Chapter 3
The Modern Art of Identification of Natural Substances in Whole Plants

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Keywords (Bio-)analytical methods • Mass spectrometry • Separation methods • Structural • Elucidation • Supercritical liquid extraction

1 Introduction

Although the oxidative reaction is essential for life, this process is responsible for important stresses causing serious cellular damage to DNA, lipids, and proteins. These damages can result in cancers, cardiovascular, or neurodegenerative diseases. Among the armory available against Reactive Oxygen Species (ROS), antioxidant secondary metabolites of plants can be involved in cell defense. One of the main sources of natural antioxidants is the dietary intake. It is well known nowadays that antioxidants are important constituents of vegetables and fruits. For example, vitamin C (ascorbic acid) contained in high amounts in citrus fruits (oranges, lemons, mandarins, etc.,) is able to neutralize ROS and so participate in cell defense, but also protects against rancidity which affects the color or/and aroma of foodstuffs. Furthermore, quite a lot of natural antioxidants are also of interest for anti-aging cosmetics.

Thus, the identification of natural antioxidants is an increasingly important subject. Their isolation from plants and their structural elucidation require the use of several techniques and the development of different analytical methods including extraction procedures. These procedures have to be adapted at each stage (extraction, separation, detection, etc.,) based on the physicochemical properties (solvent solubility, polarity, hydrophobicity, etc.,) of the antioxidants considered.

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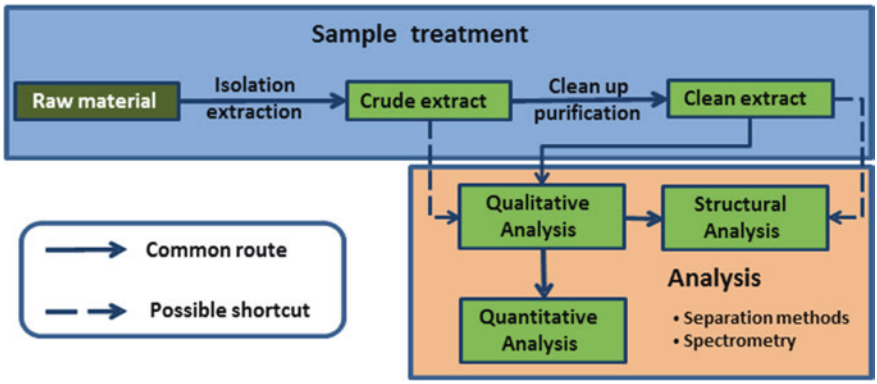

Fig. 1 General scheme of plant analysis. Shortcuts are possible depending on the quality or the purity obtained after sample treatment

This chapter provides an overview of the current techniques used to identify interesting natural products (see also Explanatory Box 1). Whenever it is possible, examples of applications are given for some antioxidants. Following the scheme presented in Fig. 1, the chapter focuses on their isolation (extraction and purification), separation, and detection for qualitative or quantitative analysis. The last part is dedicated to hyphenation between separative methods and mass spectrometry for the identification of secondary metabolites in plant extracts.

Explanatory Box 1: (Bio-)Analytical Techniques

Natural product research relies heavily on a barrage of powerful analytical techniques. The latter enable, for instance, the identification and characterization of a given compound, including structural elucidation. The latter is often far from trivial, as many natural products contain one or several chiral centers. Analytical techniques commonly employed in this context include elemental analysis to determine the elemental composition of a given compound (e.g., content of carbon, nitrogen, hydrogen, sulfur), (high resolution) mass spectrometry (HR-MS) to determine its molecular weight, aspects of purity, and, if applicable, specific isotope splittings, UV/Vis and IR spectroscopy to identify the presence of specific functional groups and various types of one-dimensional or two-dimensional nuclear magnetic resonance (NMR) to investigate the structure of the compound.

Further methods may be used depending on the particular physicochemical properties of a compound, for instance polarimetry or circular dichroism in case of chiral compounds, refractometry in the case of liquids, electron

spin resonance (ESR) in the case of radicals, and many transition metal ions and electrochemical methods in the case of redox active compounds. Indeed, electrochemical methods such as Cyclic Voltammetry and Differential Pulse Polarography have recently been at the forefront of investigations into the redox behavior of many secondary metabolites, their (*in vitro*) interactions with biomolecules, and the redox sensitivity of biological targets (proteins, enzymes).

At the same time, analysis is paramount when it comes to the purity of a given natural product. Again, methods such as HR-MS and NMR may be used, together with various types of chromatography. Here, high-performance liquid chromatography (HPLC) and gas chromatography (GC) are commonly used methods also mentioned in the text.

This 'analysis' of the natural compound itself is matched by the analysis of biological activity and biochemical mode(s) of action. Here, rather different methods are employed. Biological activity is usually determined in rapid screens based on isolated proteins and enzymes, cells, microorganisms, viruses, or even small animals (such as nematodes). In the last couple of years, in silico screening has become important, as computational methods become more and more predictive and hence competitive. Such virtual screens enable researchers to reduce the number of real biological screens and to focus on a dramatically reduced number of carefully selected compounds. At the same time, the methods available to track down intracellular processes triggered by various natural and unnatural compounds, such as specific cellular signaling pathways, have become more sophisticated. It is now possible to use a whole barrage of staining and labeling techniques to investigate individual cellular responses, from proliferation, differentiation, and apoptosis to the up- and down-regulation of individual proteins using fluorescently labeled antibodies. As we will see in some of the following chapters, it is also possible to use fluorescent staining to detect cellular components, such as various Reactive Oxygen Species, posttranslational cysteine modifications, Ca^{2+} influx, and content of reduced glutathione to name just a few. These methods are based on fluorescence and microscopy. Other methods used to track down compounds in cells include Energy Dispersive X-ray Analysis (EDX), which is indicative of the presence of certain elements and can now also be applied to biological samples without the need of any labeling. These methods are complemented by other proteomic and genomic analytical techniques, such as isotope labeling in combination with mass spectrometry, Western Blots to quantify (time resolved) changes in protein levels, and stains indicative of the mitochondrial membrane potential $\Delta\Psi_M$.

The development of analytical techniques to track down and characterize natural products is currently attracting considerable attention. At the same time, screening techniques to determine the biological activity of

compounds are becoming ever more sophisticated. They are joined by highly effective methods for ‘intracellular diagnostics’ and target evaluation. Here, areas such as proteomics, genomics, chemogenomics, and life cell imaging are growing rapidly.

2 Extraction of Antioxidants from Plants

Extraction is one of the most imperative steps for the analysis of natural products involving chromatography. The procedures involved are so that the molecules of interest initially contained in the plant tissues are solubilized in a liquid phase. The resulting solution can be more or less directly injected into the chromatographic system or submitted to other analytical pretreatments (e.g., liquid–liquid extraction, solid phase extraction, etc.,) depending on the quality required of the purification. The ideal extraction procedure must be simple, rapid, and efficient. Nevertheless, the active molecules must be carefully preserved from possible degradation.

2.1 Conventional Extraction Procedures

Since the earliest times, man has always performed extraction procedures of active molecules from plants for medicinal purposes. For many drugs, he had to prepare brews by maceration in cold water or decoction, infusion, or percolation of plants using warm or boiling water. Increasing the temperature generally improves the efficiency of the extraction procedure because heat increases the diffusion and solubility of the molecules in the solvent. During the process, the solvent penetrates the tissues and dissolves the metabolites. A pre-grinding of the plant (especially for very hard and dense samples such as nuts or bark) is usually carried out to facilitate the disruption of the cell walls and to increase the surface exchange between the solid matrix and the solvent (Heldman and Hartel 1997). The solution moves through the solid matrix to its surface and becomes dispersed in the solvent (Fellows 2000). The solid matrix can be eliminated afterwards by filtration, decantation, etc. The solvent plays a key role in this process and consequently it must be carefully chosen regarding the hydrophilic–lipophilic balance of the metabolite to be extracted. The Folch extraction is undoubtedly one of the most popular solid liquid extraction procedures (Folch et al. 1957). The tissue is mixed with chloroform/methanol (2/1, v/v) in the following proportion: 1 g in 20 ml of solvent mixture. The whole mixture is shaken for 15–20 min at room temperature allowing the extraction of apolar antioxidants such as carotenes and carotenoids. By contrast, alcohols (methanol, ethanol) or hydro-alcoholic mixtures give better results for more polar compounds such as polyphenols (Jokic et al. 2010).

The traditional extraction procedures also include some slightly more complex methods such as hydrodistillation or Sohxlet extraction. The latter is probably the

most regarded among all the extraction methods and it often constitutes the reference for comparison between other available procedures (accelerated solvent extraction, microwave-assisted extraction, etc.,). It is a solid–liquid extraction procedure presenting the benefits of a constant source of renewed solvent by distillation. The extracting solvent is thus never saturated and the extraction yield is improved as the sample is progressively depleted of its molecules of interest.

In spite of its efficiency, this method requires time (several hours even in its automated version) and high amounts of solvent (hundreds of milliliters). Moreover, the constant heat (use of boiling solvent for hours) may also induce the thermal degradation of active molecules reducing the activity of the final extract (Wang and Weller 2006). However, the classic Sohxlet remains an efficient low-cost method because it only requires laboratory glassware and an electric heater. Furthermore, the extract composition can be easily modulated according to the nature of the solvent used (Zarnowski and Suzuki 2004). Finally, in some cases, the highest yields are even obtained with a Soxhlet apparatus. This was observed, for example, for the methanolic extraction of protocatechuic, *p*-hydroxy-benzoic, vanillic, and ferulic acids in *Sambucus nigra L.* inflorescence (Waksmundzka-Hainos et al. 2007).

2.2 Improved Extraction Methods

Alternative extraction methods were introduced to overcome the inconveniences of the traditional methods. They were developed in order to prevent active products from thermal degradation, to improve the extraction yields, but also to reduce solvent consummation, time, and costs.

2.2.1 Supercritical Fluid Extraction

In certain ways, the supercritical fluid extraction (SFE) is a form of solid–liquid extraction where the extracting solvent is not a liquid at atmospheric pressure but a supercritical fluid. Such a fluid is at an intermediate physical state between liquid and gas (see Explanatory Box 2). It adopts the density of a liquid, whereas its viscosity and diffusion rate correspond to a gas. Consequently, the diffusion and solubility of the molecules in the solvent (i.e., the supercritical fluid itself) are more efficient. Reaching a supercritical state supposes that the temperature and pressure of the fluid are raised over their critical value (Fig. 2a). For carbon dioxide, which is probably the most used supercritical fluid, both parameters are relatively low: 31 °C and 74 bars, respectively. These conditions are obtained and maintained, thanks to a complex equipment (Fig. 2b) comprising a pump delivering the high-pressurized fluid (e.g., a liquid chromatography pump), temperature controllers, pressure regulators, and special SFE cells. Thus, this extraction method is more expensive than traditional extraction methods.

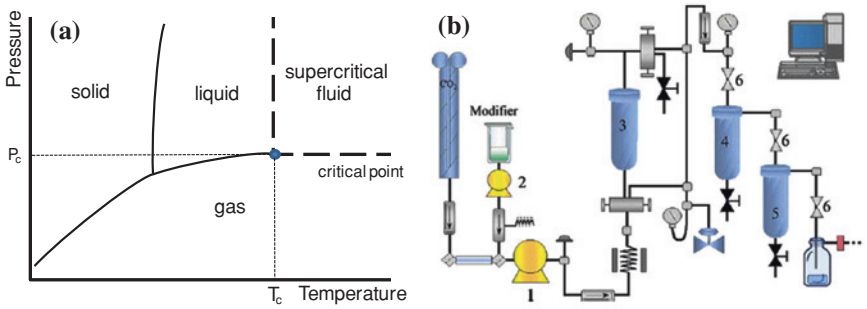

Fig. 2 **a** Physical state of a fluid as a function of pressure and temperature (P_c = critical pressure, T_c = critical temperature). **b** Scheme of a SFE equipment. Diagram of a supercritical fluid extraction pilot plant equipped with two fractionation cells. *1* CO_2 pump, *2* modifier pump, *3* solid samples extraction cell, *4* fractionation cell 1, *5* fractionation cell 2, *6* valve, reprinted with permission (Herrero et al. 2006)

Explanatory Box 2: Supercritical Fluids

Supercritical fluids in general, and supercritical carbon dioxide (CO_2) in particular, have recently attracted considerable attention in natural products research. These materials occur once a liquid reaches its 'critical point' which is defined by a specific temperature and pressure and can be illustrated in a so-called 'phase diagram'. Such diagrams are widely known and contain areas where a given compound is either solid, liquid, or gaseous (i.e., occurs in one of the traditional states of matter). The critical point, however, marks a specific temperature–pressure point beyond which a rather different phase occurs. Instead of evaporating to form a gas, the liquid turns 'supercritical'. Most common liquids do not turn supercritical under normal everyday conditions. Water, for instance, may well turn supercritical, but only at high temperature and under substantial pressure. CO_2, in contrast, turns supercritical at just over 300 K (at around 30 °C) and 72 atmosphere pressure. It is therefore an ideal supercritical fluid for practical applications, as it is easily produced and also chemically fairly inert. Curiously, CO_2 is unusual as it does not occur in a liquid phase under normal pressure at all, but sublimes at −78 °C.

Such supercritical materials show properties of liquids as well as gases, and are very 'temperamental' once either their temperature or pressure are changed. Indeed, various properties of these supercritical fluids reside between the ones of classical fluids and gases, such as density, viscosity, and diffusivity. Hence it is possible to exploit the liquid-like and gas-like properties of such supercritical materials for a range of practical applications.

It is possible, for instance, to use supercritical CO_2 for the extraction of caffeine from coffee beans or of essential oils from natural sources. Here, the ability to 'extract' caffeine or oils is clearly associated with the liquid-like behavior of supercritical CO_2, while the subsequent removal of the 'solvent' (as a gas and without any residues) takes advantage of the gaseous behavior of the CO_2. It is even possible to use supercritical CO_2 for the separation of compounds, as it is easily possible to modify its density by 'fine-tuning' temperature and pressure.

SFE processes as follows. The sample is introduced into the extracting vessel. The supercritical fluid, regulated at the desired pressure and temperature, passes through the sample and dissolves the molecules of interest. The dissolving power of the fluid is lowered by decrementing the applied pressure and the extracted molecules in the collector. In the case of sophisticated equipments, several collectors may be associated in series to improve the efficiency of the sample fractionation and when SFE is carried out at industrial scale, the CO_2 is possibly recycled. Finally, CO_2 is a gas at room temperature, so its complete elimination from the extract is easily achieved. Finally, a dry plant extract is obtained without any organic solvent residues.

As mentioned above, carbon dioxide is the most popular fluid. Supercritical CO_2 preferentially extracts apolar compounds, but by changing its density by modulating the applied pressure and temperature, its extracting power can be optimized for other metabolites as well. This technique is widely used to extract phytonutrients such as caffeine, (Park et al. 2007) aroma, (Blanch et al. 1995; Polesello et al. 1993; Augusto et al. 2003) spices, (Illes et al. 1999), and lipids (Eggers 1996) but also antioxidants such as astaxanthine (Lopez et al. 2004), carotenes (Illes et al. 1999; Longo et al. 2012), tocotrienols, tocopherols (Illes et al. 1999), and resveratrol (Benova et al. 2010). Furthermore, because SFE is performed in the absence of light and oxygen, it prevents possible degradation or isomerization of these compounds during the extraction step. Active metabolites poorly soluble in CO_2, such as phenolic and glycosidic compounds, however, cannot be extracted from the plant limiting the interest of SFE for many applications. A small percentage of a polar organic modifier (up to 20 %) is miscible to the supercritical CO_2. The addition of a co-solvent (e.g., methanol, ethanol, acetonitrile) increases the solubility of polar compounds (Valcarcel and Tena 1997) but sometimes reduces the selectivity of the extraction. In addition, the use of an organic modifier requires a slightly higher temperature to reach the supercritical state and this could be disadvantageous for thermally labile compounds. In addition, the dried extract, finally obtained may still contain traces of organic solvent residues.

The use of subcritical water is a possible alternative to CO_2 for the extraction of polar metabolites. Subcritical water extraction (SWE) is generally carried out

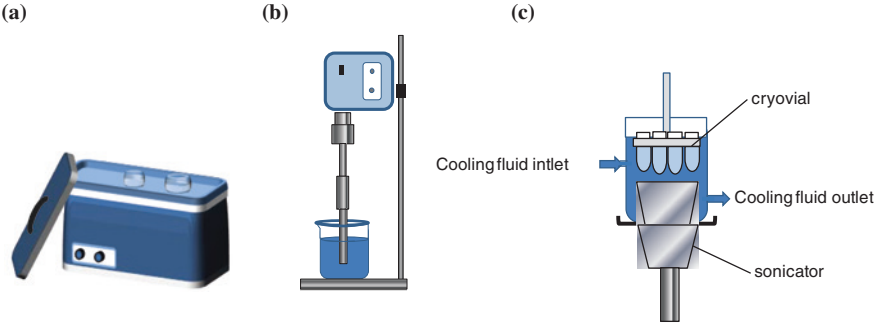

Fig. 3 Devices for ultrasonic assisted extraction. **a** Ultrasonicbath. **b** Ultrasonicprobe. **c** Cup-horn

at high temperature (100 to 374 °C). At the same time, the high pressure applied (10–60 bars) maintains its liquid state. Increasing the water temperature lowers its dielectric constant allowing the solubilization of less polar molecules. Consequently, the polarity of water can be easily adjusted from a polar to an apolar-like solvent just by varying both parameters. Thus SWE has been used for the extraction of catechins and proanthocyanidins (Garcia-Marino et al. 2006). For this application, SWE is more efficient than a solid–liquid extraction performed in a water–methanol mixture (25/75, v/v). The extraction of anthraquinone of *Morinda citrifolia* by SWE is also more efficient than ethanol or ultrasound-assisted extraction for the extraction (Pongnaravane et al. 2006). Although SWE improves the extraction ability for polar compounds, it remains unsuitable for thermolabile molecules (Lang and Wai 2001). Furthermore, although SWE only uses a 'green' solvent, maintaining the high temperature and and the pressure is energy consuming.

2.2.2 Ultrasonic Assisted Extraction

The ultrasonic assisted extraction (UAE) is a fairly simple and easy-to-use method. Sample and solvents are mixed together in a vessel and the mixture is then either placed in an ultrasonic bath or directly submitted to ultrasound using a diving probe (Fig. 3). The cup-horn sonicators are ideal for small volume multisampling extractions. In a study concerning the ultrasound assisted extraction of ginseng saponins from ginseng roots, Wu et al. (2001) compared the performance between an ultrasonic bath and a horn probe. They obtained similar results in terms of extraction yield and rate with both devices.

The extraction mechanism has already been described in detail (Luque-Garcia and Luque de Castro 2003; Soni et al. 2010). Sound waves at frequencies higher than 20 kHz are applied. The sound waves travel into the matter in which the mechanical vibrations generated induce expansion and compression cycles. The expansion creates bubbles in the solvent. They grow and finally collapse when they reach a critical volume. Rapid gas and vapor compression inside the bubbles

produce a very high temperature and pressure (Suslick 1994). When coming close to a solid matrix, the bubbles become asymmetric and when they collapse, their implosion produces high-speed jets of liquid impacting the solid surface. These high-speed jets lead to the detachment and expulsion of small particles of matrix facilitating the transfer of the molecules in the extracting solvent.

In order to optimize an UAE, several parameters have to be considered. In a study on extraction of oil in flaxseeds by ultrasound, Zhang et al. (2008) showed that the higher the ultrasonic power, the higher the yields. This parameter, however, has also to be regulated as the yields may decrease above the optimal power. The extraction time is shortened and an excess of sonication can even decrease the quality of extracts. The same goes for temperature (Wang and Weller 2006). Very often, all these parameters are easily optimized using an experimental design. For example, the highest antioxidant activity of polyphenols [(−)-epicatechin, procyanidin B2, chlorogenic acid, and procyanidin B1] extracted from unripe apples are obtained for an ultrasonic power of 519.39 W, an extraction time of 30 min, and an extraction temperature of 50 °C in ethanol 50 % (Yue et al. 2012).

The main benefits of UAE over conventional Soxhlet extraction are higher efficiency (higher yields in less time) (Zhang et al. 2008) and the leaching of thermolabile compounds. Compared to other advanced techniques such as SFE or accelerated solvent extraction (ASE), the pressure is generally lower. Hence, the UAE equipment is cheaper than that for SFE and ASE. Because UAE can be carried out with polar or apolar solvents, it allows extracting compounds whatever their polarity, whereas supercritical CO_2 is rather limited to apolar molecules. Thus UAE is used increasingly for the extraction of natural compounds in plants. The mechanical activity of the ultrasound improves the diffusion of solvents into the tissue and facilitates the disruption of the cell wall. The solubilization of the metabolites is thus favored. The extraction of tirucallane-like triterpenes from *Juliania adstringens* was achieved with ethanol as solvent (Makino et al. 2004). Hromadkova et al. performed the extraction of the hemicellulose components of buckwheat hulls in alkali solutions (Hromadkova and Ebringerova 2003). The literature also mentions applications concerning antioxidants. For example, two ultrasonic extraction methods were optimized by Adam et al. (2009) for the isolation of esculetin, scopoletin, 7-hydroxycoumarin, rutin, xanthotoxin, 5-methoxypsoralen, and quercetin from plants such as *Mentha* species, *Ruta graveolens L.*, *Achyllea millefolium L.*, *Plantago lanceolata L.*, and *Coriandrum sativum L.* Glycosylated phenolic antioxidants such as myricitrin-3-rhamnoside, quercitrin-3-rhamnoside, europetin-3-rhamnoside, or kaempferol-3-rhamnoside were extracted from *Acacia confusa* flowers and buds (Tung et al. 2011). In this case, the best results were obtained applying 12 sonication cycles of 10 min each.

2.2.3 Accelerated Solvent Extraction

Accelerated solvent extraction (ASE) is a solid–liquid extraction process that bears similarities to SFE. Indeed, this patented technique (Dionex) is performed

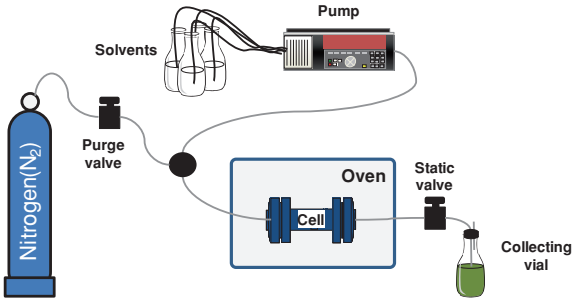

Fig. 4 Equipment for accelerated solvent extraction (*ASE*). The sample is weighed and placed into the extraction cell. The cell is filled with solvent, pressurized, and heated in the oven. The hot solvent extracts the metabolites by direct contact with the sample by both static and dynamic action. After the extraction is complete, the solvent is removed from the cell to the collecting vial with the assurance of compressed nitrogen. The sample is ready for further analysis

at elevated temperatures, usually between 50 and 200 °C and at pressures between 10 and 15 MPa to maintain the solvent in a liquid state below its critical conditions. Subcritical water can also be used in an ASE apparatus (Fig. 4) (Eskilsson et al. 2004) but generally ASE is carried out with common organic solvents (pure or mixtures). The use of adapted stainless cells (Dionium^{TM}) also allows the sample pretreatments at relatively extreme pH (0.1 M of H_2SO_4, HCl or KOH) (Dorich et al. 2008).

Thus, ASE allows extracting apolar as well as polar compounds depending on the choice of the solvent. For example, Mulbry et al. (2009) optimized an oil extraction process for algae studying several compositions of solvent starting from hexane to methanol–chloroform mixtures. They showed that ASE yields higher values for total oil content than the traditional Folch method. Other studies showed that the same goes for oxysterols in food (Boselli et al. 2001) or carotenoids in plants (Denery et al. 2004). Compared to techniques performed at atmospheric pressure (Soxhlet or UAE), extracts are obtained in a very short time and with a little solvent consumption. The extraction time is reduced from several hours (for Sohxlet) to a few tens of minutes and ASE requires around ten times less solvent. In addition, as for all pressurized solvent extraction methods, the extraction efficiency is improved. The temperature, however, must be carefully controlled, otherwise there is still a risk of decomposition for thermolabile metabolites. Solvent composition, temperature, applied pressure, and the number of cycles are parameters associated to the optimization of the extraction yields (Cicchetti and Chaintreau 2009). These parameters can easily be optimized, thanks to experimental design. Using response surface methodology (RSM), Hossain et al. optimized the composition of rosemary, marjoram, and oregano extracts. The optimum temperature (allowing the highest antioxidant activity for the extract) was 129 °C and the optimal percentage of methanol in the hydro-alcoholic solvent was around 56 % for marjoram and rosemary, whereas it was only 33 % for oregano (Hossain et al. 2011). This study concerns several phenolic antioxidants including rosamarinic, caffeic, and gallic acids.

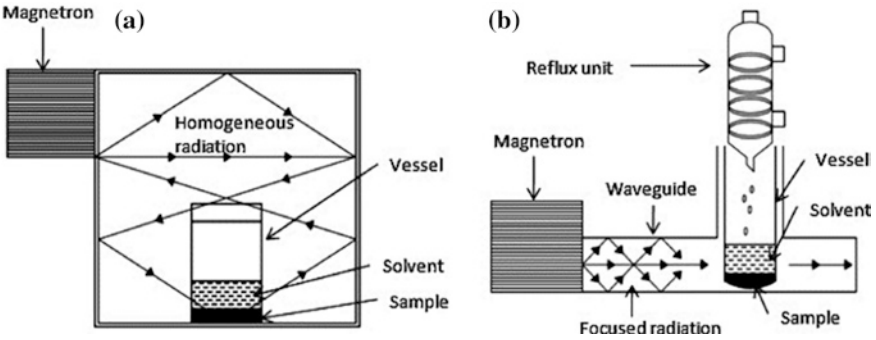

Fig. 5 Apparatus for microwave assisted solid extraction **a** Closed-type microwave system and **b** open-type microwave system. Reprinted with permission (Chan et al. 2011)

2.2.4 Microwave Assisted Solid Extraction

In the landscape of the extraction of organic compounds, microwaves appeared in the middle of the 1980s (Ganzler et al. 1986). Extraction is carried out in a microwave oven whose power (600–1000 W) and frequencies (~2,450 MHz) are the same as for domestic ovens. Commercially available MASE systems (Fig. 5) use either closed extraction vessels allowing a pressure and temperature control or open vessels topped with a condenser (Chan et al. 2011; Mandal et al. 2007). Both systems have their own advantages and drawbacks (Table 1).

Whatever the apparatus, a part of the electromagnetic energy absorbed by the extracting material is converted into heat energy via two distinct and simultaneously occurring mechanisms: (1) the friction between molecules due to the electrophoretic migration of ions under the changing magnetic field and (2) the constant realignment (more than 10^5 times per s) of the dipolar moment of molecules (solvent and/or metabolites) with the magnetic field. Both clearly indicate that only dielectric material or solvents are subjected to microwaves. The main advantage of the MASE over the methods does not only lie in its rapidity but also on its heating process. Compared to classic heating, microwaves are able to directly reach the heart of the sample. Thus, this technique is not only a surface method and through heating the method guarantees better extraction efficiency.

Several parameters strongly affect the final quality and the yield of the extract. The solvent must be carefully chosen taking into account its absorbing properties and the solubility of the extracted metabolites. Generally, organic solvents with high dielectric constants (i.e., methanol, ethanol) exhibit the highest performances. Very often, small amounts of water in the extracting solvent improve cell penetration and the heating of the plant matrix. For example, the optimum solvent composition for the extraction of chlorogenic and geniposidic acid from *Eucommia ulmodie* is aqueous methanol 80/20 (v/v) (Li et al. 2004). By contrast, a solvent with a low dielectric constant (i.e., hexane) is often less efficient in MASE compared to the corresponding Soxhlet procedure (Alfaro et al. 2003). Such a solvent,

Table 1 Advantages and drawbacks of microwave assisted solid extraction (Mandal et al. 2007)

MASE system	Advantages	Drawbacks
Open vessel	• Safety (operating at atmospheric pressure) • Possible addition of reagents during the extraction process • Solvent excess easily removed • Large samples can be processed • Easy cooling down and no depressurization • Low cost • Possible fully automation • Suitable for thermolabile species	• Less precision than closed-vessel systems • Throughput is lower in most open systems • Extraction times usually longer than for closed vessels
Closed vessel	• High temperatures reached allowing the decrease of the treatment time • Loss of volatile substances during irradiation is avoided • Less solvent is required (no evaporation occurs) • Less hazardous during acid extractions (fumes are confined in the closed vessel) • High throughput (a few ten samples simultaneously)	• Risks of explosion at high pressures • Treatment of limited amount of material • Some material (PTFE) does not withstand high temperatures • No addition of reagents or solvents during the procedure • Cooling down after the treatment and before the opening of the vessel (to avoid loss of volatile compounds)

however, can be mixed to a good microwave absorbing solvent to improve the solubilization of apolar compounds. For example, the mixture of ethanol (the absorbing solvent) and hexane 1/3 (v/v) was the optimal composition for the extraction of solanesol from tobacco leaves (Zhou and Liu 2006). In this case, hexane helps the solubilizing of this apolar metabolite. Zhao et al. (2012) recently used graphite powder sealed in glass tubes as microwave absorbing agent. The samples, the solvent, and the absorption microwave tubes are transferred into closed vessels and then submitted to microwave heating. Such an approach allows the use of nonpolar solvents (i.e., hexane, cyclohexane, isooctane, petroleum ether, etc.,) in microwave assisted extraction. The solvent volume is also an important parameter. Even if the solvent to sample mass ratio can strongly vary from an application to another, ratios in the range of 10:1 (ml/mg) to 20:1 (ml/mg) are usually the optimal proportions. Yet whatever the case, the solvent volume has to be sufficient to completely immerse the solid sample. Finally, the constitutive water of the plant can also be used as extracting solvent itself. This kind of procedure is named solvent-free microwave-assisted extraction (SFMAE) and is generally carried out at atmospheric pressure. Here, nothing else (i.e., no additional solvent) is required besides the samples' natural moisture. This moisture serves as heating source leading to the disruption of the cell walls and to the release of analytes. Many applications

have already been carried out using this approach, e.g., for essential oils (Lucchesi et al. 2004a, b). SFMAE, however, can also be performed under pressure. Michel et al. (2011a) developed and optimized (extraction time, irradiation power, number of cycles) the pressurized solvent-free microwave assisted extraction (PSFME) technique for antioxidative compounds contained in sea buckthorn (*Hippophaë rhamnoides L.*) berries. Compared to other extraction techniques (including ASE), PSFME leads to the most active and richest extract regarding its phenolic content. Molecules such as quercetine and its glycosylated derivatives were successfully extracted, including isorhamnetin, which could not be extracted reasonably with any other techniques.

As for the other described methods above, the extraction time must also be carefully optimized. A too short or too long extraction time strongly affects the yields. The extraction time is generally shorter than for other methods. Usually, a few tens of minutes are sufficient but the extraction time can sometimes be reduced to a few tens of seconds. Thereafter, overexposure to heat often leads to thermal degradation of the active molecules. The same goes for the microwave power. In fact, the best conditions of extraction are a compromise between time and power. High power requires low extraction times and vice versa. The consequences, however, obviously are not the same. Indeed, rapid exposure at high microwave power induces a rapid cell wall disruption leaching not only the analytes but also unwanted impurities, whereas long time exposure at low power is more selective in the extracted compounds (Mandal et al. 2007). In some cases, the microwave power was found less decisive. For example, Gao et al. (2006) showed that the extraction yields of flavonoids from *S. medusa* were not significantly affected by the microwave power above 400 W. They used a 1,200 W-microwave power allowing a shorter extraction time and higher efficiency than a dynamic solvent extraction without microwave assistance.

The particle size of the matrix is also a crucial parameter. As for other procedures, reducing the particle size provides a higher surface of contact with the solvent improving the extraction efficiency. Furthermore, in MAE, having small particles increases the efficiency of penetration of the microwave into the plant tissues. Usually, fine grinding of the solids is ranged between a few hundred micrometers to a few millimeters. The smallest particles pose the most difficulties regarding their removal from the final extract.

Generally, because of the efficient heart heating and the short extraction time avoiding thermal degradation, MASE leads to very interesting results in terms of yields and sample activity. For example, Uquiche et al. improved the recovery (up to 45.3 %), quality (higher content in unsaturated fatty acids), and stability to oxidation of Chilean hazelnut oils after a microwave pretreatment prior to the pressing step. They assigned the higher stability to the oxidative deterioration to a possible inactivation of the oxidative enzymes (Uquiche et al. 2008). Prior to their quantitative analysis by liquid chromatography coupled to mass spectrometry, Sánchez-Ávila et al. achieved the extraction of some pentacyclic triterpenes of interest from olive leaves (erythrodiol, uvaol, oleanolic, ursolic and maslinic acids) by microwave assisted extraction in only 5 min (Sanchez-Avila et al. 2009), whereas it took

more than 5 h by maceration and around 20 min by UAE. MASE also showed excellent results for the extraction of antioxidants such as polyphenols. It has been successfully applied to the extraction of hydroxycinnamic acids and flavanols from green tea leaves leading to better results than the corresponding conventional hot water extraction (Nkhili et al. 2009).

Microwaves can also be combined with other techniques. For example, coupling microwave with ultrasonic extraction is complementary and may present many advantages. Performing a simultaneous ultrasonic/microwave assisted extraction (UMAE), Lou et al. showed that the extract of burdock leaves exhibited higher antioxidant and antibacterial activities than the corresponding maceration extract. Moreover, UMAE only requires 30 s. In these extracts, they also evidenced a higher total content in phenolic compounds (chlorogenic, *o*-hydrobenzoic, caffeic, ferulic, *p*-coumaric acids and rutin) than in the extract achieved by maceration (Lou et al. 2011).

2.3 Concluding Remarks on Extraction

Sample extraction is a critical step of any qualitative or quantitative analysis by separative methods. Besides, the same applies to the activity of the final extract. It is hard to foresee, however, which would be the best extraction methods. In spite of hard heating and time consumption, Soxhlet remains a good basic approach. Among the improved extraction methods, none of them appears to be superior to another. They all have their own advantages and drawbacks and whatever the methods, the parameters governing the final quality of the extract have to be carefully optimized. Finally, the chosen methods really depend on the application, the molecules, and many other parameters. Indeed, for example, the highest extraction yields of protocatechuic, *p*-hydroxybenzoic, and gallic acids from *Polygonum aviculare* foliage were obtained by MASE in a closed system, whereas the highest yield for ferulic acid was obtained by UAE (Wang and Weller 2006). Many other similar examples are found in the literature. As already mentioned, several extraction approaches can be combined together. In addition, they can even be coupled online with purification techniques. For example, Yang et al. (2007) coupled continuous ultrasound assisted extraction, solid-phase extraction, and liquid chromatography for the determination of danshensu sodium salt and four tanshinones in the roots of *Salvia miltiorrhiza bunge*.

3 Clean-Up and Purification Techniques

The solid–liquid extraction methods are more or less selective regarding several parameters, such as the nature of the matrix (leaves, roots, bark, etc.,) and the conditions applied (temperature, pressure, etc.,). The same goes for the qualitative

and/or quantitative analysis of the produced extracts by a separation with gas or liquid chromatography. The specificity of detection and the selectivity of these methods strongly depend on the systems chosen, including the nature of the stationary and mobile phases, and obviously the detector. The extracts generally still contain many unwanted molecules that may interfere with the analytes of interest during the analytical step and there is a real need for their removal.

3.1 Clean-Up Techniques

Very often, a clean-up procedure needs to be carried out in order to eliminate the interfering contaminants from the extracts before their analysis. Moreover, this step may also be a good opportunity for pre-concentration to improve their limit of detection. Many techniques can be implemented for this purpose. Among these techniques, the liquid–liquid extraction (LLE), solid-phase extraction (SPE), and microextraction (SPME) are widely used.

3.1.1 Liquid–Liquid Extraction

Liquid–liquid extraction is one of the traditional ways of sample purification used to reduce matrix interferences. The sample in solution is put in contact with a second nonmiscible solvent, vigorously mixed, and then decanted until the return to two independent liquid phases. According to their affinity for the two solvents, the compounds split between the two liquid phases. The clean-up procedure consists in the elimination of the contaminants which preferentially stand aside from the analytes of interest in one of the phases. In practice, one of the phases is usually an aqueous plant extract, while the other phase is composed of a less polar organic solvent. The parameters that strongly affect the final results are the nature of the organic solvent, the pH (which changes the charge state of weak acidic or basic analytes and thus their water solubility), the temperature, and the presence of complexing compounds in the extract. The compound solubility is governed by their chemical nature and the polarity of the solvent. The polarity however, can be very different from one compound to another, therefore it is difficult to develop an LLE procedure suitable to recover all plant actives. Nevertheless, this fact may even be used as a benefit. For example, phenolic compounds have been extracted with petroleum ether, ethyl acetate, or diethyl ether allowing the removal of lipids and unwanted polyphenols (Fuleki and Francis 1968). Many other examples of a clean-up procedure can be found in the literature (Garcia-Salas et al. 2010). For example, Muñoz-González et al. (2012) carried out a liquid–liquid extraction of phenolic acids using ethylacetate in acidic condition prior to their analysis by gas chromatography coupled to mass spectrometry (GC-MS). A similar approach was conducted for the extraction of resveratrol and other stilbenoids from beer (Jerkovic et al. 2008) before their analysis by liquid chromatography coupled to mass spectrometry (LC-MS)

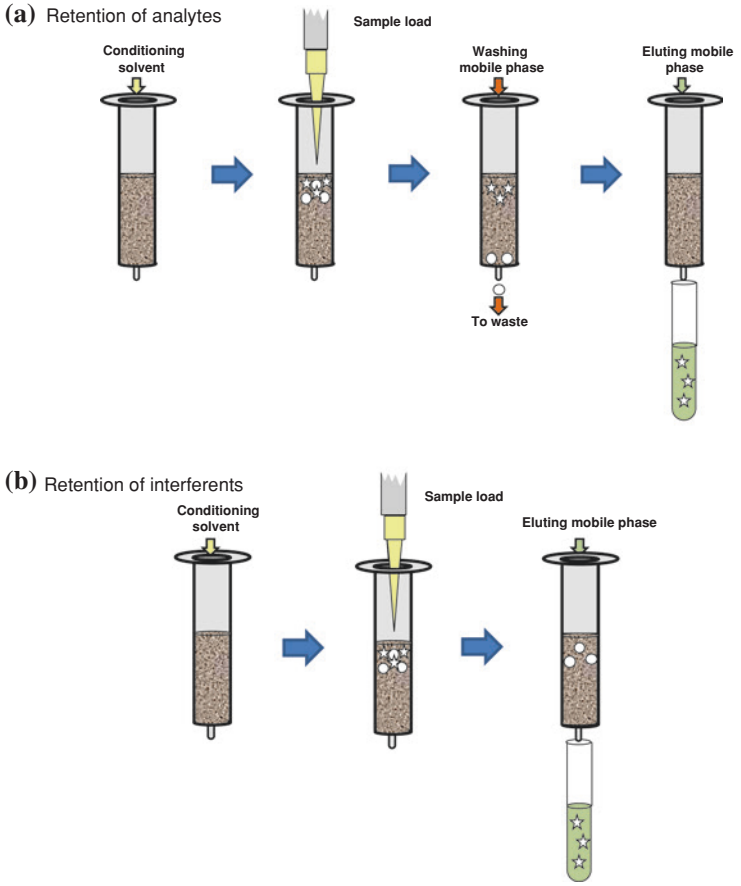

Fig. 6 Principle of the SPE: **a** for analytes more retained than the interfering compounds and **b** for analytes less retained than the interfering compounds

The main drawback of LLE is the requirement for expensive and hazardous organic solvents. High volatile solvents such as dichloromethane or hexane are particularly interesting to carry out evaporation to dryness, but are unfavorable regarding cost and the environment.

3.1.2 Solid-Phase Extraction and Solid-Phase Microextraction

Solid-phase extraction is strongly inspired from the open column system used for years in the laboratories of organic chemistry. The main difference is its size reduction (small cartridges) and the quality of the packing material. The two principles of extraction are explained in Fig. 6.

The SPE cartridge is first conditioned with solvents for good wetting of the adsorbent material. The solvent is adapted to allow the anchorage of the molecule

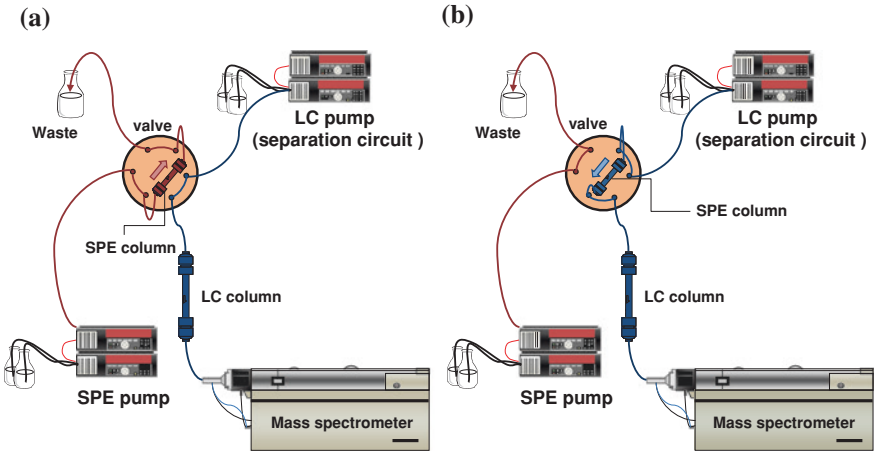

Fig. 7 On-line SPE: **a** The sample is loaded on the SPE column for preconcentration with the assistance of the SPE circuit (*brown*), while the analytical circuit is equilibrating (*blue*). **b** After a 1/6th turn of the valve, the SPE column is back flushed and the sample is injected onto the column for analysis

of interest in the stationary phase. The solvent flows through the adsorbent either by gravity, overpressure applied at the top of the cartridge, or depression at its bottom regarding the equipment. The sample is then loaded onto the top of the cartridge. The cartridge is sometimes dried after penetration of the mixture. The next step then depends on how the analyte of interest and the contaminants behave toward the adsorbent. If the analytes have a higher affinity for the stationary phase than the interfering molecules, the cartridge is washed with a solvent allowing only the elution of the impurities. In a second step, the molecules of interest are recovered with a small volume of a solvent possessing strong elution power. In the second scheme, the analytes have weaker affinity for the stationary phase than the interfering molecules. The compounds of interest are eluted with a small solvent volume without releasing the unwanted molecules.

Currently, SPE is undoubtedly the most popular sample clean-up procedure because of its simplicity and versatility. Indeed, as in liquid chromatography, there is a large set of commercially available stationary phases exhibiting many different adsorbing properties. The clean-up can be performed on silica ensuring the retention of polar solutes or reversed phases (e.g., octyl- or octadecylsilyl supports) for the retention of apolar compounds. Strong as well as weak cation or anion exchangers are also available for the retention of ionic and ionizable compounds. Some providers even propose mixed beds composed of a reversed phase and ion exchangers or porous graphitized carbons as adsorbent. There are also many different cartridge models available for the treatment of a few microliters to a few ten milliliter sample volumes including 96-deep well plates for high throughput extraction. SPE can be performed either manually, by automated systems (sample preparation robots) or even online with liquid chromatography (Fig. 7). Generally,

online SPE is mostly performed for their determination of natural compounds in biological fluids such as plasma or urine. For example, an online SPE-LC-MS/MS method was developed for the determination of salidroside (one of the major phenolic glycosides of *Rhodiola sp*. displaying antioxidant activity) in rat plasma (Chang et al. 2007). The cost of SPE is strongly connected to the equipment. Nevertheless, SPE can be performed manually at low cost using a cartridge (prices ranging from 2 to 10 €) with a syringe adapter or with an SPE chamber (around 1,000 €) and a vacuum pump. The cartridges can also be recycled after use.

SPE has been used widely in the field of antioxidants. For example, tocopherols were extracted from vegetable oils on porous polymer SPE cartridges by Beldean-Galea et al. (2010) before their separation with capillary gas chromatography. Highly polar compounds may also be concerned. Thus, vitamin C, (−) epicatechin, and (+) catechin were extracted from juice using end-capped reversed-phase columns before analysis by high-performance liquid chromatography coupled to a diode array detector and a mass spectrometer (Shui and Leong 2004). Elimination of sugars from coffee pulp extracts was carried out on C18 cartridges in order to purify polyphenols before their analysis by normal-phase HPLC and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Ramirez-Coronel et al. 2004). SPE has also been employed for resveratrol (Malovana et al. 2001). SPE cartridges can even be filled with molecularly imprinted polymers (MIP). These polymers exhibit the highest selectivity toward a targeted compound or a family of compounds compared to traditional adsorbents. For example, Claude et al. (2008) synthesized a very selective MIP for solid-phase extraction of the 18-β-glycyrrhetinic acid, a triterpene acid, from the liquorice roots. The described SPE protocol allows the elimination of 93 % of the matrix (including the fatty acid fraction) during the washing step and 98 % of the 18-β-glycyrrhetinic acid is recovered during the elution step. A second example is the fabrication of highly selective MIPs for the specific preconcentration of the flavonoids (quercetine and rutin) from white and red wine achieved by Theodoridis et al. (2006).

When the compounds of interest are particularly diluted in a complex matrix and rather volatile, the preconcentration by solid phase microextraction (SPME) becomes more suitable (Hinshaw 2003). Furthermore, SPME eliminates most of the drawbacks associated with extracting organic solvents. This technique is based on the partition of the analytes between a liquid or a gas phase and an adsorbent polymer laid on a silica fiber. This procedure is applicable to solid, liquid, and gaseous samples. SPME is involved in various domains such as the extraction of volatile organic compounds (Larroque et al. 2006) or pesticides (Sagratini et al. 2007; Cortes-Aguado et al. 2008; Schurek et al. 2008) in environmental chemistry. SPME is performed either online or offline prior to analysis by GC or LC. The principle of the offline SPME is depicted in Fig. 8.

The extraction efficiency mainly relates to several physicochemical parameters. Temperature, adsorbing time, and choice of the adsorbent (Table 2) need to be carefully optimized. Low molecular weight or volatile compounds usually require a 100 μm polydimethylsiloxane (PDMS)-coated fiber, whereas an 85 μm polyacrylate-coated fiber is more suitable for very polar analytes. The user should be aware that the fiber may be rapidly saturated with concentrated samples and

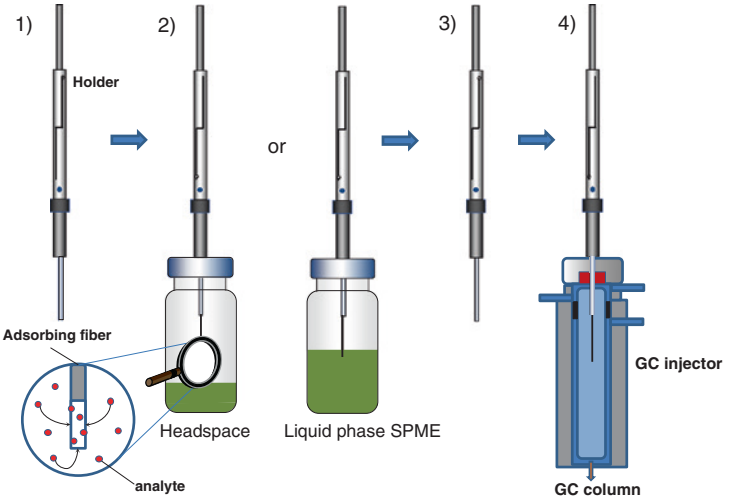

Fig. 8 Principle of manual offline solid phase microextraction (*SPME*): *1* The adsorbent fiber is retracted inside of the needle of the SMPE holder. *2* The needle of the holder is introduced through the rubber *top* of the vial and the fiber is pulled out until the molecules of interest are adsorbed. Two modes are possible: headspace (adsorption from the gas phase) and liquid phase (direct adsorption from the liquid phase). *3* The adsorbent fiber is retracted. *4* The needle of the holder is put through the rubber septum of the GC injector, the fiber is pulled out of the needle. Heat of the GC injector causes adsorbed molecules to vaporize. They are then transferred into the column where they are separated

Table 2 Fibers of common use in SPME and their application domains (PDMS: Polydimethylsiloxane; DVB: Divinylbenzene)

Nature of the adsorbent	Coating (μm)	Applications
PDMS	100	LC and GC (volatile and semi-volatile compounds)
Carbowax-DVB	65	GC (polar volatile and semi-volatile compound)
PDMS-DVB	60	LC and GC (volatile and semi-volatile alcohols or amines)
Carboxen-PDMS	75	GC (trace of volatile compounds)
DVB-Carboxen-PDMS	30–50	GC (volatile and semi-volatile compounds)
Polyacrylate	85	LC (polar compounds)

that compounds may compete with each other for the adsorption. This is a particularly sensitive issue for quantitative analysis as the analytes exhibiting the highest affinity for the sorbent and may affect the accurate quantification of the other compounds (Gorecki et al. 1999).

Stirring and pH are also associated parameters. SPME can be performed by soaking the fiber in the liquid extract. This technique, however, is usually applied to the extraction of volatile compounds from nonvolatile samples isolated in the headspace or gas portion of a sample vial.

Many applications are found in the literature concerning natural compounds. These applications mainly concern volatile natural compounds and their concentration by headspace solid-phase micro-extraction (HS-SPME) performed prior to a GC analysis. Numerous SPME methods have been developed in food chemistry for the analysis of aroma (Sides et al. 2000; Kataoka et al. 2000; Coleman and Dube 2005) and spices (Perez et al. 2007). HS-SPME is also suitable for the analysis of essential oils (Kovacevic and Kac 2001; Paolini et al. 2008). It has also been applied to volatile organic sulfur aroma compounds in black-and-white truffle (Pelusio et al. 1995), and in beer (Xiao et al. 2006). Some applications have been also developed in SMPE for a separation by liquid chromatography. For example, an automated in-tube solid phase microextraction coupled capillary LC-MS has been implemented for the determination of catechins and caffeine in tea by Wu et al. (2000). Polyphenols in wine (Flamini 2003) or phytohormones in plant extracts (Liu et al. 2007b) can also be analyzed by LC after an SPME step.

Several other related techniques may also be mentioned such as stir bar sorptive extraction (SBSE), liquid–liquid microextraction (LLME) or liquid-phase microextraction (LPME). Some applications were also developed in the field of natural compounds with many examples in the field of food flavor chemistry (Jelen et al. 2012; Xiao et al. 2006).

3.2 Purification

For structural elucidation by NMR or biological tests, a scale-up isolation of the natural active molecules needs to be carried out in order to obtain them in larger amounts and in higher purity. In the laboratory, several techniques may be involved (Sticher 2008). Flash chromatography remains a basic approach. Although flash chromatography is less resolute than most instrumental techniques, it exhibits the advantage of easy implementation at low cost.

3.2.1 Planar Chromatography

Preparative planar chromatography or thin-layer chromatography (TLC) can be involved in the purification process by increasing the thickness of the stationary phase layer (0.5 to 2 mm) compared to analytical plates. One of the main advantages of preparative TLC lies in the possibility of recovering the separated compounds by scratching the TLC plate. Then the compounds are dissolved and the silica stationary support is eliminated by filtration. This approach however remains limited to a few milligrams of material.

Overpressure layer chromatography (OPLC) is one of the forced flow TLC techniques. It requires specific equipment (Fig. 9) but the separation is faster and the resolution is highly improved (Nyiredy 2001). OPLC can be used either for analytical or semi-preparative purposes (Bryson and Papillard 2004; Tyihak et al. 2012).

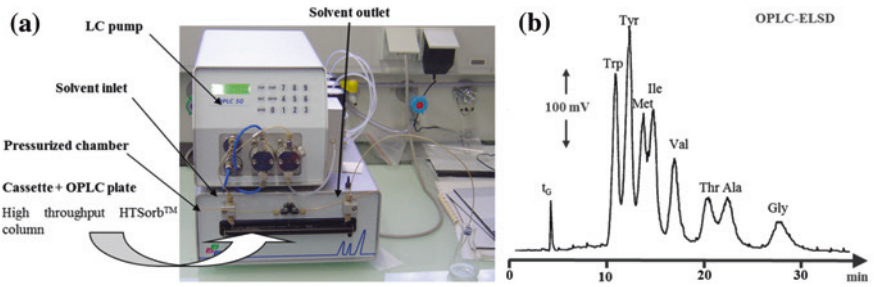

Fig. 9 Overpressure layer chromatography (*OPLC*). **a** Apparatus: The OPLC plate is spotted with the liquid sample. This step can be performed manually or with an automatic TLC Sampler. The OPLC plate is put in a cassette. The assembly is inserted in the pressurized chamber and a high counter pressure is applied onto the cassette (up to 50 bars). The LC pump flows the mobile phase through the plate to carry out the separation. Detection is ensured either by an offline procedure (UV lamp, coloration with reagents) or with online detection. **b** Fractionation of 8 amino acids by OPLC coupled to an ELSD: mobile phase: acetonitrile/water/acetic acid (75/25/0.1, v/v/v). On-line Injection (Rheodyne 7125 valve): 20 µL of a solution at 300 mg/L of each amino acid. OPLC plate: 5 × 20 cm backed aluminum with a silica gel (thickness particle size 11 µm. Online Detection: Evaporative Light Scattering Detector (*ELSD*) Sedex 75 (SEDERE) (pressure = 2.2 bars, Temperature = 45 °C, Photomutiplicator = 8) (Chaimbault, unpublished results)

The OPLC preparation unit allows a transposition from the analytical to a few ten mg semi-preparative scale. OPLC has been used for the purification of active compounds from a plant extracts as for example the main components (thymol, carvacrol, (−)-linalool, diethyl-phthalate, and α-terpineol) of *Thymus vulgaris L.* essential oil (Moricz et al. 2012a). Another similar application is isolation by OPLC of some antibacterial components from chamomile (*Matricaria recutica L.*) flower extract before their offline identification by GC–MS (Moricz et al. 2012b).

3.2.2 Preparative Liquid Chromatography

Generally, instrumental methods of purification are superior to the others in terms of resolution and separation speed. Among instrumental methods, preparative LC (prep-LC) is one of the most commonly used. This is probably due to the fact that purification can be achieved by transposing a chromatographic analytical profile into a semi-prep or prep LC method. The overload of the column however often leads to critical peak deformations. Consequently, the chromatographic resolution decreases with a risk of remixing of the separated compounds. Nevertheless, this technique allows the purification of a few milligrams to a few ten milligrams (semi-prep LC) to hundred milligrams of the desired compounds (prep-LC). The variety of commercially available supports (silica, reverse phase, ion exchange, etc.,) makes the prep-LC a versatile method for the isolation of apolar, polar, and charged compounds. The same LC equipment can sometimes be used for analytical or preparative applications depending on the column size. The amount of stationary phase used is much higher so that the column diameters are usually in

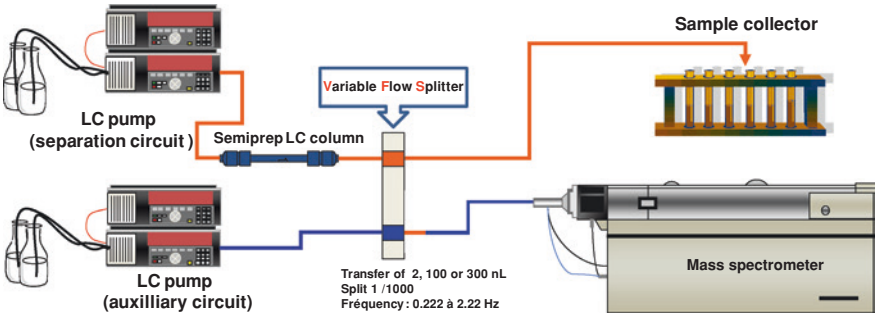

Fig. 10 Principle of prep or semiprep LC with online detection by mass spectrometry using a variable flow splitter (*VFS*). The MS detector can be replaced by an ELSD (Himbert et al. 2003). The frequency of the VFS is adjusted to such frequency that only a small amount of the extract (e.g., 1/1,000) is sent to MS for identification. The main part of the purified fraction is sent to the collector

range from 10 to 100 mm. The average particle size of semi-prep LC stationary phases is typically between 5 and 10 µm and may reach 100 µm for prep-LC. The main drawback of the prep-LC is the high solvent consumption. Indeed, the flow rate in semi-prep LC is usually in the range of 2 mL/min to a few tens mL/min all along the separation process. Semi-prep and prep LC can be coupled with the common detection methods (UV-Visible absorbance, fluorescence) but also with evaporative light scattering detection (ELSD) (Liu et al. 2007a) and mass spectrometry using a passive or active splitting of the mobile phase (Variable Flow Splitter, Fig. 10) (Nichols and Foster 2009; Himbert et al. 2003). MS is particularly interesting as it provides online control of the purity. Semi-prep and prep LC have been employed in many applications concerning plants. For example, Liu et al. (2007a) isolated ginsenosides from crude extracts of *Panax notoginseng* by semi-preparative HPLC coupled with an ELSD. The sample load was 300–400 mg and the final purity of each isolated compound was more than 97 %. Jiao et al. (2007) purified tricin from an antioxidant product derived from bamboo leaves. Around 3 g of tricin was prepared from 174 g of a crude fraction.

3.2.3 Countercurrent Chromatography

Countercurrent Chromatography (CCC) is an interesting alternative to prep-LC for the purification of natural molecules. CCC is now a generic term including several types of chromatography based on the same principle. The two main techniques are the centrifugal partition chromatography (CPC) developed by Nunogaki (Murayama et al. 1982) and the high-speed countercurrent chromatography (HSCCC) set up by Hito (Ito et al. 1982). The main difference between CPC and HSCCC is the instrumental design (which will not be discussed in this chapter). CCC is an instrumental LLE process allowing hundreds of automatic successive extractions, thanks to a special column possessing one or more cartridges interlinked by channels.

The fractionation consists of a continuous-flow partition of compounds between two immiscible liquid phases. The first liquid phase is the stationary phase, whereas the second behaves as the mobile phase. As for LLE, the solutes are separated according to their partition coefficients between the two solvent phases based on their hydrophilic–lipophilic balance. Evidence is increasing that the nature and volumes of both liquid phases strongly influence the final chromatographic resolution. In CPC, the liquid stationary phase is immobilized, thanks to the centrifugal force applied by the spinning motion of the coil column. The mobile phase is passed through the stationary phase by an LC pump. The main advantage of CCC over prep LC is that no solid stationary support is required to perform the separation. Thus, there is no irreversible sample adsorption and the recovery is close to 100 %. Moreover, the role of the two liquid phases can be switched during a run. The liquid stationary phase becomes the mobile phase and vice versa allowing the highly retained compound to be eluted from the column.

CCC is particularly recommended when samples contain solid particles or high-molecular constituents and when the solutions contain high concentrations of acids, bases, or salts in order to prevent the LC column from plugging and to avoid any interferences (Berthod et al. 2009).

To check the purity of the collected fractions, offline controls can be carried out by TLC (Sannomiya et al. 2004) or LC. Yet, online detections are possible using the same detectors as for LC. Thus, the detection can be achieved by UV (including a diode array detector), (Peng et al. 2005; Goncalves et al. 2011) evaporative light scattering detection (Cao et al. 2003; Ha et al. 2011) and obviously mass spectrometry with a passive T split (Gutzeit et al. 2007) or a variable flow splitter (Toribio et al. 2009).

CPC provides successful analytical chromatographic separations but because of its high charge capacity, CCC is above all an excellent purification technique. The preparative scale strongly depends on the rotor volume (Srivastava 2008). Purifications between a few milligrams to several hundred grams can be considered under certain conditions, the purification of a few kilograms can also be achieved (Sutherland and Fisher 2009). Insufficient resolution can be improved using multidimensional CCC (2DCCC) (Lu et al. 2007). Furthermore, the hyphenation of CCC with LC allows fast online checking of fraction purity (Liang et al. 2011; Michel et al. 2011b). This kind of coupling is not so obvious and requires specific experimental setups to overcome the differences in pressure and flow rate in the CCC and LC circuits.

To the present day, many applications on analysis and purification of natural molecules in plants have been published (Marston and Hostettmann 2006). Water soluble as well as insoluble metabolites may be addressed by this technique (Wanasundara et al. 2005). For example, Schræder et al. developed an HSCCC method for the isolation of phytosterols and they obtained sitostanol and β-sitosterol fractions with purity higher than 99 % (Schroder and Vetter 2011). Yu et al. (2011) proposed a CPC method for the purification of coniferyl ferulate from the extracts of *Angelica sinensis* oil (Fig. 11). The purity of the fraction was checked by LC UV and the identification was carried out by MS, 1H, and 13C NMR (Yu et al. 2011).

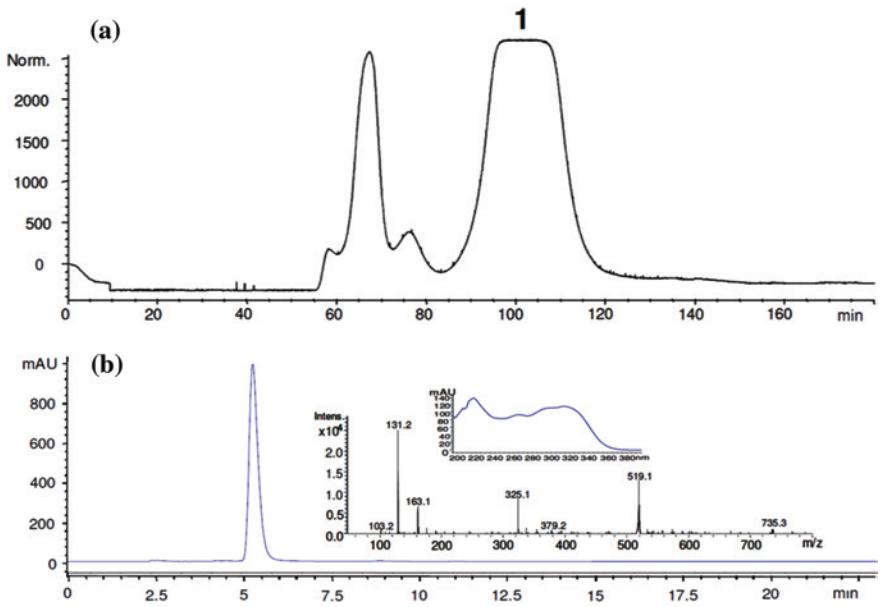

Fig. 11 High-performance CPC chromatogram of a coniferyl ferulate rich fraction (**a**), HPLC chromatogram of peak fraction 1 (**b**) with UV spectra and MS data. HPCPC solvent system: n-hexane-ethyl acetate-ethanol-water (5:5:5:5, v/v) in elution; station phase:upper organic phase; mobile phase:lower aqueous phase; descending mode; flow rate:2.0 ml/min; rotor speed 1,500 rpm, detector wavelength: 318 nm. The HPLC condition was adapted from an HPLC analysis performed on a Zorbax ODS C18 column (250 × 4.6 mm I.D., 5 mm) with a Zorbax ODS C18 guard column (12.5 × 4.6 mm I.D., 5 mm); mobile phase: 1 % aqueous acetic acid (**a**) and acetonitrile (**b**) using a gradient program of 50 % in 0 to 15 min, 50 to 100 % B in 15 to 18 min, and 100 % B in 18 to 23 min. The flow rate was 1 ml/min and the injection volume was 10 ml. The column was operated at 25 °C. ESI-MS conditions are as follows: drying gas N_2, 10 l/min; temperature, 350 °C; pressure of nebulizer, 40 psi; source voltage, 4.0 kV; Scan range, 50–800 m/z, positive mode (Yu et al. 2011)

Many applications are related to flavonoids and other polyphenols (Gutzeit et al. 2007; Lu et al. 2007; Michel et al. 2011b). Among the highest polar metabolites, glycosylated compounds such as glucosinolates (Toribio et al. 2007) or saponins (Shirota et al. 2008) have also been purified by CPC.

4 Separative Methods

After extraction, the sample or fraction content needs to be qualitatively and quantitatively characterized. At the very minimum, the purity must be checked but very often a plant extract remains a complex mixture that needs to be analyzed further in more detail. Among the analytical arsenal available, chromatographic

techniques (including capillary electrophoresis) are essential because they allow the separation of the different constituents of complex samples before their identification (currently, structural identification is regularly carried out online by mass spectrometry and even by NMR) and quantification by a variable set of more or less specific detectors.

Chromatography techniques are known for long. Gas chromatography (GC) was first developed by Martin and Synge (Nobel prize laureates in 1952) in 1941 (Martin and Synge 1941) and high-performance liquid chromatography (HPLC) got its real kick-start in the 1970s. Since they were created, the relentless technological progress continuously improves chromatography in many aspects. Whatever the physicochemical properties of the molecules (i.e., apolar vs. polar, neutral vs. ionic, small molecules vs. polymers, etc.,), nowadays there is always a system suitable for their separation. In the same way, the set of detectors available for chromatography has considerably advanced and with time, these detectors have detectors have become more and more sensitive. Both aspects contribute to an increasing interest in chromatography.

This part gives an overview of the different techniques available for the analysis of natural products and describes current trends in this area. One of these trends is the systematic coupling of separative methods with mass spectrometry. The hyphenation of separative methods with MS is the subject of the final part of this chapter.

4.1 Choice of the Separative Technique

Separations of analytes are always achieved according to their differences in physicochemical properties. Two approaches are possible:

- Chromatography is a forced flow separation technique relying on the partition of the analytes between the stationary and the mobile phase. The compounds exhibiting high affinity for the stationary phase are more retained than the others. By choosing the nature of the stationary phase or changing the physicochemical properties of the mobile phase, chromatographists modulate the retention so that separation is achieved with the required resolution.
- In capillary electrophoresis (CE), separation is obtained by the differential migration of the analytes under the influence of an electric field. One could think that this technique is restricted to ions but several variants have been developed to achieve the separation of neutral compounds and enantiomers as well (Altria 1996b).

The choice of the right separative technique (including the detector) is key to a successful analysis. The technique is chosen in accordance with the physicochemical properties of the analytes. Their chemical modification by a specific reagent is possible to turn them compatible with the analytical method (derivatization). Such derivatization, however, generally induces more drawbacks than benefits.

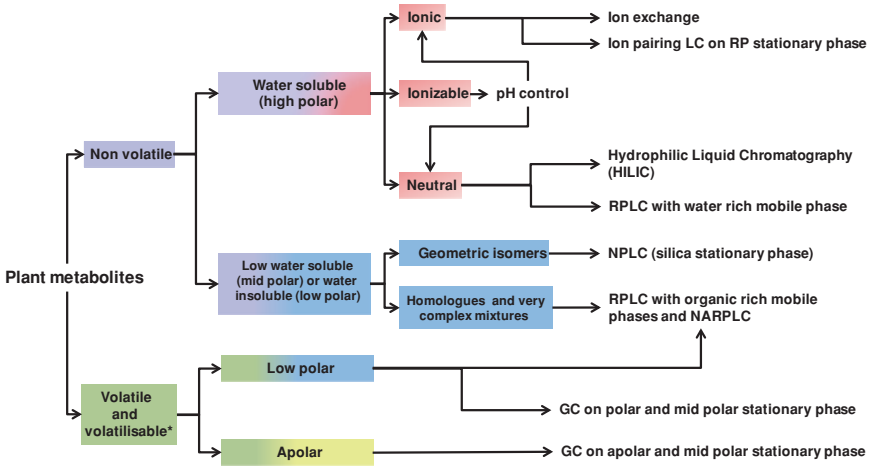

Fig. 12 Selection of the chromatographic system according to the physicochemical properties of the analytes. Principal combinations are indicated and the possible use of supercritical fluid chromatography (*SFC*) or TLC is not indicated. *RP* reversed phase. *NARP* nonaqueous reversed phase. *NP* Normal phase. *Asterisk* for thermo resistant compounds

In chromatography, small molecules (MW < 3,000 Da) are separated depending on their physicochemical properties. Many different types of chromatography are applicable to their separation (Fig. 12). Obviously, there are often alternative solutions to any given problem but this kind of decision tree remains a good starting point to the development of a future separation method.

4.2 Gas Chromatography

4.2.1 Generalities

Gas Chromatography (GC) is one of the most commonly used analytic techniques in the laboratories. The mobile phase (carrier gas) consists in an inert gas (e.g., helium or nitrogen) and the stationary phase consists in a column either packed with a solid support or coated with a high boiling polymer. The latter behave as an immobilized liquid. Nowadays, analyses are performed using coated capillary columns because they are much more efficient than packed columns.

Retention is a function of the partition of compounds between the mobile and stationary phases. The higher the temperature, the more the compound is in the gas phase. Consequently, the retention time decreases as the temperature increases because the analyte interacts less with the stationary phase. It is easily understandable that this technique is rather suitable for volatile thermostable compounds. As the carrier gas is inert, the selectivity toward compounds is principally based on the nature of the stationary phase although parameters such as the temperature,

film thickness of stationary phase, and column length may also be considered for the development of separation. In chromatography, retention relies on the concept that "birds of a feather flock together." Separations on apolar supports (e.g., 100 % methylsiloxane polymer and methylsiloxane polymers with 5 % of phenyl groups) are governed by the temperature profile and the apolar character of the molecules. These supports are employed for all general-purpose applications. They are appreciable for biological samples because they are resistant to contamination and for analysis of low volatile compounds because they are stable at high temperature (up to 325 °C in isotherm and 350 °C in the temperature program). These columns, however, are less performing for distinguishing analytes according to their polar groups. Typically, the analysis of free fatty acids (or fatty acid methyl esters) varying in their unsaturation number and position requires polar stationary phases (e.g., polyethylene glycol) for separation. By contrast, polyethylene stationary phases do not resist high temperature (T_{max}: 250 °C). Such columns can be replaced by the newly developed ionic liquid stationary phases that withstand higher temperatures (Sun and Armstrong 2010). Depending on the application, the column polarity can be progressively increased with the percentage of phenyl or cyanopropyl groups in the methylsiloxane polymer. Chiral stationary phases are also commercially available and they can be implemented for separation of volatile enantiomers in plants (Konig and Hochmuth 2004).

Apart from the separation considerations, the instrumentation itself also plays a key role. Generally, plant analysis involves a preanalytical extraction procedure so that the final extract is dissolved in a liquid medium. The solvent must be volatile enough to ensure the total spontaneous transformation of the sample into a gas phase. Then, the gas phase is transferred into the column. The sample is introduced under pressure at the inlet of the column via the injector.

Several different GC injectors can be implemented in plant analysis (del Rio et al. 2007) but the split/splitless injector remains probably the most used because of its versatility. The split mode can be used for injection of highly concentrated samples, whereas the splitless mode is selected for trace analysis. The sample introduction into the injector is achieved with a syringe (1 to 2 μL) for liquid extract or as previously mentioned, by desorption of an SPME fiber (Kim et al. 2003; Nunes et al. 2006). The injector temperature must be high enough to ensure the instantaneous volatilization of the sample and its correct transfer into the column. Inappropriate temperatures lead to excessive analyte discrimination during injection and to peak broadening during analysis.

The molecules are detected at the outlet of the column. Even if mass spectrometry is currently probably the most widely application used detection method, the flame ionization detector (FID) remains of interest because it is universal for hydrocarbon compounds (Buchmabauer et al. 1997; Hasanloo et al. 2008; Rather et al. 2012). Other detectors may present some interest in plant analysis such as the thermo-ionic detector also called NPD because it specifically detects nitrogen- (i.e., alkaloids) and phosphorus-containing compounds. This detector is most often used for the specific quantification of nitrogen- and phosphorus-containing pesticides in plants (Tekel et al. 2001; Hirahara et al. 2005). The same applies to the

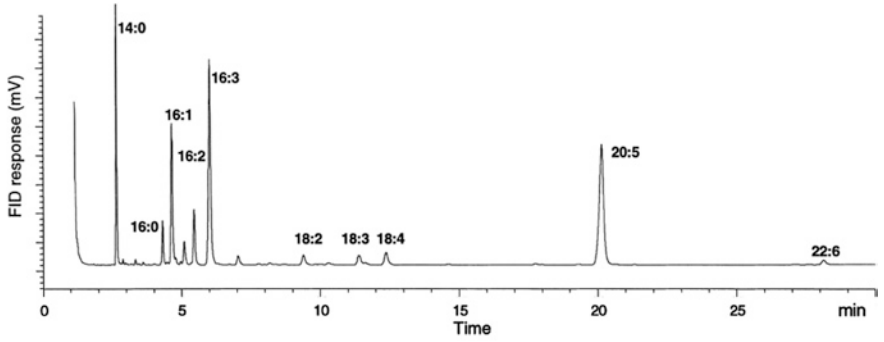

Fig. 13 Gas chromatogram of a mixture of FAMEs in a microalgae-oil extract. Column: DBwax (60 m × 0.32 mm ID), Carrier gas: helium; flow rate: 3 ml/min. Injection split: 1/20. Column temperature: 180 °C during 10 min, then increased to 230 °C by 20 °C/min. The final temperature was kept for 20 min. Detection: flame ionization detector (*FID*). Reprinted with permission from Viron et al. (2000)

electron capture detector and its use for the quantification of chlorinated pesticides (Ismail et al. 1993; Cao et al. 2008).

4.2.2 Applications to Plant Analysis

Gas chromatography has been involved in plant analysis for a long time. Obviously, flavors and fragrances are commonly analyzed by this technique (De Medici et al. 2006; Verhoeven et al. 2012). Apolar compounds such as phytosterols (Grunwald 1970; Du and Ahn 2002; Dhara et al. 2010), tocopherols, and tocotrienols (Du and Ahn 2002; Ruperez et al. 2001), and fatty acids (free or as fatty acid methyl esters) (Fig. 13) (Hasanloo et al. 2008; Seppanen-Laakso et al. 2002; Ng 2002; Viron et al. 2000) are also analyzed by GC-MS. Carotenoids are less volatile and thermodegradable. Therefore, gas chromatography is not really suitable for their analysis (Rodriguez-Amaya 2001). They are rather analyzed by liquid and supercritical fluid chromatography.

Some small polar metabolites can be analyzed by GC. Zou et al., who developed a simple and rapid procedure for determination of caffeine in beverages by GC/MS. The limit of detection of this method was 0.001 mg/L (Zou and Li 2006). GC coupled to mass spectrometry is also used for identification and quantification of alkaloids such as cocaine and its metabolites (Jenkins et al. 1996).

In the past, gas chromatography was also used for analysis of water-soluble metabolites such as sugars and amino acids. Although they are nonvolatile, it is possible to analyze these thermodegradable molecules after a derivatization step (e.g., methylation, silylation, acetylation, etc.,). Some more or less specific reagents are employed to substitute the labile hydrogen atoms in the molecules. Deprived of intermolecular hydrogen bonds, these metabolites become volatile

and more resistant toward heat. This approach is still used in laboratories but tends to be abandoned with the progress in analytical chemistry. New methods are now available in liquid chromatography. For instance, with ELSD or electrospray MS, amino acids can be analyzed in their native form (i.e., underivatized) by LC (Chaimbault et al. 1999; Petritis et al. 1999).

4.2.3 Current Trends

GC technology is still undergoing further development. As previously mentioned, one of the current tendencies is its systematic coupling with MS detection. In the recent years, research has also been focused on fast GC and comprehensive multidimensional GC (GC × GC) allowing GC runs to go faster (high throughput analysis) and ensuring that the exploration of particularly complex samples is improved, respectively.

- *Fast-GC*: the world shortage of helium and the relentless miniaturization of the apparatus facilitated the development of fast GC. Both points are more or less linked together. On one hand, hydrogen replaces helium as carrier gas. Hydrogen is particularly interesting because of its high fluidity. Thus, H_2 flows more rapidly through the column. Additionally, the chromatographic efficiency is improved. On the other hand, the size reduction of columns in all dimensions (i.e., short and/or wide bore columns) also enhances the chromatographic speed and efficiency with decreased mobile phase consumption. In fast GC, the internal diameter is decreased from 250 μm (commonly used with He) to 100 μm. Consequently, the flow rate of the carrier gas is decreased by a factor of 2.5 to 3.5 but maintaining it in such condition requires higher inlet pressure. This inconvenience can be easily overcome with H_2. Finally, the reduction of column inner diameter and the use of the hydrogen as carrier gas reduces analysis time at constant chromatographic resolution (Korytar et al. 2002). An increase of speed by factor of 3–5 can easily be achieved with fast GC. Regarding the sensitivity, the low load ability of the narrow-bore column involves a reduction of the injected volume to avoid peak broadening. Nevertheless, in fast GC, the analytes elute as narrower and, hence, higher peaks resulting in improvement in the limits of detection. Fast GC has advantageously been applied to plant analysis. Mondello et al. (2003) developed a fast- GC analytical method for fats and oils. They compared the fast GC chromatographic profile transposed from a GC method performed on a polar column (Supelcowax-10) of classic dimension (30 m × 0.25 mm ID × 0.25 μm film thickness) using helium as carrier gas. They obtained a similar chromatogram using the same column in a narrow-bore version (10 m × 0.1 mm ID × 0.1 μm film thickness) and H_2 as carrier gas except that the analysis time is reduced by a factor of 5 (15 min instead of 75 min). In another study carried out by the same team, a fast GC analysis of citrus essential oils was carried out with a speed gain of almost 14-fold in comparison with traditional GC procedures (Mondello et al. 2004). Around 60 volatile metabolites including terpenes were analyzed in only 3.3 min.

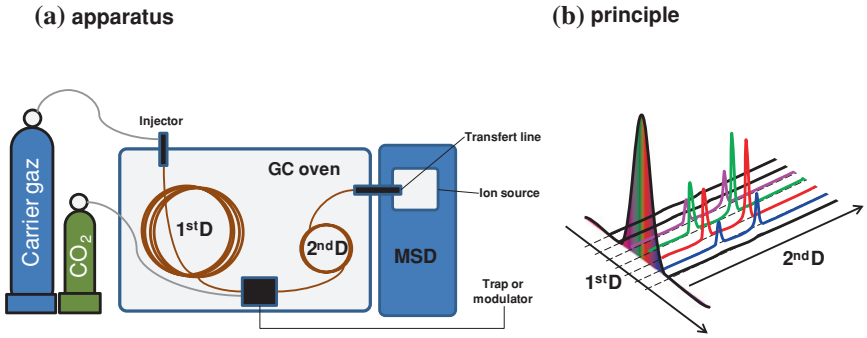

Fig. 14 Multidimensional GC (GC × GC with MS detection). **a** Scheme of a GC × GC apparatus. **b** Principle of separation using two dimensions: The sample is injected onto the first column (first D) and the compounds eluted are trapped at its outlet thanks to a time-programmed modulator (frozen by CO_2 expansion). When the modulator is warmed, the trapped molecules are injected onto the second dimension where co-eluted analytes are resolved

- *GC × GC*: One dimension is very often insufficient to separate all the compounds of interest in particularly complex samples. Thus, chromatographists sought to improve the resolution. One possibility is to increase the column length, yet this approach is rapidly limited. The other approach consists in coupling chromatographic columns so that the analysis is performed in two dimensions. This approach requires specific equipment (Fig. 14) involving a key body, which is mostly a cryogenic moving trap (Marriott and Kinghorn 1997) or a dual-jet modulator (Beens et al. 2001) depending on the apparatus. Basically, this device captures the eluted molecules at the outlet of the first column (first dimension) during a programmed time. Meanwhile, the analysis is performed on the second column (second dimension). When the analysis in the second dimension is finished, the trap releases the trapped molecules onto the second dimension. Additionally, the cryo-modulation provides an increase in the S/N ratios by a refocusing effect within each analysis carried out on the second dimension. The functioning of the system supposes that (1) the stationary phases of both dimensions are different (first dimension apolar and second dimension polar or vice versa) and (2) that the column size of the second dimension is much smaller than the first dimension so that the second dimension analysis is performed in a few seconds (e.g., 5–10 s). The larger part of the separation is ensured by the first dimension and the separation of co-eluted compounds is achieved by the second dimension. The operation of such a system requires a careful optimization (column lengths, trapping time, temperature, etc.,) and severe parameter controls. The resulting chromatogram appears as a 2D contour plot (Fig. 15) with a color scale corresponding to the respective peak heights of analytes. GC × GC is very often coupled to mass spectrometry detection but analysis can be carried out with flame ionization and electron capture detection as well (Augusto et al. 2010; Corporation 2004).

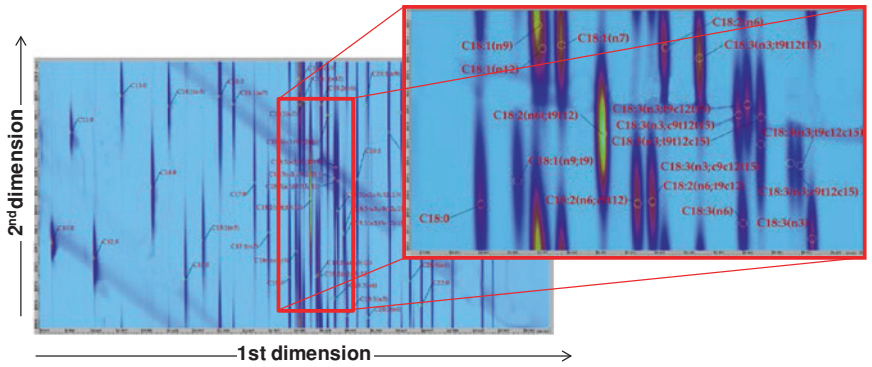

Fig. 15 GC × GC-FID 2D chromatogram of the standard mixture of 45 FAMEs. The first dimension is polar: BPX-70 column (30 m × 0.25 mm × 0.25 μm). The second dimension is apolar: ZB5-MS column (10, 5 and 2 m × 0.25 mm × 0.25 μm). Temperature program: 120–230 °C at 20 °C/min 230–260 °C (10 min) at 20 °C/min. Carrier gas flow rate in the first dimension = 0.5 and 12 mL/min in the second dimension. Modulation time = 1.40 s. Adapted from de Geus et al. (2001)

Historically, multidimensional GC has been developed for the petrochemical industry (Phillips and Beens 1999; Bertsch 2000; Bertoncini et al. 2005) and environmental chemistry, including the analysis of pesticide residues (Banerjee et al. 2008). In the field of natural compounds extracted from plants, GC × GC above all has been used for the characterization of volatiles in essential oils in many species such as *Rosa damascena* Mill. (Ozel et al. 2006), *Origanum micranthum* (Gogus et al. 2005), *Eucalyptus camaldulensis* (Ozel et al. 2008), fresh and aged tea plant (Tranchida et al. 2010) or roasted and unroasted fruit from *Pistacia terebinthus* (Gogus et al. 2011). The other main family of natural compounds concerns fatty acids (de Geus et al. 2001; Manzano et al. 2011) and fatty alcohols as methyl derivatives (Manzano et al. 2011). At last, polar metabolites (e.g., shikimic acid, citric acid, many sugars, etc.,) have been also analyzed as trimethylsilylated derivatives by GC × GC in a metabolomic study concerning *Populous tremula* (Jover et al. 2005).

4.3 Chromatographic Methods Using a Liquid Mobile Phase

4.3.1 Generalities

Whenever the metabolites of interest are nonvolatile or thermolabile, the separation methods involving a liquid mobile phase are undoubtedly the most appropriate. These methods include thin-layer chromatography (TLC), liquid chromatography (LC), and supercritical fluid chromatography (SFC), which in many respects shows similarities with LC.

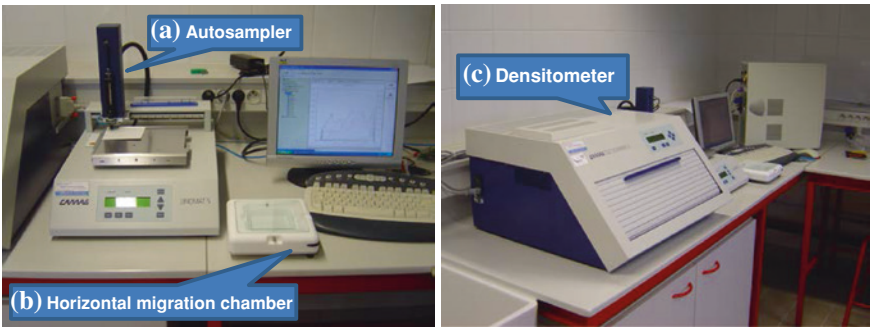

Fig. 16 HPTLC basic instruments from CAMAG. **a** Autosampler: The sample is deposited onto the HPTLC plate using a syringe. The sample is band wide sprayed (suitable for automatic detection). The spraying gas is generally nitrogen. The number of bands and their width as well as the sprayed volume and deposition speed are programmed depending on the number of deposited samples, nature of solvent (volatility), and the concentration of analytes, respectively. **b** Horizontal migration chamber: separations can be carried out from two sides in unsaturated, saturated, and sandwich configuration but single-sided developments are also possible. **c** UV–Visible scanner for densitometric evaluation of TLC/HPTLC chromatograms. The scanner can work either in absorbance, reflectance, or fluorescence mode

4.3.2 Thin-Layer Chromatography and High-Performance Thin-Layer Chromatography

In thin-layer chromatography, also called planar chromatography, separations are carried out on a flat stationary phase supported by a glass plate or aluminum foil. The most used stationary phase is likely silica gel but applications can be also developed on reversed phase, (Poukensrenwart et al. 1992) ion exchange (Luo et al. 1998; Karuna and Sashidhar 1999), and even chiral materials for enantiomer separation (Srivastava et al. 2008). High-performance TLC is the instrumental version of TLC. Their main differences lie in the stationary phase particle sizes, plate dimensions, and migration mode. The TLC plate contains 5–20 μm particles constituting a 250 μm film thickness and the migration is vertical. HPTLC is performed on 100 μm thick films constituted of 5 μm particles and separation takes place in a horizontal development chamber (Fig. 14). HPTLC exhibits a two times higher efficiency than TLC over a short distance of migration (up to about 10 cm) (Nyiredy 2001). Except for OPLC (described in Sect. 3.2.1), the mobile phase migrates through the stationary phase by capillary action. Sample application determines the quality of the analysis. Although HPLC provides higher resolution power (i.e., higher numbers of theoretical plates) than TLC or HPTLC, many aspects of planar chromatography remain of interest in the area of plant analysis. Furthermore, when the resolution power is not the limiting factor, HPTLC and HPLC lead to very comparable results (Farina et al. 1995; Sharma et al. 2007; Urakova et al. 2008). Due to its offline principle, the (HP)TLC system

offers versatility and a low cost analysis. The sample deposition is either manual or automated (Fig. 16). Here, the use of an automatic sampler leads to more reliable results in quantitative analysis. The sample preparation is simple and there is no risk of clogging by small solid particles (no previous filtration is required). Samples and standards are processed together facilitating the identification of compounds. Furthermore, several analyses may be run simultaneously lowering analysis time and the consumption of the mobile phase. Last but not least, HPTLC also allows multidimensional separations simply by rotating the plate by one quarter turn and performing further separation with another mobile phase (Waksmundzka-Hajnos et al. 2008).

TLC plates constitute open systems. Thus, the basic detection is cheaper than for LC: a visual detection is often sufficient (direct or indirect for non-UV-absorbing compounds after a post-run derivatization with appropriate reagents). Sophisticated detectors, however, are also available commercially, such as UV-visible and fluorescence densitometers (Fig. 16). These scanners allow accurate quantification of metabolites with the help of calibration curves. At last, it is now even possible to perform offline MS detection on TLC plates either by MALDI-TOFMS (Wilson 1999; Crecelius et al. 2003) or electrospray-MS (Morlock and Jautz 2008).

TLC and HPTLC are perfect approaches for roughing out and formulating basic knowledge on a given complex sample. Many applications can be found concerning natural compounds in pharmacy, cosmetics, food chemistry, etc. Several applications concern antioxidants such as anthocyanidins (Farina et al. 1995), phenolic compounds (Sharma et al. 2007), and flavonoids (Cvek et al. 2007), caffeoyl derivatives such as chlorogenic acid (Urakova et al. 2008) or curcumin (Ashraf et al. 2012). HPTLC analysis allows the separation of high polar compounds such as glycosylated metabolites (e.g., phenylpropanoid glycosides from *Ballota nigra L.*) (Janicsak et al. 2007) or apolar natural molecules such as pentacyclic triterpens (e.g., taraxerol in *Clitoria ternatea*) (Kumar et al. 2008) and sterols (Shanker et al. 2008).

4.3.3 Liquid Chromatography

High-Performance Liquid Chromatography (HPLC or more simply LC) is undoubtedly the most widespread separation technique. This popularity is likely due to its unique versatility in terms of application and its high resolutive power. Indeed, almost every class of molecules can be analyzed by LC. Furthermore, the recent progress in LC results in a relentless improvement in efficiency (i.e., increase in the theoretical plate number) with a simultaneous increase in analytical speed. Thus, for example, Ultra Performance Liquid Chromatography (UPLC) is a high throughput LC technique exhibiting separative power comparable to capillary GC. As for GC, comprehensive multidimensional LC (LC $\times$ LC) allows the analysis of particularly complex samples increasing the peak capacity of a single chromatographic run.

(a) General aspects

Whatever the LC technology (classic LC or fast LC), the stationary phase is composed of more or less porous spherical particles packed in a column. The retention mechanism depends on the nature of the support. In the field of small molecules, two primary physicochemical characteristics are involved in the LC separations: polarity and electrical charge. Based on the concept that "birds of a feather flock together," water-soluble molecules are highly retained by polar supports [normal phase (NPLC) and hydrophilic interaction liquid chromatography (HILIC)]. Inversely, low-polar compounds are highly retained on apolar stationary phases [reversed-phase liquid chromatography (RPLC)]. Ionic molecules can also be separated using ion-exchange chromatography but ion-pairing chromatography on RP supports or mixed bed supports (e.g., RP mixed with ion exchange supports) may also be implemented to perform separation of organic ions. The separation is based on the differential interaction between the analytes and the stationary phase. Thus, the choice of the stationary phase is key to success in the separation but contrary to GC, the composition of the mobile phase (eluent) is also of importance. In RPLC and HILIC for instance, the retention and selectivity is modulated by the hydrophilic–lipophilic balance of the mobile phase and additional parameters such as pH, ionic strength, and even the temperature often have to be adjusted to achieve successful separation.

All the separation modes above evoked may be used for natural compound analysis. Low-polar compounds can be analyzed either by NPLC or RPLC. NPLC is particularly interesting for the separation of geometrical isomers and several reports have described for a long time the use of silica as adsorbent for the analysis of tocopherol and tocotrienol isomers (Weber 1984; Balz et al. 1993; Tangney et al. 1979; Chase et al. 1994). Their retention is ensured by the use of a mobile phase containing a high proportion of apolar solvent (e.g., 98.8 % hexane) and a small proportion of polar modifier (e.g., 1.2 % isopropanol) (Weber 1984). Separation of apolar compounds can also be achieved on porous graphitized carbon (PGC). This support additionally enables the separation of geometric isomers, but contrary to silica PGC, is above all an apolar adsorbent. The retention on PGC, however, is very sensitive to the position of electron-rich groups. Therefore, PGC is often used for geometric isomers. For instance, Rhourri-Frih et al. (2012) investigated the potential of PGC for separation of triterpenoids in natural resins. Here, PGC exhibited a unique selectivity toward ursolic, betulinic, and oleanolic acids. RPLC is the most widespread separation mode because of its versatility and robustness toward complex mixtures. Indeed, the full range of metabolite polarity can be analyzed with RP columns. For example, Abidi (1999) proposed the resolution of *cis*/*trans* isomers of tocotrienols on RP support. The elution of apolar metabolites requires a high percentage of organic modifiers up to 100 % (nonaqueous reversed-phase liquid chromatography) in the case of carotenes (Lesellier et al. 1989) or fat soluble vitamins (Gentili et al. 2012). The retention of metabolites of intermediary polarity is achieved by increasing the aqueous fraction in the mobile phase. Many applications concerning

antioxidants and other classes of plant compounds may be cited. In this field, Wichitnithad et al. (2009) developed a simple and sensitive isocratic RPLC-UV method for the simultaneous quantification of curcuminoids (curcumin, desmethoxycurcumin and *bis*-desmethoxycurcumin) in commercial turmeric extracts with sub-µg/mL limits of detection. In order to investigate whether flavonoids and stilbenes of red wine benefit health, Stecher et al. (2001) developed a RPLC method for the determination of rutin, quercetin, myricetin kaempferol, *cis*- and *trans*-resveratrol. Rutin is a quercetine glycoside (α-L-rhamnopyranosyl-(1–6)-β-D-glucopyranose). This polar metabolite is less favorably retained in RPLC than the other polyphenols analyzed. As a consequence, the authors used an aqueous methanol gradient elution starting from a water-rich mixture allowing a single-run analysis of compounds exhibiting different polarities. Hydrophilic compounds can be retained in RPLC with a water rich (up to 100 %) mobile phase, however, polar supports are very often more appropriate for their separation. A few ten percents of water are required in the mobile phase for their elution turning the NPLC into HILIC. Although the NPLC and HILIC stationary phase may be the same (e.g., silica, diol or amino propyl supports), their separation mechanisms are somewhat different. In HILIC, water (coming from the mobile phase) forms a dynamic layer adsorbed onto the stationary phase. The polar analytes are separated by their differential partitions between this adsorbed layer and the mobile phase (Wang and He 2011). Depending on the nature of the stationary phases, an anion (e.g., amino bonded support) or cation (e.g., silica) exchange mechanism can complete the resolution. Many polar plant metabolites such as sugars and oligosaccharides (Remoroza et al. 2012; Leijdekkers et al. 2011), amino acids, and small peptides (Tolstikov and Fiehn 2002), water-soluble vitamins (thiamine, riboflavin, nicotinic acid, nicotinamide, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid) (Karatapanis et al. 2009) have been separated in this way. Ionic compounds can be separated using ion-exchange or ion-pairing chromatography on RPLC support (IP-RPLC). For example, Antonopoulos et al. proposed a separation of enzymatically digested κ-carrageenans using an anion exchange mechanism (Antonopoulos et al. 2004a) and Arnault et al. (2003) developed an IP-RPLC method for the simultaneous analysis of alliin, deoxyalliin, allicin, and dipeptide precursors in garlic products.

The success of LC may also be attributed to a very extensive range of available detectors. The refractive index detector (RID) is universal but limited to simple concentrated samples analyzed under isocratic conditions. In fact, the basic detection of LC is UV–visible (UV–Vis) absorbance. UV–Vis detection [including diode array detectors (DAD)] is widely used in the field of natural compounds as many of them possess a chromophore. This is the case for antioxidants such a resveratrol (Lamuelaraventos et al. 1995), flavonoids (Colombo et al. 2006), curcumin (Wichitnithad et al. 2009), tocopherols (Seker et al. 2012), etc. Whenever a molecule has a flat conjugated polycyclic chromophore, fluorescence may be used for their sensitive and specific detection. For example, fluorescence detection has been used for a long time for the analysis of tocopherols and tocotrienol (Abidi 1999). Alternative detections can be implemented for compounds lacking a chromophore.

Electrochemical detection (ECD) is an extremely selective and sensitive detection technique for metabolites possessing reducable or oxidizable functional groups (e.g., *o*- or *p*-quinones, ketones, aldehydes, etc.,). Their detection is ensured by the electrical current resulting from oxidation or reduction reactions in the detector cell. Such a detection method allows the quantification of sugars, (Corradini et al. 2012) and amino acids (Marioli and Sereno 1996; Agrafiotou et al. 2009). The use of ECD is obviously not reserved to nonabsorbing compounds and it may possibly also be applied to the detection of catechins (Maoela et al. 2009) resveratrol (Kolouchova-Hanzlikova et al. 2004) and many other antioxidants in medicinal plants. Furthermore, as for many specific detectors, ECD provides a huge sensitivity. In their comparative study, Subagio et al. (2001) found detection limits for ECD about 75 times lower than for UV in the case of catechins. Detection limits of 3 and 15 μg/L were obtained by ECD for *trans*-resveratrol and *cis*-resveratrol, respectively (Kolouchova-Hanzlikova et al. 2004). Another alternative is the evaporative light scattering detector (ELSD) or the corona charged aerosol detector (Corona CAD). Both detection principles are based on the nebulization of the mobile phase. This starting point requires the use of mobile phases composed of highly volatile solvents (including acids or bases added for pH and ionic strength adjustment). The principle of detection is different in both cases. With ELSD, the aerosol is composed of neutral micro droplets. The solvent is dried in a drift tube and the solid particles of analytes scatter light according to their size (mass sensitive). The scattered light is detected by a photomultiplier at fixed angle from incident light (Dreux et al. 1996; Megoulas and Koupparis 2005). Consequently, ELSD may be considered as a universal detector. It is more sensitive than the RID and almost not affected by gradient elution (as long as the volatility of the mobile phase is respected). ELSD has been used widely for the detection of natural compounds lacking a chromophore, such as lipids (e.g., triglycerides, fatty acids, steryl glucosides, etc.,) (Stolyhwo et al. 1985; Marcato and Cecchin 1996; Moreau et al. 2008), carbohydrates (Herbreteau et al. 1992; Antonopoulos et al. 2004b; Nogueira et al. 2005) and amino acids (Chaimbault et al. 2000; Petritis et al. 1999). Nevertheless, ELSD was also used for UV-absorbing compounds, such as phenolic terpens (Bicchi et al. 2000) or resveratrol-like antioxidants (e.g., astringin, piceid, and isorhapontin) (Co et al. 2012). With Corona CAD, the aerosol is composed of charged micro droplets. In some respects, the Corona CAD may be considered as an atmospheric pressure chemical ionization-mass spectrometer (APCI-MS) without any mass analyzer. The Corona CAD detector detects any nonvolatile or semi-volatile analytes under the condition that these compounds are ionizable. The Corona CAD is generally found to be three to 6 times more sensitive than the ELSD (Vervoort et al. 2008), even 12 times for complex for lipid samples (Hazotte et al. 2007), but the ELSD is less sensitive toward an increase in the salt concentration in the mobile phase (Vervoort et al. 2008). The area of application for Corona CAD is the same as for ELSD. Many applications have been carried out for analysis of lipids (Moreau 2006; Lisa et al. 2007) and oligosaccharides (Inagaki et al. 2007; Asa 2006).

(b) Current trends

As for GC, the key words in LC development are: faster, more sensitive, and still more resolute. Hence the consequences are the same as in GC. More and more applications have been developed with mass spectrometry; considerable efforts have been put into the development of new stationary phases and instruments for fast LC and multidimensional LC (LC × LC) are now operational for applications to very complex matrices.

- *Fast LC*: High throughput LC started in 1999 with the development of monolithic columns (Guiochon 2007). Due to their wide pores, monolithic columns can work at high flow rates (up to 10 mL/min) without generating high backpressures. So, they were introduced as an alternative to conventional packed columns (4.6 mm I.D. columns packed with spherical particles within a diameter range of 5 to 10 μm). In addition to the analytical speed, the length of column is not pressure limited meaning that several columns may be coupled together to improve peak resolution (Novakova et al. 2004). Monolithic columns are constituted of a single porous rod of silica (functionalized or not). The largest pores (2 μm-sized macropores) allow the rapid circulation of the mobile phase at low pressure while the smallest pores (130 Å-sized mesopores) provide a sufficient exchange area to ensure analyte retention. Thus, monolithic columns exhibit similar chromatographic resolution as conventional columns but in a shorter analysis time. A substantial interest of monolithic columns is the use of conventional LC equipment. Thus many applications have been transposed easily in the past on this support. Flavonoids and isoflavones from tomato were analyzed in less than 10 min on a reversed-phase monolithic column (Biesaga et al. 2009) and soy extracts (Rostagno et al. 2007). Monolithic columns, however, are slowly being abandoned in favor of (1) ultra performance liquid chromatography (UPLC) and (2) core-shell particles.
  The size reduction of particles (sub 2 μm diameters) highly improves column efficiency (i.e., by an order of a magnitude order with hundred thousand theoretical plates per meter) and thus leads to better resolution. Consequently, the first means to speed-up separation is the shortening of the column length (50 mm instead of 150 mm for conventional support). Furthermore, the use of smaller particle size leads to a possible increase in the flow rate almost without any peak broadening. The downsize is a dramatical increase in backpressure. This problem can be overcome in two ways.
  The first solution consists in developing appropriate analytical equipment (pumps, injectors, columns) with strong resistance to overpressure reaching 1,000 bars (or even higher) (Gritti and Guiochon 2012) and high-speed detectors. This approach called ultra performance liquid chromatography (UPLC) has been followed by many instrument manufacturers and HILIC, ion-exchange, reversed and normal stationary phases are now available for applications under ultrahigh pressure conditions (Nunez et al. 2012). Thus, all previously developed methods on conventional material may be transposed to UPLC. An example of phenolic metabolite analysis via UPLC is presented in Fig. 17. Nováková et al. (2010)

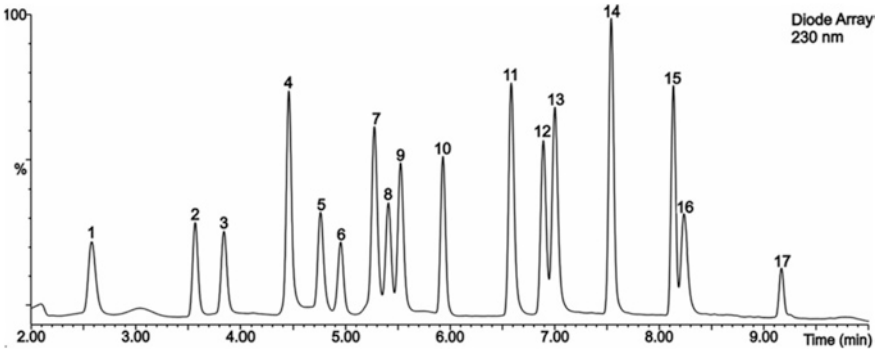

Fig. 17 UPLC–UV chromatogram of a mixture of the following 17 phenolic acids detected at 230 nm: *1* gallic acid, *2* 3,5-dihydroxybenzoic acid, *3* protocatechuic acid, *4* chlorogenic acid, *5* gentisic acid, *6* 4-hydroxybenzoic acid, *7* caffeic acid, *8* vanillic acid, *9* syringic acid, *10* 3-hydroxybenzoic acid, *11* 4-coumaric acid, *12* sinapic acid, *13* ferulic acid, *14* 3-coumaric acid, *15* 2-coumaric acid, *16* salicylic acid and *17* trans-cinnamic acid. Reprinted with permission (Gruz et al. 2008)

simultaneously determined caffeic and chlorogenic acids, umbelliferone (coumarin), and nine other flavonoid derivatives at a few nmol/L level in Chamomile (*Matricaria recutita L.*) flowers and tea extracts by UPLC–MS/MS in around 20 min. Lerma-García et al. (2010) quantified vegetable sterols (ergosterol, cholesterol, campesterol, stigmasterol, β-sitosterol, and lanosterol) in oil with limits of detection below 0.07 μg/mL by LC-MS in less than 5 min.

The second solution to overcome high pressure is the use of fused-core particle packed columns. Their most significant advantage over sub 2 μm packed material is their relatively larger permeability. Thus, this kind of support allows at least twice faster and more efficient separation of analytes than conventional columns at half the backpressure compared to the UPLC column. The use of fused-core particles is relatively new in chromatographic separation of natural compounds. In this area, 20 phenolic compounds (gallic and caffeic acid derivatives, flavonoids including their glycosylated metabolites) were analyzed in tea, mate, and coffee in less than 5 min by UPLC-UV-fluorescence (Rostagno et al. 2011). Using an RP fused-core support, Olszewska (2012) quantified ten flavonoid aglycones with high sensitivity (LODs from 0.115 to 0.525 ng injected) in *Ginkgo biloba*, *Betula pendula* and a variety of *Sorbus* species.

- LC × LC: Comprehensive multidimensional LC has been developed exactly for the same reasons as GC. Even if LC has become more and more efficient in the last couple of years, one dimension remains often insufficient to explore the composition of complex samples. Consequently, there is a need to increase the peak capacity of the analysis. This is especially the case with the appearance of ‘omic’ disciplines such as proteomics or metabolomics. The general principle of 2D-LC is the same as in GC. The basic equipment, however, is obviously different. The use of a cryo-modulator is not applicable and this device is

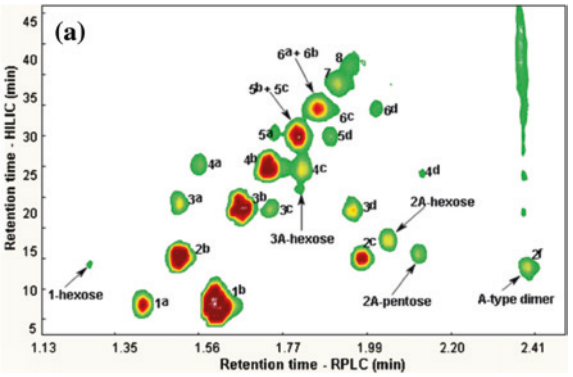

Fig. 18 Fluorescence contour plot obtained for the HILIC × RP-LC analysis of cocoa procyanidins. Numbers correspond to the degree of polymerization of procyanidine isomers. 'a' indicates A-type procyanidin. Reprinted with permission (Kalili and de Villiers 2009)

replaced by a more or less complex set of valves fitted with loops (Groskreutz et al. 2012). The monolithic columns (Lubda et al. 2001) and more recently the fused-core columns (Dugo et al. 2008) are advantageously used for rapid analysis in the second dimension. One of the main problems is related to the choice of the orthogonal separation modes because of possible solvent incompatibility (the first dimension mobile phase becomes the second dimension injection solvent). Possible solutions have been provided in the literature (Stoll et al. 2007; Cesla et al. 2009). For example, LC × LC was applied to the determination of polyphenols in wine (Donato et al. 2011), cocoa (Kalili and de Villiers 2009) (Fig. 18) and flavonols of green tea using a combination of the HILIC mode in the first dimension and the RPLC mode in the second dimension (de Villiers et al. 2010). In the case of the 2D-LC analysis of cocoa procyanidins, the peak capacity is around 3,500 (Kalili and de Villiers 2009).

4.3.4 Supercritical Fluid Chromatography

Whatever the domain of application, supercritical fluid chromatography (SFC) is less commonly used than LC. Yet, this technique exhibits some advantages in separative sciences. SFC is a hybrid technique using a supercritical fluid as mobile phase. Physicochemical properties of such a fluid have already been evoked in Sect. 2.2.1 for SFE. The principal supercritical fluid for chromatography is carbon dioxide. In SFC, CO_2 exhibits smart solubilizing properties, low viscosity and high diffusivity. SFC is usually 10 to 20 times faster than conventional LC (Rosset et al. 1991) and also provides higher efficiency. New stationary LC phases such as core-shell can be implemented to perform separation enhancing its chromatographic performances even more (Lesellier 2012). Thus, SFC can be considered as the first fast chromatographic technique for nonvolatile compounds. Supercritical CO_2, however, possess a low dielectric constant and consequently rather acts as normal-phase LC. Furthermore, SFC is applicable to compounds of low to moderate polarity. Temperature and pressure affect the solubility of the analytes

in supercritical CO_2 and thus modulate the retention and the selectivity in SFC. Additionally, a small percentage of polar modifiers (e.g., methanol) can be added in the mobile phase to elute more polar compounds. SFC is compatible with GC detectors such as FID, NPD, electron capture (Richter et al. 1989), Fourier transform infrared (FTIR) (Calvey et al. 1995) but also with LC detectors like UV–Vis, ELSD (Dreux and Lafosse 1997) and even mass spectrometry (Herbreteau et al. 1999).

SFC replaces advantageously GC for nonvolatile and thermolabile compounds. Some applications in the area of plant natural product research can be found in the literature. Not surprisingly, most of the separations are concerning apolar and low-polar metabolites such as *cis*- and *trans*-carotene (Lesellier et al. 1991), tocopherols (Jiang et al. 2003), fat-soluble vitamins (Turne et al. 2001), allicin in garlic extracts (Calvey et al. 1994) or carnosic acid from rosemary (Ramirez et al. 2004). Most of the time however, natural compounds are commonly extracted by SFE and analyzed by LC.

4.4 Capillary Electrophoresis

Capillary electrophoresis is a typical method for ion analysis. Contrary to the previously described methods requiring forced flow elution, electrophoresis uses a completely different mechanism of separation based on the migration of charged species subjected to an intense electric field. The separation takes place in a fused silica capillary which is generally a few ten cm to one meter long and has an internal diameter varying from 50 up to 100 μm. Except for capillary electrochromatography, the separation does not involve partition of molecules between a stationary phase and a mobile phase. The difference of mobility enabling the separation (electromigration) of analytes is solely due to their difference in charge over size ratio (Altria 1996a). Thus, the highest charge, results in the highest mobility, and, inversely the highest hydrodynamic radius (size) results in the lowest mobility. The capillary is filled with ion strength and pH controlled buffers ensuring conductivity during the analysis and a constant charge state for weak acidic or basic compounds. The inner wall of the capillary is lined with more or less deprotonated silanols (depending on the pH) and to maintain electroneutrality, cations of the buffer build up near the surface. When the separating voltage is applied across the tube, the cations naturally migrate toward the cathode. This phenomenon called electroosmotic flow (Altria 1996a) is superimposed over the electromigration so that cation analysis is speeded up. For anion analysis, it is better is to invert the flow using cationic surfactants.

Capillary electrophoresis has been applied to the analysis of natural compounds either in food chemistry or in phytochemistry. Many applications concern the analysis of polyphenols in propolis (Volpi 2004; Hilhorst et al. 1998) or in plant extracts (Kristo et al. 2002; Crego et al. 2004; Vaher and Koel 2003) with UV detection or mass spectrometry (Gomez-Romero et al. 2007; Hurtado-Fernandez et al. 2010).

A current trend in CE is the miniaturization and in this area. Several authors have already proposed CE-microchip methods to identify phenolic compounds in green tea (Hompesch et al. 2005), in wine (Scampicchio et al. 2004), pears and apples (Blasco et al. 2005) using an amperometric detection.

Micellar electrokinetic chromatography (MEKC) is an interesting variant extending capillary electrophoresis to the separation of neutral compounds. In this technique, ionic surfactants are added to the separation buffer at a concentration so that corresponding micelles are formed. Under the influence of the electric field, the micelles migrate toward the counter electrode. Neutral compounds are partitioned between the buffer and the micelles. When they enter the micelle they migrate at its velocity and when they are out, they move at the electroosmotic flow speed. Depending on this equilibrium, neutral compounds are separated (Otsuka and Terabe 1996). This approach has also been implemented for natural compounds in plants. Zhu et al. (2008) achieved the separation of six flavonoids (tangeretin, nobiletin, hesperetin, naringenin, hesperidin, and naringin) in less than 15 min with sub-ng/mL LOD using a conductivity detector. They applied this for the detection of these substances in extracts of *Fructus aurantii Immaturus* (Zhu et al. 2008). Jiang et al. developed a MEKC-UV method for the simultaneous determination of ten bioactive flavonoids in food containing propolis and *Ginkgo biloba*. They added 16 mM β-cyclodextrin to their buffer to complete the separation. The LOD were ranging from 0.15 to 1.36 μg/mL (Jiang et al. 2008). Lee et al. quantified glucoraphanin in MEKC-UV DAD at 230 nm (Lee et al. 2010). The glucoraphanin migrates around 5 min with a theoretical plate number of 380,000/m allowing its quantification in complex vegetal matrices (broccoli seeds, florets, and Brussels sprouts). Many other applications involving MECK can be found in the literature (El Deeb et al. 2011) and its possible coupling with atmospheric pressure photoionization-mass spectrometry (Marchi et al. 2009) offers new opportunities in the field of plant analysis.

Capillary electrophoresis is a rapid analytical method exhibiting high separation efficiency, low solvent consumption, versatility, and simplicity (Cifuentes 2006) but some specific features have hindered its development in the past. One of its main drawbacks is the difficulty to couple capillary electrophoresis with mass spectrometry. The first reason is due to electric compatibility between apparatus but it can easily be overcome by plugging both apparatus to common ground. The second difficulty was the scan speed of mass spectrometers which was too slow for the high peak efficiency of CE. This is now resolved with the new generation of mass spectrometers developed for fast LC. The last point concerns the (low) flow rate incompatibility between the MS ion sources (i.e., electrospray) and CE. Several solutions have been provided to overcome this problem among which are the sheath flow interface, the sheathless interface and sheathless interface fitted with a gold wire electrode (Cai and Henion 1995). The fine adjustment of these interfaces, however, was generally hard to solve. Recently, a universal plug-and-spray adapter derived from Moini's work (Moini 2007) was developed offering new opportunities for further user-friendly developments in CE-MS.

New technological advances, as well as novel instrument configurations could reinforce CE-MS robustness in the future and would stimulate its development especially for plant matrix analysis.

5 Analysis by Hyphenated Techniques

The most significant advances in analytical chemistry and particularly in separative sciences are probably due to the relentless progresses in their hyphenation with spectrometry. Obviously, gas chromatography was associated for a long time to infrared (Pichard et al. 1990) and mass spectrometry but in the last 15 years, the huge technological change lies in the coupling with liquid phase separation techniques. Mass spectrometry is the most widespread technique in this field and its expansion is undoubtedly not over yet. Nuclear Magnetic Resonance (NMR) can also be coupled with liquid chromatography. Very often, LC-NMR is associated with LC-MS for structural elucidation, particularly for isomers which exhibit similar mass spectra. For example, an LC-DAD-MS/SPE-NMR method was developed for the structural elucidation of iridoid glycoside isomers from *Harpagophytum procumbens* (Seger et al. 2005). LC-NMR-MS was also used for the separation and characterization of secoisolariciresinol diglucoside isomers in flaxseed (Fritsche et al. 2002) or antioxidants (carnosol, carnosaldehyde, epiisorosmanol, carnosic, and 12-methoxycarnosic acid) in rosemary extracts (Pukalskas et al. 2005). The use of NMR however remains limited probably due to its high costs (including deuterated solvents) and rather low sensitivity compared to MS. Moreover, for many applications, mass spectrometry is sufficient to resolve the questions raised. This last part of this chapter therefore focuses on hyphenation with mass spectrometry.

5.1 Why Hyphenation with Mass Spectrometry?

The hyphenation between separative methods and structural spectrometry techniques (i.e., FTIR, NMR and MS) always increases analytical peak capacities in a single run and in several respects; mass spectrometry appears to be the perfect detection method for chromatography. Indeed, the ideal detector should exhibit the following qualities:

- **It must be universal**. In mass spectrometry, the critical parameter is ion production so that a mass-over-charge ratio is detected. Currently, the commercially available ion sources are covering all areas of molecular size (from small molecules to polymers) and polarity (from apolar compounds to ions) (Fig. 19). The only requirement is the compatibility between the separative methods and those ion sources. Electron impact (EI) and chemical ionization

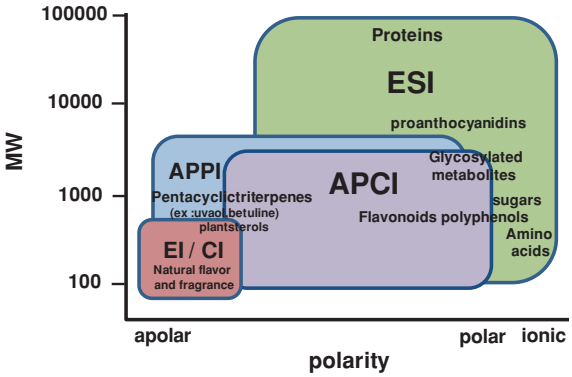

Fig. 19 Range of use of the MS ion sources as a function of molecular weight and polarity. Readers are cautioned that the areas are only given as tendencies. EI: electron impact, CI: chemical ionization, ESI: electrospray ionization, APCI: atmospheric pressure chemical ionization, APPI: atmospheric pressure photoionization. Adapted from Syage et al. (2008), De Hoffman et al. (1994)

(CI) is compatible with GC because of the vacuum required in both sources. Atmospheric pressure ionization sources (API) are compatible with separation techniques requiring a liquid mobile phase.

- **The ideal detector must be** sensitive and stable over time. Moreover, it must provide signals proportional to the analyte concentration (amount) in the sample. Mass spectrometry is commonly used for the quantification of analytes at sub-ppb levels thanks to selected ion monitoring (SIM) or multiple reaction monitoring (MRM) in tandem MS (Cao et al. 2006). The signal stability, however, clearly relates to the nature of the sample. Biological samples tend to clog the mass spectrometer optoelectronics resulting in a more or less progressive decrease in the signal. This drawback can be reduced substantially by a clean-up procedure of the sample before its analysis. The decrease in signal during quantitative analysis is also compensated by the use of internal standards (ideally stable isotope labeled products).
- **The ideal detector must not** induce artifacts. In MS, physical artifacts generally originate from bad settings or a compressed gas supply. Chemical artifacts are often due to the well-known ion suppression phenomenon which may have several sources. The presence of less volatile compounds can decrease droplet evaporation. The matrix can compete with the analytes during the ionization process and affects the amount of the analyte ions in the gas phase reaching the detector (Annesley 2003). The importance of the ion suppression is also depending on the ion source. Thus, APCI and APPI are less subjected to ion suppression due to the matrix effect than ESI (Marchi et al. 2009; Jessome and Volmer 2006). Nevertheless, the problem is often overcome using a sample clean-up such as SPE or equivalent.

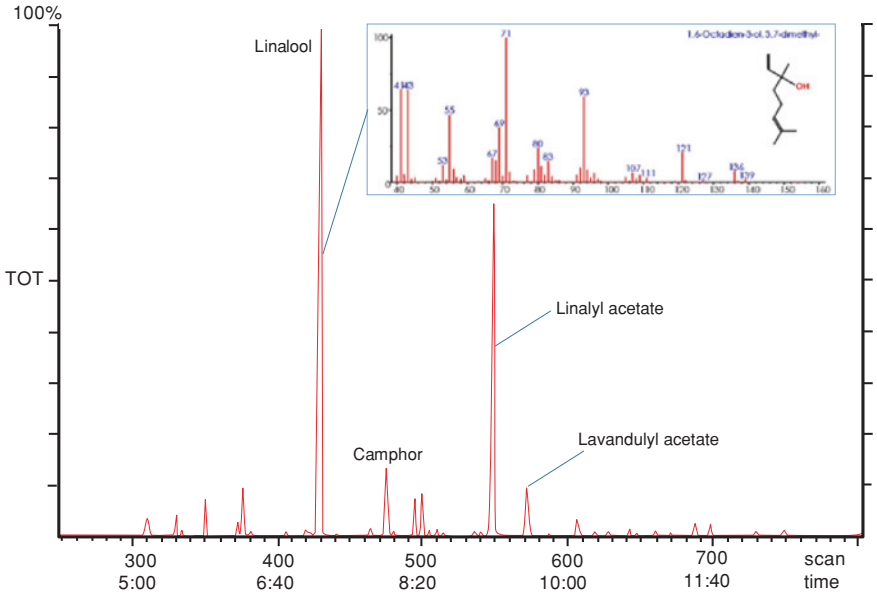

Fig. 20 Analysis of lavender oil by GC-EI-MS. Linalool (and other indexed compounds in this figure) was identified after request to spectrum library (e.g., NIST library). The accuracy of the identification is assessed by statistic scores in terms of purity, fit and reversed fit between the experimental and the library spectra

- **The ideal detector should** provide structural information. Interestingly, structural information is precisely the rationale of mass spectrometry (Fig. 20). In GC-EI-MS, the structural elucidation can also be assisted by spectrum libraries. Readers are cautioned, however, that the final attribution is under the responsibility of the chemist. Indeed, libraries tend to provide identifications on request. The chemist, however, must check whether the identification ultimately also makes sense. For all other ionization techniques, usually no libraries are available because of the lack in reproducibility of spectra within the apparatus. Efforts are currently underway to provide a solution to this problem. Here, intern libraries may be constructed from standards in order to identify compounds in complex mixtures. With very mild ionization methods such as ESI, structural information is either obtained from the in-source fragmentation of the pseudo molecular ions (i.e., the protonated or deprotonated molecules) (Cole 1997) or by tandem mass spectrometry. With the new generation of MS devices, obtaining of structural information is also facilitated by software which is able on request to automatically trigger MS/MS experiments above a predefined threshold.

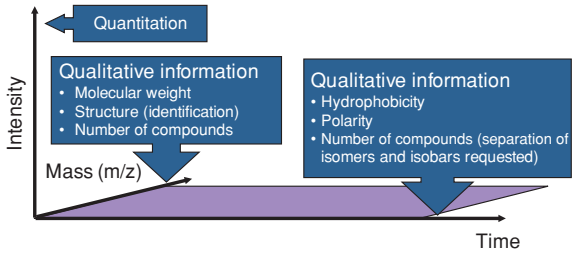

Fig. 21 Principle of separative methods hyphenated to mass spectrometry. The peak capacity of a single run is increased thanks to multidimensional analysis (at least two dimensions, even more with tandem MS). Both dimensions are complementary and each one counterbalances the lack of information of the other

- **The ideal detector should** be able to distinguish co-eluted compounds and to deconvolute their signals. During a run, the specific ion currents corresponding to each compound can be extracted from the total ion current. The problem of co-elution is therefore resolved unless co-eluted analytes are isomers (even in high resolution MS) or isobaric (a problem with low resolution MS). If the mass spectrometer is not fitted with an ion mobility device, in case of isomers, a separate separative method may be included (Fig. 21).
- **The ideal detector should** be specific and only detect the desired compounds. Here, the main question is how to be both universal and specific. The mass spectrometer succeeds in such a challenge. It is universal in full scan and becomes specific in Selected Ion Monitoring (SIM) and even more in tandem mass spectrometry thanks to the Multiple Reaction Monitoring (MRM) mode. In that particular case, all compounds that do not show the required daughter ion(s) resulting from the targeted mother ion are not detected. As previously mentioned however, the compounds transparent to the detection are still eluted from the column and may induce ion suppression (i.e., decrease in signal intensity due to ionization competition between analytes and the unwanted compounds in the matrix).
- **The ideal detector should** be inexpensive, easy to use, and not destructive. These points are more or less in agreement with MS detection. The MS detector is destructive. It is so sensitive, however, that only a small part of the sample is required for detection in semi-prep LC. Except for GC-MS, the use of a splitting system allows collection of the main fraction of the sample at the column outlet. The price and the ease of use strongly depend on the apparatus. GC-MS instruments are obviously cheaper and more user-friendly than Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (LC-FTICRMS).

5.2 Interfacing Mass Spectrometry with Separation Techniques

One sensitive issue concerning hyphenation is how compatible mass spectrometry is with regard to the separative techniques. Two problems may be taken into consideration. The first requirement is ion production without which MS cannot be performed (MS carries out mass-over-charge ratio measurements). The second problem is high vacuum required to analyze the ions produced. The different separative methods do not exhibit the same degree of compatibility in this field and the same applies for ion production techniques.

Electron impact and chemical ionization also require high vacuum to be implemented. Consequently, these ion sources are fully compatible only with capillary GC. The mobile phase does not bring too much matter into the source and small excess gas is easily purged from the source by the efficient pumping system (e.g., turbo molecular pumps). The GC column is directly plumbed to the ion source and the interface just consists of a heated transfer line between the GC oven and the ion source. The role of the transfer line is to keep the compounds in the gas phase when they leave the oven.

The coupling with liquid separative methods historically was more problematic until the emergence of the API sources (ESI: electrospray ionization, APCI: atmospheric pressure chemical ionization and APPI: atmospheric pressure photoionization). Atmospheric pressure ionization has compelled MS manufacturers to develop dedicated interfaces allowing the evacuation of a large gas volume so that a sufficient vacuum is maintained for MS analyzers. In a first stage, the only real requirement in MS coupling is a volatile mobile phase. It is also well known, however, that the composition of the mobile phase strongly influences ionization efficiency in ESI, APCI, and APPI (Kostiainen and Kauppila 2009). Therefore, all the LC modes (RPLC, NPLC, etc.,) do not exhibit the same degree of compatibility with API-MS (Table 3). For example, apolar solvents do not suit ESI because of their low conductivity. Thus NPLC, contrary to APPI, leads to poor results when it is directly coupled to ESI. By contrast, although salts and ion-pairing agents may cause ion suppression, ion-exchange chromatography (Chen et al. 2008, 2009) and ion-pairing reversed-phase liquid chromatography (Chaimbault et al. 1999; de Person et al. 2008) remain sufficiently compatible with ESI-MS. RPLC and HILIC are the most universal LC modes for hyphenation. The only circumspection is to avoid too concentrated buffers for pH or ionic strength control. HILIC is preferable to RPLC for highly polar compound analysis with ESI because the retention of such compounds is favored in an organic-rich aqueous mobile phase, which in turn improves ion desorption in the source (Wang and He 2011).

Compatibility of the mobile phase (including the flow rate) with the ion source alone is not sufficient to achieve the highest sensitivity of detection. The result also depends on the physicochemical properties of the analytes themselves. In fact, the sensitivity is strongly related to the difference of proton affinity between analytes and the mobile phase solvents and additives in the gas phase (in APPI, the

Table 3 Compatibility of electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) in LC-MS

	Ion sources			
Chromatography	ESI	APCI	APPI	Comments
NPLC	±	++	+++	Low ionization rate in ESI with mobile phases containing high percentages of apolar solvents (e.g., hexane)
RPLC	+++	+++	+++	Volatile additives such as acetic or formic acid, ammonia, ammonium acetate, and format can be used for pH control at a few ten mM concentrations APPI: methanol is preferred to acetonitrile and acidic or basic buffers tend to decrease the MS signal (Kostiainen and Kauppila 2009)
HILIC	+++	+++	+++	Same remarks as for RPLC
IP-RPLC	++	−	−	IP-RPLC chromatography means little for APCI and APPI as both ionization processes are rather applicable to neutral and low-polar compounds. Additionally, strong acidic as well as basic additives decrease ionization efficiency. Volatile IP agents are causing ion suppression but can be used with ESI-MS: • IP agent for cation separations: perfluorocarboxylic acids from trifluoroacetic (TFA) to pentadecafluorooctanoic acid. TFA and heptafluorobutyric acid (HFBA) usually cause severe ion suppression whereas the longest homologues may improve the MS signal (Chaimbault et al. 1999; de Person et al. 2008) • IP agents for anion separations: *N*,*N*-dimethyl hexylamine, trialkyl amines (e.g., triethylamine, tributylamine) (Pruvost et al. 2001; Storm et al. 1999)
IEC	+	−	−	Ion evaporation is less efficient in aqueous mobile phases than in organic-rich aqueous eluents. Mobile phases require highly concentrated saline buffers that induce ion suppression

NPLC normal-phase liquid chromatography, *RPLC* reversed-phase liquid chromatography, *HILIC* hydrophilic interaction liquid chromatography, *IP-RPLC* ion-paring reversed-phase liquid chromatography, *IEC* ion-exchange chromatography

nature of the dopant also plays a key role). Thus, in a comparative study between APCI and APPI conducted in order to determine the amount of pentacyclic triterpenes (e.g., α and β-amyrin, friedelin, erythrodiol, etc.,) in bark samples by LC-MS, Rhourri-Frih et al. found that APPI was overall more sensitive than APCI in positive ion mode (LODs in the range of 0.005 to 0.015 mg/L for APPI and

0.002 to 0.84 mg/L for APCI in SIM). APCI showed the greatest sensitivity for acidic triterpenes (ursolic, betulinic, and oleanolic acids) in negative ion mode. As expected, ESI failed to ionize low-polar compounds such as betulin or uvaol (see also Fig. 19) even though the methanol mobile phase was fully compatible with this ion source (Rhourri-Frih et al. 2009). Furthermore, Rauha et al. (2001) demonstrated the importance of optimizing the composition of the mobile phase according to the ion source. The authors optimized the composition of the mobile phase for ESI, APCI, and APPI-MS detection of flavonoids [(+)-catechin, isorhamnetin, vitexin, isoquercitrin, luteolin-3′-7-diglucoside]. Under optimized conditions, the limits of detection were of the same order of magnitude for ESI, APCI, and APPI in positive and negative ion mode even if overall, ESI in negative mode resulted in better LODs.

The analysis of antioxidants by LC-MS does not show any particular difficulties and many applications can be found in the literature involving the different API sources. The ion source is generally used in good agreement with Fig. 19. Carotenoids (Hao et al. 2005) and fat-soluble vitamins (Stoggl et al. 2001) are analyzed in LC-MS with APCI or APPI. For example, Stoggl et al. (2001) achieved quantification in the femtomole range of vitamin E and some other tocopherol derivatives in food and phytopharmaceutical preparations by HPLC-APCI-MS/MS. Recently, six fat-soluble vitamins (A, E, D2, D3, K1 and K2) and six water-soluble vitamins (B1, B2, B3, B6, B7 and B12) were analyzed simultaneously in LC-APPI-MS/MS. This study demonstrates that APPI is clearly a versatile ion source allowing ionization of both polar and apolar analytes. Obviously, electrospray is ideally suited for detection at low levels (ng/g of fruits or vegetable) of water-soluble vitamins (Gentili et al. 2008). Polyphenols are usually detected as deprotonated molecules (due to the phenol groups) either by APCI (Justesen 2000) or ESI (Cho et al. 2004; Calderon et al. 2009). Calderon et al. (2009) developed a screening method for antioxidants in vegetal material by RPLC-ESI-MS. In this study, the authors identified catechins, epicatechins, their dimers and trimers (proanthocyanidins), some gallic acid derivatives, and a few other glycosylated flavonoids (derived from quercetin) in cocoa.

The other separative methods described in this chapter can also be coupled to MS. Supercritical fluid chromatography is very often interfaced with APCI (Dost and Davidson 2000; Manninen and Laakso 1997; Taylor 2009) but it can also be coupled with ESI-MS (Salvador et al. 1999). For example, Matsubara et al. (2012) developed a highly sensitive and rapid profiling method for carotenoids and their epoxidized products by SFC-ESI-MS/MS in 20 min with detection limits in the femto-molar range order. Capillary electrophoresis is most often coupled to MS with ESI as ion source but it can be also coupled with atmospheric pressure photoionization-mass spectrometry (Marchi et al. 2009). APPI shows even a higher robustness than ESI even toward low and nonvolatile buffers (Mol et al. 2005; Hommerson et al. 2009). The best sensitivities, however, are obviously still obtained with volatile buffers at low salt concentrations (Kostiainen and Kauppila 2009; Hakala et al. 2003). At last, as previously mentioned (Sect. 4.4), HPTLC can be interfaced with MALDI-TOFMS and even electrospray-MS.

6 Conclusions

This chapter has tried to provide an overview of the current techniques used for extraction, purification, and analysis of plant secondary metabolites. Many techniques may be implemented at any level but it is impossible to provide general rules on their choice for new applications. Indeed, there are very often several solutions to achieve a satisfying analysis and the right choice of a technique strongly depends on the starting material and the metabolites of interest. Nevertheless, it must be admitted that the current state of art in analytical chemistry probably provides an adequate solution to perform the development of the individual methods required. Furthermore, continuous research in this field will undoubtedly offer new and precise exploration tools for complex samples, such as plant extract. Besides, the 'peak capacity' race has already started with the following *leitmotiv*: always faster, more informative, and more sensitive. The democratization of hyphenation between separative methods and structural elucidation techniques—and in this context notably mass spectrometry—is only the first step. The expansion of rapid separation and comprehensive multidimensional techniques is ongoing and the relentless technological progress will undoubtedly improve matters further.

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Author Biography

Patrick Chaimbault (born 1969) is currently the Dean of the Department of Chemistry in Metz (University of Lorraine). He has been trained as chemist at the University of Orléans (France) and holds an MSc in Chemistry and Physicochemistry of Molecules of Biological Interest. In 2000, he obtained his PhD focusing on the analysis of underivatized amino acids by liquid chromatography coupled to mass spectrometry. After three years of experience as analytical manager in the pharmaceutical industry, he started his academic career. In September 2002, he became Assistant Professor in Analytical Chemistry at the University of Orléans. In 2007, he was habilitated to supervise research (French diploma: Habilitation à Diriger les Recherches). He moved to the University of Metz in November 2008 where he currently holds the position of Professor of Chemistry.

Patrick develops methods concerning the sample preparation and the analyses of complex biological materials (plants, animal cells, body fluids, etc.,) by Mass Spectrometry (MALDI-TOFMS, MALDI-FTICRMS, ESI-MS). He is an expert in coupling separative techniques (GC, LC, Capillary Electrophoresis) with Mass Spectrometry using API sources (ESI, APCI, APPI). To date, he has published over 40 articles in this field. Patrick is currently in charge of the analytical chemistry topic (Identification, Isolation and Analysis) included in the natural products project “NutriOx”.