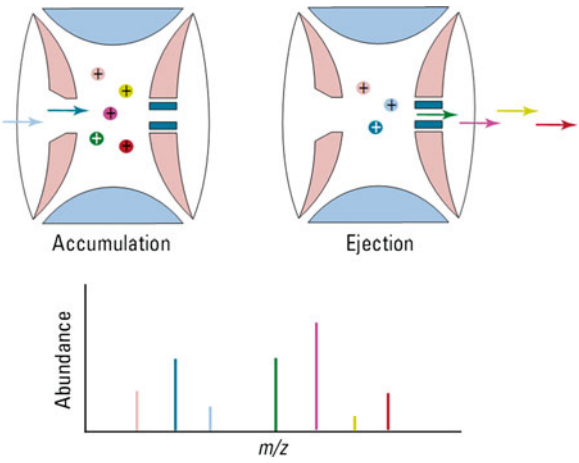



**Fig. 1.11** Scheme of operation of an ion trap (Copyright 2001 Agilent Technologies, Inc. reproduced with permission)

and fragment it, thus obtaining the so-called "fragmentation spectrum" or MS/MS or $MS^2$ spectrum. The fragmentation is induced by collisions with helium atoms, making it possible to control the energy of collisions by controlling the speed of the ions in the trap.

#### 1.3.5.3 Resolution

The ability of a mass spectrometer to distinguish between similar masses is usually expressed as resolution, $R$, defined as:

$$R = m/\Delta m \tag{1.7}$$

being $m$ the nominal mass of the first peak, and $\Delta m$ the difference between two adjacent peaks that are resolved.

The resolution that is needed in a mass spectrometer depends largely on the application. For example, to distinguish between ions of the same nominal mass but different exact masses, a high resolution equipment is needed.

### *1.3.6 Electronic Olfactometry*

Although several approaches of an experimental "electronic nose" were made in the late 50s, it was in 1982 when Dodd et al. (1982) announced the beginning of a new technology: electronic olfactometry. These authors reported the design of an electronic nose based on a set of reversible and semi-selective sensors with different chemical properties. Selectivity was achieved by applying the pattern recognition techniques to the responses obtained with these sensors.

In 1993, Gardner and Bartlett (Gardner 1993) defined the electronic nose as an instrument composed by a set of electrochemical sensors with partial specificity associated with a pattern recognition system capable of recognizing both simple and complex odours. Compared to the human olfactory system, this is also composed by a system capable of detecting odours (human olfactory receptors) and by a data processing system (the brain), which requires training or learning and memory storage. In fact, in both cases, the goal is to relate the perceived aroma with a response that, once stored in the memory, will act as a model for further analysis. In the case of the electronic nose, that memory are databases which has been created from samples previously analyzed by the instrument and from external information that allows the identification of the sample. In this way the recognition system is "trained" to obtained optimal classifications that allow odour identification. Another similarity with biological systems is that the perceived aroma is processed in a global form, without identifying each constituent, so that the classification is based on the perceived aromatic similarity when the pattern is recognized.

#### 1.3.6.1 Components of an Electronic Nose

Electronic noses include three major parts: a sample delivery system, a sensing system and a data analysis system, where both signal pre-processing and pattern recognition techniques are included.

The sample delivery system enables the generation of the headspace (volatile compounds) of a sample, which is the fraction analyzed. The system then injects this headspace into the sensing system of the electronic nose. The sample delivery system is essential to guarantee constant operating conditions.

On the other hand, the sensing system is the "reactive" part of the instrument. When in contact with volatile compounds, the sensors react, which means they experience a change of electrical properties. The main difference between electronic nose systems is the nature of the sensors used. Then, the main goal is to find sensor arrays with broad selectivity profiles but similar at the same time. This sensor array should generate a pattern recognition model capable of distinguishing between different samples. On the other hand, the sensor array responses to a specific compound should be as independent as possible (in a statistical sense) to gather as much information as possible about the compound. Depending on the type of measures in which sensor response is based, sensors can be classified as (1)conductivity sensors, such as MOS, conducting polymers and MOSFET, (2) piezoelectric sensors, such as QCM and SAW and (3) optical sensors.

MOS sensors are based on conductance changes induced by the absorption or reaction of gases on their surface. These sensors are formed by depositing a thick film of the metallic oxide into a ceramic piece that is electrically heated. They are mainly produced using $SnO_2$, ZnO, $In_2O_3$, $WO_3$, $Fe_2O_3$, $Ga_2O_3$ and $TiO_2$, being $SnO_2$ the oxide most widely used. In most cases, and in order to change sensor sensitivity, doping materials such as Pd or Pt are added. An example of a one of

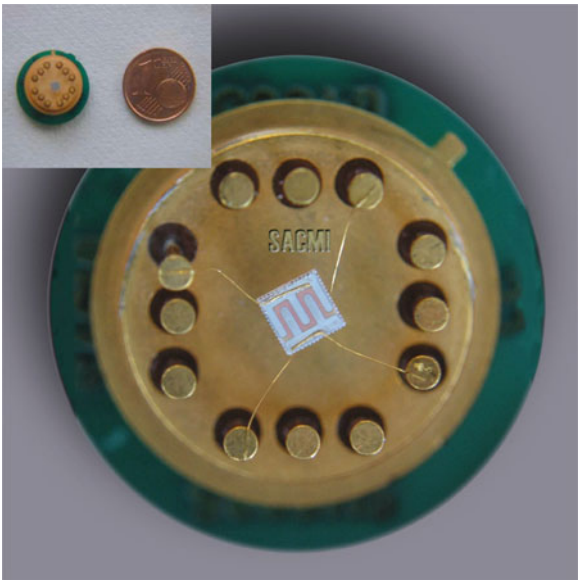



**Fig. 1.12** Example of a MOS sensor used in this study (reprinted from M.J. Lerma-García et al., Ingredienti Alimentari 45 (2009) 12 by courtesy of Chiriotti Editori srl)

the MOS sensors used in this thesis is shown in Fig. 1.12. Metal oxides are an intrinsically *n*-type bulk semiconductors. Oxygen is always present in the eluent of the sensor arrays and upon exposure to oxygen, the oxygen is physi- or chemisorbed onto lattice vacancies in the semiconductor. The loss of the electron results in a decrease in conductance thus increasing resistance. When an odorant is present, the adsorbed oxygen species reacts and is removed from the surface allowing the electron to flow back into the conduction band thereby increasing the conductance (and decresing resistance). These sensors are capable of working at high temperatures (200–400 °C) and its sensitivity is quite high (5–5,000 ppm), but they usually present significant drift and contamination problems.

Finally, data analysis system treats all data provided by sensor arrays. The chemometric techniques used can be classified into two groups: signal pre-processing and pattern recognition techniques.

Signal pre-processing techniques are used to compensate possible deviations from sensor signals, and also to reduce variations between the measure of different samples. The techniques applied in these cases include:

- Handling of the baseline, which is a transformation based on the initial values of the sensors.
- Normalization of responses. Scaling is able to adjust the values of the responses of the sensors to prevent that some variables have more weight than others in the result obtained. The normalization methods are able to avoid experimental variations and reduce computational errors when applying pattern recognition techniques (Gardner 1993).

- Selection of the areas of the sensor response that provide discriminatory information without eliminating essential information. If all the data extracted from a sensor array was used, large matrices will be obtained; for this reason it is convenient to reduce them. Many authors have used different algorithms for this purpose (Hermle 1999), although commercial equipments normally used one data per sensor.

On the other hand, the automated pattern recognition system is the pattern recognition algorithm, which is created to extract useful information from sensors. There are a variety of pattern recognition methods available. The choice of the method depends on available data and the type of result that is required. Data treatment by multivariate techniques is one of the most employed methodologies to analyze electronic nose signals, as multivariate analysis generally involves data reduction. It reduces high dimensionality in a multivariate problem where variables are partly correlated, allowing the information to be displayed in a smaller dimension. Typical multivariate techniques include principal component analysis, feature weighting, ANN, cluster analysis, and discriminant function analysis. In a supervised pattern recognition technique input patterns are learned and associated with an odor class. In an unsupervised pattern recognition technique, the multidimensional configuration space from the preprocessor is converted into a feature space. The unknown chemical is identified by comparison to a knowledge base, from a previous learning scheme. More information on pattern recognition techniques can be found in the Sect. 1.3.7.

## *1.3.7 Data Statistical Treatment*

### 1.3.7.1 Supervised Classification Analysis[1]

In the supervised classification analysis, models capable of predicting if an object belongs to a determine category are constructed. For this purpose, a data matrix that contains at least one variable that indicates the category to which each object belongs (categorical variable) is constructed. This categorical variable constitutes the variable to be predicted. Data matrix also contains one or more scale variables that describe object characteristics. These variables are used as predictors.

To construct a model, it is necessary to have a data set with known information of both, category and predictors (although data belonging to a category could be assumed). The object assignment to the categories should be exhaustive (all objects belong to a category) and mutually exclusive (any object belongs to more than one category). These objects form the training set, which is used to construct

[1] This section has been adapted and translated with permission from Editorial Síntesis, S.A from "G. Ramis Ramos and M. Celia García Álvarez-Coque, Quimiometría, Chap. 8: Análisis Clasificatorio".

the classification model. Once constructed, the model is used to predict the category of new objects from the measurement of predictors. The prediction of an evaluation set allows the validation of the model, which will be applied to predict the category of unknown samples (Ramis Ramos 2001).

There are different supervised classification techniques, such as discriminant analysis, which can be linear (LDA) or quadratic, and the ANN technique.

*LDA*. In LDA, an algorithm that searches functions or discriminant vectors, i.e, linear combinations of the manifest variables that maximize the variance between categories, while minimizing the intra-category variance, is used. To construct the model, it is necessary to assign the objects of the training set to a given category. To do this, a categorical variable is added to the data matrix containing as many categories as needed. The LDA estimates the coefficients $a_1$, $a_2$, …, $a_m$ of the linear discriminant function, $f$, which is able to predict the object belonging s to one or another category:

$$f = a_1x_1 + a_2x_2 + \ldots + a_mx_m \tag{1.8}$$

The discriminant functions are constructed one at a time, looking for directions in space that maximize the expression:

$$\lambda' = \frac{SC_D}{SC_I} \tag{1.9}$$

where $SC_D$ is the sum of squares of the Euclidean distances between objects belonging to different categories in the direction of the discriminant function, and $SC_I$ is the sum of squared Euclidean distances between objects belonging to the same category, also in the direction of the discriminant function. From the $q$ categories, $q - 1$ discriminant functions are obtained (although if the number of predictor variables, $N$, is lower than $q$, $N - 1$ discriminant functions will be obtained). The discriminant functions are obtained in decreasing order of their value $\lambda$' and maintaining orthogonality between them.

The $\lambda$' function varies widely with the number of objects and the separation between them. Therefore, instead of maximizing $\lambda$', is often minimized Wilks' lambda, which is defined as:

$$\lambda_W = \frac{1}{1 + \lambda'} = \frac{SC_I}{SC_I + SC_D} \tag{1.10}$$

This function takes values between 0 and 1. Categories with a clear separation gave $\lambda_w$ values close to 0, while largely overlapping categories gave $\lambda_w$ values close to unity (Ramis Ramos 2001).

*ANN*. An ANN is a systematic procedure of data processing. On a very simplified and abstract level, ANN is based on the cognitive process of the human brain, and is able to accurately predict categorical and scale variables. An ANN is not based on an explicit algebraic model, as LDA. It is based in a set of activation units, called nodes, perceptrons or artificial neurons, which are connected together in a network.

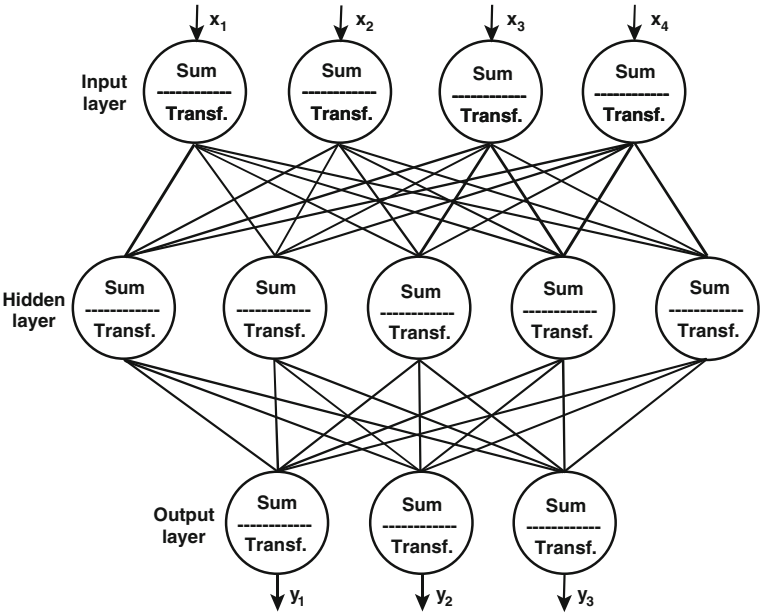



**Fig. 1.13** Scheme of a neural network designed to predict three responses from four predictor variables (adapted with permission from Editorial Síntesis, S.A from G. Ramis Ramos and M. Celia García Álvarez-Coque, Quimiometría, from Fig. 8.12 of p. 192)

An artificial neuron contains two algorithms, one of them calculates the weighted sum of the values that come through the inlet connexions, and the other, called transfer function, generates a response or output that is communicated to other neurons. The network is able to "learn" mainly by adjusting the "weights" of the connections between neurons, until the network as a whole provides sufficiently precise predictions.

A diagram of a simple network, with neurons arranged in three rows or layers, called input layer, hidden layer and output layer, is shown in Fig. 1.13.

The input layer has as many entries as manifest variables, generating as many outputs as nodes in the output layer. The hidden layer has usually one or two more neurons than the input layer. Finally, the output layer has as many neurons as categorical variables to predict.

To get the network to function properly, it is necessary to subject it to a learning or training phase. When the network is trained, the neurons in the input layer obtained information of the values of the manifest variables for the new object. The results of the input layer are processed again in the hidden layer, and the results generated by it are reprocessed in the output layer. The results of the output layer are the probabilities that the new object belongs to each the categories for each of the categorical variables (Ramis Ramos 2001).

### 1.3.7.2 Linear Regression[2]

Linear regression is an explanatory model which aims to "explain" the P value of dependent variables (response variables) from the information provided by Q independent variables (predictors). Once the model is obtained, expressed as algebraic equations, the final objective is to predict future values of the dependent variables from the independents (Mongay 2005).

*Univariate models*. The simplest model is "simple linear regression," with a dependent variable and an independent, and which is reduced to:

$$y = b_0 + b_1 x + e \tag{1.11}$$

being $\hat{y} = b_0 + b_1 x$ the adjust linear function, where $b_0$ and $b_1$ are sample regression coefficients.

However, Q independent variables could be needed to explain the experimental data. In this case, the model corresponds to a multiple linear regression or MLR, which fits a simple equation with Q independent variables:

$$y = b_0 + b_1 x_{i1} + b_2 x_{i2} + b_3 x_{i3} + \ldots + b_Q x_{iQ} + e_i = b_0 + \sum_{q=1}^{Q} b_q x_{iq} + e_i \tag{1.12}$$

which is the general expression of the univariate linear model, where $e_i$ is the error associated with the i-th observation when the model is accepted, $x_{iq}$ is the i-th observation of the independent variable $X_q$, and $b_0$, $b_1$, …, $b_Q$ are the Q + 1 parameters to be determined (Mongay 2005).

*Multivariate models*. In these models, there are P dependent variables ($P > 1$). These models do not pose major problems than the transformation of all vectors of the model matrices (Mongay 2005).

MLR is next described, since is the regression model used in this thesis.

One of the critical operations in MLR is the selection of predictor variables to be included in the model. The model should include a single predictor variable to represent each of the sources of variance of data, that being relevant, are also correlated with the response. If all significant sources of variance are not taken into account, "underfitted" models, which made predictions affected by systematic error, will be obtained. In the opposite case, when the model included more sources of variance than those strictly necessary, "overfitted" models are obtained. In this case, model predicions will be affected by an excessive error (Ramis Ramos 2001).

[2] The information included in this section has been obtained and translated with permission from Editorial Síntesis, S.A from "G. Ramis Ramos and M. Celia García Álvarez-Coque, Quimiometría, Chap. 9: Calibración y regresión múltiple y multivariante" and from the Servei de Publicacions de la Universitat de València from "C. Mongay Fernández, Quimiometría, Chap. 15 : Regresión lineal múltiple.

A simple procedure for variable selections is to initially include in the model all variables that have some probability of influencing the response. Regression coefficients are obtained, and then the variable associated to the lowest regression coefficient is eliminated. The process is repeated until all variables have non-negligible values of the regression coefficient. However, this technique should be applied with caution, since the presence in the model correlated variables reduces its regression coefficient. Therefore, when there is a group of strongly correlated variables, only one variable should be selected to represent the group.

On the other hand, there are more rigorous selection procedures known as "forward", "backward" and "stepwise". These procedures are usually found in statistical packages, and are very helpful when a large number of variables are handled, and when it is not easy to assign the variables to specific sources of variance. In the three methods, the variables are entered or removed from the model following a criterion of "in–out". Thus, in the "forward" algorithm, linear correlations of all variables with the response are calculated, and it is chosen as candidate the variable with the highest $r^2$ value. The variable is only introduced if it exceeds the entrance threshold of the following $F$-test:

$$F = \frac{SC'_{exp} - SC_{exp}}{\frac{SC'_{res}}{n-p}} \tag{1.13}$$

where $SC'_{exp}$ and $SC_{exp}$ are the sums of squares of the variance explained by the models constructed including and excluding the variable candidate, respectively, and $SC'_{res}$ is the sum of squares of the residual variance of the model constructed including the candidate variable. If the first variable entered the model, it is considered as the next candidate the variable that has a higher correlation with the model residuals, that is, the variable which is more correlated with response after removing the variance due to the first variable. The second variable entered the model if it also exceeds the entrance threshold. The process continues with a third candidate, and ends when there are no variables with a partial correlation with the response significantly different from zero, and that also exceeds the entrance threshold. In the "backwards" algorithm, all variables are initially introduced in the model, and then are sequentially eliminated according to the $F$ test. The first variable to be eliminated is the variable that has the lowest correlation with the response. The process ends when there are no variables in the model that satisfy the rejection threshold. Finally, in "stepwise" algorithm, variables are sequentially introduced, as in the "forward" algorithm. However, the entrance of a new variable modifies the significance of those variables which are already present in the model. For this reason, after the inclusion of a new variable, a rejection threshold is used to decide if one of the other variables should be removed from the model. The process terminates when there are no variables entering or being eliminated from the model (Ramis Ramos 2001).

Once the model is obtained, it is necessary to evaluate the goodness of fit. This can be estimated based on the coefficient of multiple determination, $R^2$, and also

by the sum of squared residuals. However, if the sum of squares is calculated considering only the objects of the calibration set, the ability of the model to accurately predict new objects, not included in this set, could be excessively optimistic. To solve this problem, cross-validation techniques are used, such as leave-one-out, which is the most common used. Leave-one-out validation involves the use of a single object from the original sample matrix as the validation set, and the remaining objects as the training set. This is repeated such that each object in the sample matrix is used once as the validation set. In addition to this, the prediction capability of a model could be also evaluated using an independent evaluation set, which will determine the percentage of correctly predicted objects (Ramis Ramos 2001).

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