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Headspace-Solid Phase MicroExtraction and Gas Chromatography Mass Spectrometry Applied to Determination of Volatiles in Natural Matrices

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ABSTRACT

Headspace – solid phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry-ion trap detector (GC-MS-IT) has been used to characterize volatile compounds in several natural matrices. A great number of fibres with different polarities are commercially available, allowing the screening of the highest possible number of components. HS-SPME has several advantages, such as the condensation of extraction, concentration and sample introduction into a single step. In this extraction technique, a medium polarity fibre for flavours (carboxen/polydimethylsiloxane or divinylbenzene/polydimethylsiloxane) is usually used in order to achieve equilibrium between non-polar and polar compounds, thus increasing the screening range. The traditional methodologies using organic solvents, namely dichloromethane (DCM), allow the determination of a considerable number of volatile and semi-volatile compounds in natural matrices. In this review, the main theoretical points involved in GC-MS analysis will be discussed. The application of HS-SPME and other extraction methodologies (hydrodistillation, Soxhlet and solvent extraction), coupled to GC-MS-IT, to several natural matrices (macroalgae, *Rumex induratus*, *Brassica oleracea* L. var. *costata* DC., *Catharanthus roseus* and mushrooms, with particular emphasis in these last two) will be referred. With this the usefulness of these methodologies in the screening of volatile and semi-volatile compounds will be demonstrated.

Keywords: *Brassica* spp., *Catharanthus roseus*, GC-MS, HS-SPME, macroalgae, mushrooms, natural matrices, *Rumex induratus*, volatile compounds

Abbreviations: **AAT**, alcohol acyl transferases; **ACH**, acyl-CoA thioesterase; **ACS**, acyl-CoA synthetase; **ACX**, acyl-CoA oxidase; **ACP**, acyl carrier protein; **ADH**, alcohol dehydrogenase; **AOC**, allene oxide cyclase; **AOS**, allene oxide synthase; **APCI**, atmospheric pressure chemical ionization; **APPI**, atmospheric pressure photoionization; **CAR/PDMS**, carboxen/polydimethyl-siloxane; **CCD**, carotenoid cleavage dioxygenases; **CCO**, carotenoid cleavage oxygenases; **CI**, chemical ionization; **CW/DVB**, carbowax/divinylbenzene; **DCM**, dichloromethane; **DMAPP**, dimethylallyl diphosphate; **DVB/CAR/PDMS**, divinylbenzene/carboxen/polydimethyl-siloxane; **EI**, electron ionization; **ESI**, electrospray ionization; **FT-ICR**, fourier-transform ion cyclotron resonance; **GC-IT-MS**, gas chromatography-ion trap detector-mass spectrometry; **HCA**, agglomerative hierarchic cluster analysis; **HPL**, hydroperoxide lyases; **HS-SPME**, headspace-solid phase microextraction; **IPP**, isopentenyl diphosphate; **KAT**, 3-ketothiolase; **LOX**, lipoxygenase; **MALDI**, matrix-assisted laser desorption/ionization; **MFP**, multifunctional protein containing a 2E-enoyl-CoA hydratase and a 3S-hydroxyacyl-CoA dehydrogenase; **OPDA**, 12-oxo-phytodienoic acid; **PCA**, principal component analysis; **RF**, radio frequency; **SBSE**, stir bar sorptive extraction; **SIM**, selected ion monitoring mode; **SPE**, solid-phase extraction; **TOF**, time of flight; **TSI**, thermospray ionisation

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Invited Review

INTRODUCTION

A quick check of the literature will reveal that most of the analytical studies available concern chemical classes with well-established pharmacological properties, such as polyphenols or alkaloids (Chauser-volfson and Gutterman 1997; Ferreres *et al.* 2008). However, several pharmaceutical properties, namely antioxidant activity (Singhara *et al.* 1998), antimutagenic, antibacterial, antifungal (Bakkali *et al.* 2008) among others, have been recently recognized for some volatile compounds. Analysis of the volatile profile in natural matrices is very important for different scientific areas, particularly those concerned with food analysis, cosmetics, ecology, biochemistry, phytochemistry and plant physiology.

Volatile compounds have different chemical characteristics, covering a wide range of polarity, solubility, volatility and pH. These compounds can be found in natural matrices, usually in very low amounts (ppb range), and many of them are highly unstable. Accordingly, the choice of an appropriate extraction method greatly influences the reliability and accuracy of natural products' analysis. In order to achieve practical and reliable methods for the analysis of complex matrices, several techniques have been developed, including solid-phase microextraction (SPME), extraction-distillation, solid-phase extraction, supercritical fluids, solvent and Soxhlet extraction, among others.

The coupling of different extractive methods to GC-MS-IT allows the separation and identification of distinct compounds, even when they occur in samples at trace amounts.

GC-MS, as one can easily figure, combines two powerful techniques: GC for separation of compounds and MS to their detection and identification. This feature rapidly turn this analytical approach into a most effective and useful technique.

In this review, the main theoretical points involved in GC-MS analysis will be discussed and some examples of application of GC-MS-IT on the analysis of different natural matrices will be presented.

VOLATILE COMPOUNDS

Volatiles cover a wide range of compounds with different characteristics, usually with low molecular weight, which can be metabolites derived from amino acids, fatty acids and carbohydrate compounds. Although volatiles were regarded exclusively as secondary metabolites of plants, nowadays several works have proven that, apart from plants vegetable materials (leaves, flowers, fruits and roots, among others), some volatiles are emitted from other living organisms, such as fungi (mushrooms), insects, algae or even oysters (Fujimura *et al.* 1994; Harborne 1997; Josephson *et al.* 2006; Cho *et al.* 2007). By interacting with volatiles, the olfactory sensory system is responsible for the flavour preferences that rules human diet (Vichi *et al.* 2005; Goff and Klee 2006; Edris 2007).

It is believed that volatiles have protective effects in plants, mainly due to their anti-microbial and anti-herbivore activity, acting as repellents for herbivores and pathogens. A number of these compounds also have the ability to attract arthropods that fall upon or parasitize herbivores, thus minimizing further damage to plant tissue. Volatiles can also play a role in attracting pollinators. Some of the molecules have the ability to scavenge reactive oxygen species, protecting against internal oxidative damage (Dudareva *et al.* 2004; Schwab *et al.* 2008). Furthermore, recent studies have demonstrated that these compounds play an important role in plant-plant communication. Undamaged plants can adaptively respond to the chemical information emitted by damaged neighbours (Yan and Wang 2006).

From a chemical viewpoint, volatiles can be straight-chain, branched-chain, aromatic or heteroaromatic compounds, constituted by a wide range of chemical groups, such as hydroxyl, carbonyl, carboxyl, ester, lactone, amine and thiol functions. They can be produced in different biosynthetic pathways and can be divided in terpenoids, apocarotenoids and norisoprenoids, lactones, benzenoids and phenylpropanoids, nitrogen and/or sulphur-bearing volatile compounds and fatty acids-derived compounds.

Terpenoids

Terpenoids constitute one of the most diversified families of natural products. So far, over 40000 different structures were identified in several biological systems, including a large number of medically important compounds, such as hormones and cytostatic agents (Schwab *et al.* 2008).

The universal precursors of terpenoids are the five-carbon compound isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). The mevalonate pathway was considered to be the unique biosynthetic pathway for the formation of IPP, DMAPP and the subsequent terpenes, until the discoveries by the research groups of Rohmer (Rohmer 1993) and Eisenreich (Eisenreich 1998). These works suggested that pyruvate and triose phosphate served as precursors for the formation of IPP and DMAPP *via* an alternative pathway. It was also shown that 1-deoxyxylulose, a known precursor of thiamine and pyridoxal, could be diverted very efficiently to terpenoids by *E. coli* cells. Subsequent studies by several research groups identified 1-deoxy-D-xylulose 5-phosphate as the first intermediate of the alternative terpenoid pathway. Nevertheless, details of the formation of IPP and DMAPP from this intermediate remain unknown (Rohdich *et al.* 2001) (**Fig. 1**).

These two terpene biosynthetic pathways have been studied in different taxonomic kingdoms. Animals, fungi and archaebacteria appear to use exclusively the mevalonate pathway, while eubacteria use either the mevalonate or the deoxyxylulose one (Takagi *et al*. 2000). Plants use the mevalonate pathway in the cytosol to synthesize sesquisterpenes (C_{15}), phytosterols and ubiquinone, while the deoxyxylulose pathway takes place in the plastids to generate monoterpenes (C_{10}), diterpenes (C_{20}), gibberellins, abscisic acid and carotenoids (tetraterpenes) (Pichersky *et al.* 2006; Schwab *et al.* 2008).

Apocarotenoids and norisoprenoids

Apocarotenoids are compounds involved in a great variety of cellular processes, being found in all life kingdoms. They are commonly produced by oxidative cleavage and later modification of larger carotenoid compounds. Like their precursors, apocarotenoids are non-polar isoprenoids that possess a conjugated double-bound system and, for this reason, exhibit antioxidant properties (Kloer and Schulz 2006). Furthermore, these metabolites have low threshold values and characteristic aroma notes, and have a high commercial value for flavour and perfume industries, particularly ionones, which are found in blackberry, peach and apricot, and in the odour of flowers such as violets. Furthermore, ionones are valuable for its industrial applications as starting materials for other products (Rodríguez-Bustamante and Sánchez 2007).

Depending on the carotenoid precursor and the breakdown position, a great variety of apocarotenoids can be generated. This breakdown can take place *via* either non-enzymatic or enzymatic routes. The non-enzimatic breakdown comprises photo-oxygenation, auto-oxidation and thermal degradation. On the other hand, the enzymatic breakdown can be accomplished by several enzymes, such as carotenoid cleavage oxygenases (CCO), carotenoid cleavage dioxygenases (CCD), lipoxygenases (LOX), xanthine oxidase, phenoloxidases and peroxidases.

Carotenoids can be cleaved in C9 to C13 positions and C_{13} products, known as norisoprenoids, represent the most common and widespread group (Rodríguez-Bustamante and Sánchez 2007) (**Fig. 2**).

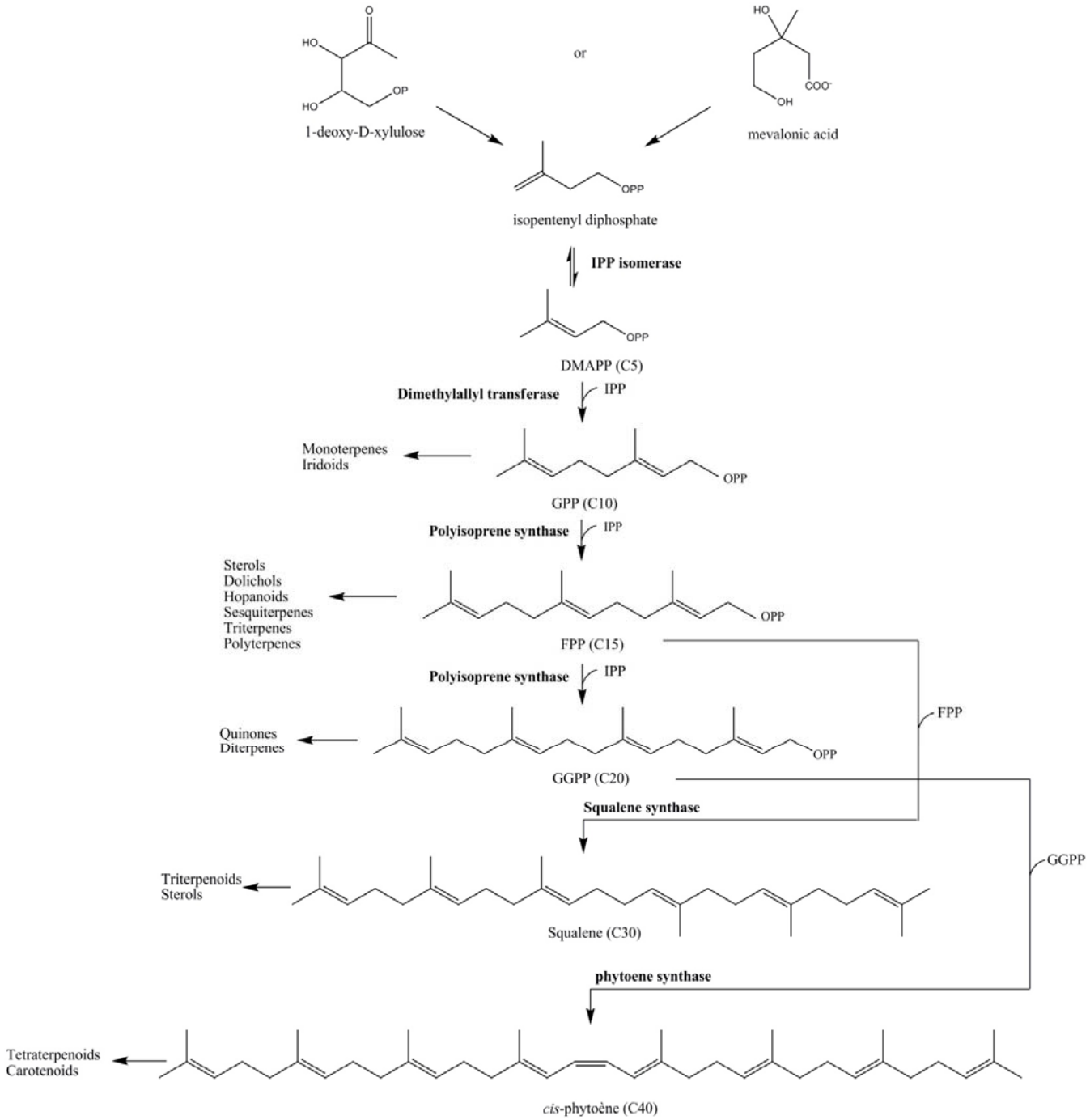

Fig. 1 Synthesis of the precursors of each class of terpenic compounds. Solid arrows indicate enzymic reactions. Abbreviations: DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

Benzenoids/phenylpropanoids

The shikimate pathway links carbohydrate metabolism to the synthesis of aromatic amino acids, which can in turn act as precursors of several primary and secondary metabolites. Phenylpropanoid metabolism provides plants with thousands of compounds that are intermediates in the synthesis of structural cell components (e.g. lignin, suberin, and other cell wall-associated phenolics), while others constitute a diverse array of pigments (e.g. flavonoids and anthocyanins, both of which are usually non-volatile). The aromatic amino acid phenylalanine is also the precursor of volatile benzenoids (C_6-C_1) and phenylpropanoids (C_6-C_3), providing the characteristic benzene ring that is further oxidized, acylated or methylated, thus yielding individual scent compounds (van Schie *et al.* 2006). Phenylpropanoids that are reduced at the C9 position (to aldehyde, alcohol, or alkane/alkene) and/or that contain alkyl additions to the hydroxyl groups of the benzyl ring or to the carboxyl group (i.e. ethers and esters) are volatile. Eugenol and safrol are characteristic compounds of this family, and are frequently the main components of several essential oils.

Benzenoid compounds (C_6-C_1) are originated from *trans*-cinnamic acid as a side branch of the general phenylpropanoid pathway. Characteristic compounds of this family known to constitute the floral scent of plants are benzylacetate, methylsalicylate, methylbenzoate, benzylbenzoate, dimethoxytoluene, phenylpropene and methyleugenol (Boatright *et al.* 2004) (**Fig. 3**).

Nitrogen- and sulphur-bearing volatile compounds

The pungent and, in some cases, unpleasant odour characteristic of some plants is due to, in a general way, volatile metabolites which incorporate sulphur or nitrogen.

A particular feature of some angiosperm families, namely Brassicaceae, is the presence of glucosinolates. These sulphur and nitrogen-containing secondary metabo-

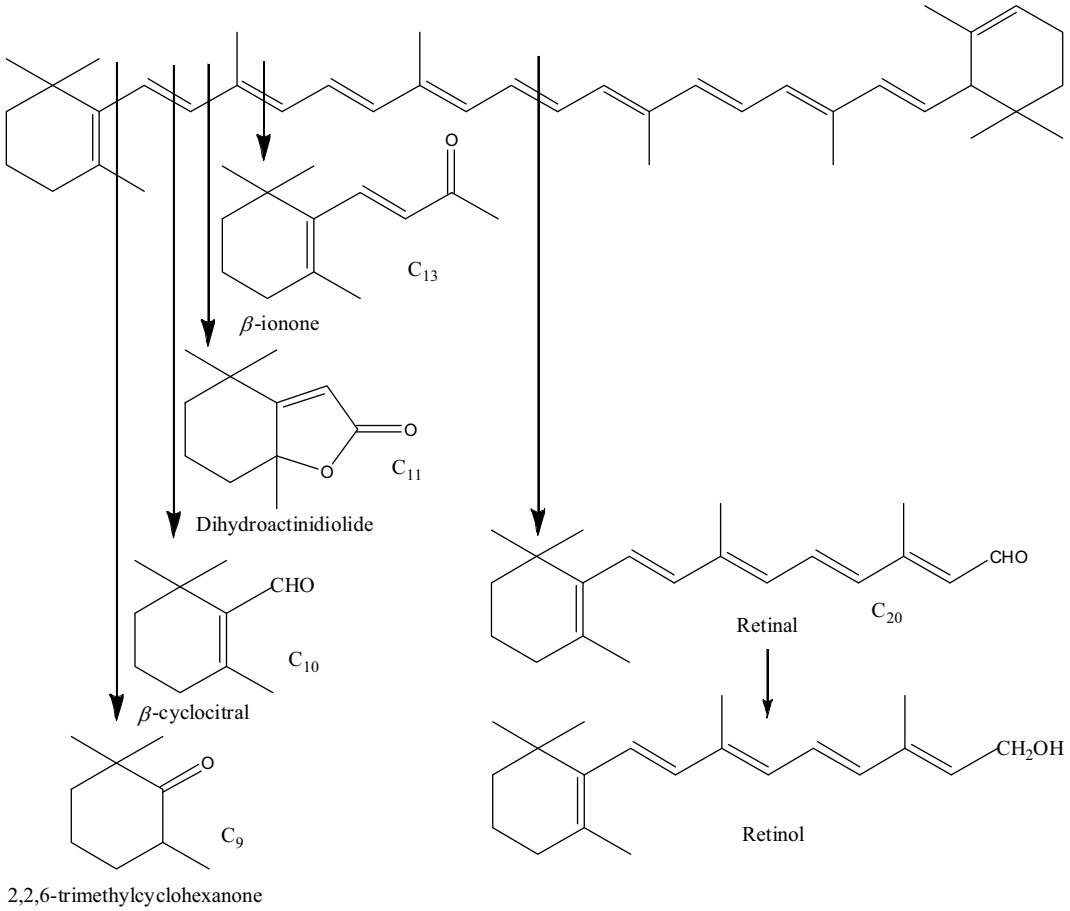

Fig. 2 Products of carotenoid cleavage.

lites are originated from amino acids. Plants that produce and accumulate glucosinolates always possess a thioglucoside glucohydrolase enzyme known as myrosinase, which hydrolyzes the glucose moiety on the glucosinolate main skeleton. The products are glucose and an unstable aglycone that can rearrange to form volatile compounds, such as isothiocyanates, nitriles, and other products, which are responsible for the pungent aroma characteristic of these plants (Halkier and Gershenzon 2006).

Pyrazines constitute a group of heterocyclic nitrogen-containing compounds, being widespread in nature and with a pronounced odour. Several authors suggest a pathway for pyrazine biosynthesis initiated by the formation of a cyclic peptide through the condensation of two amino acids. This pathway only allows the formation of 2,5-dialkylsubstituted metabolites. Another biosynthetic pathway is the condensation of a α,β-dicarbonylic compound and an amidated amino acid and allows the formation of both 2,5- and 2,6-alkyl-substituted pyrazine molecules (Beck *et al.* 2003).

Fatty acids derived compounds

Saturated and unsaturated C_6 and C_9 volatile aldehydes and alcohols are ubiquitous to all kingdoms (Akakabe *et al.* 2005). These compounds are important contributors to the "green leaf" odour, being produced by the lipoxygenase pathway and involving at least four enzymes: lipoxygenase (LOX), hydroperoxide lyase (HPL), (3*Z*,2*E*)-enal isomerase and alcohol dehydrogenase (ADH) (Schwab *et al.* 2008).

LOX catalyzes the oxygenation of fatty acids containing a (1*Z*,4*Z*)-pentadiene moiety (e.g. linoleic and linolenic acid) in a region/stereo-selective way. These LOX products are *Z*,*E*-configured hydroperoxides, which subsequently suffer enzymatic cleavages by HPL, resulting in the formation of short-chain aldehydes with 6 and 9 carbon atoms (Akakabe *et al.* 2005).

α-Oxidation leads to the enzymatic degradation of C_n fatty acids into a $C_{(n-1)}$ long chain fatty aldehyde, with concomitant release of CO_2. An α-dioxigenase-peroxidase has been suggested to operate together with aldehyde dehydrogenase and NAD^+ oxido-reductase to lead to successive fragmentations of fatty acids into shorter chain homologues (Hamberg *et al.* 1999).

Differently from α-oxidation, β-oxidation pathway mainly involves a set of several enzymatic reactions: acyl-CoA thioesterase (ACH), acyl-CoA synthetase (ACS), acyl-CoA oxidase (ACX), multifunctional protein containing a (2*E*)-enoyl-CoA hydratase and a (3*S*)-hydroxyacyl-CoA dehydrogenase (MFP) and a 3-ketothiolase (KAT), which results in the removal of C_2 units (acetyl CoA) from the parent fatty acid. This pathway has been reported across different kingdoms (plants, mammals, fungi) (Goepfert and Poirier 2007).

Successive removals of C_2 units followed by the action of an acyl CoA hydrolase leads to volatile acids with buttery and cheese-like odour. Some of these aliphatic acids can also be synthesized *de novo* by hydrolysis of an acyl carrier protein (acyl ACP). The enzymatic reduction of the parent acyl CoA leads to short- and medium chain aldehydes and alcohols. Esters can be synthesized by acyl CoA, formed during β-oxidation, and alcohols. The enzymes that catalyze the combinations of acyl CoA and alcohols are alcohol acyl transferases (AAT) (Schwab *et al.* 2008).

Jasmonates are also lipid-derived metabolites that have important functions in plants, such as mediation of the responses to mechanical trauma and pathogenesis, regulation of metabolism and reproduction. Jasmonic acid is not volatile, but some of its metabolites are, with methy jasmonate or *cis*-jasmone being good examples. This latter is involved in regulating the behaviour of some insects, repelling herbivorous ones and attracting their predators. There are also evidences suggesting that some volatiles from the jasmonic acid pathway may enable communication among plants (Liechti and Farmer 2002). **Fig. 4** represents a schematic pathway of fatty acids derived flavour compounds.

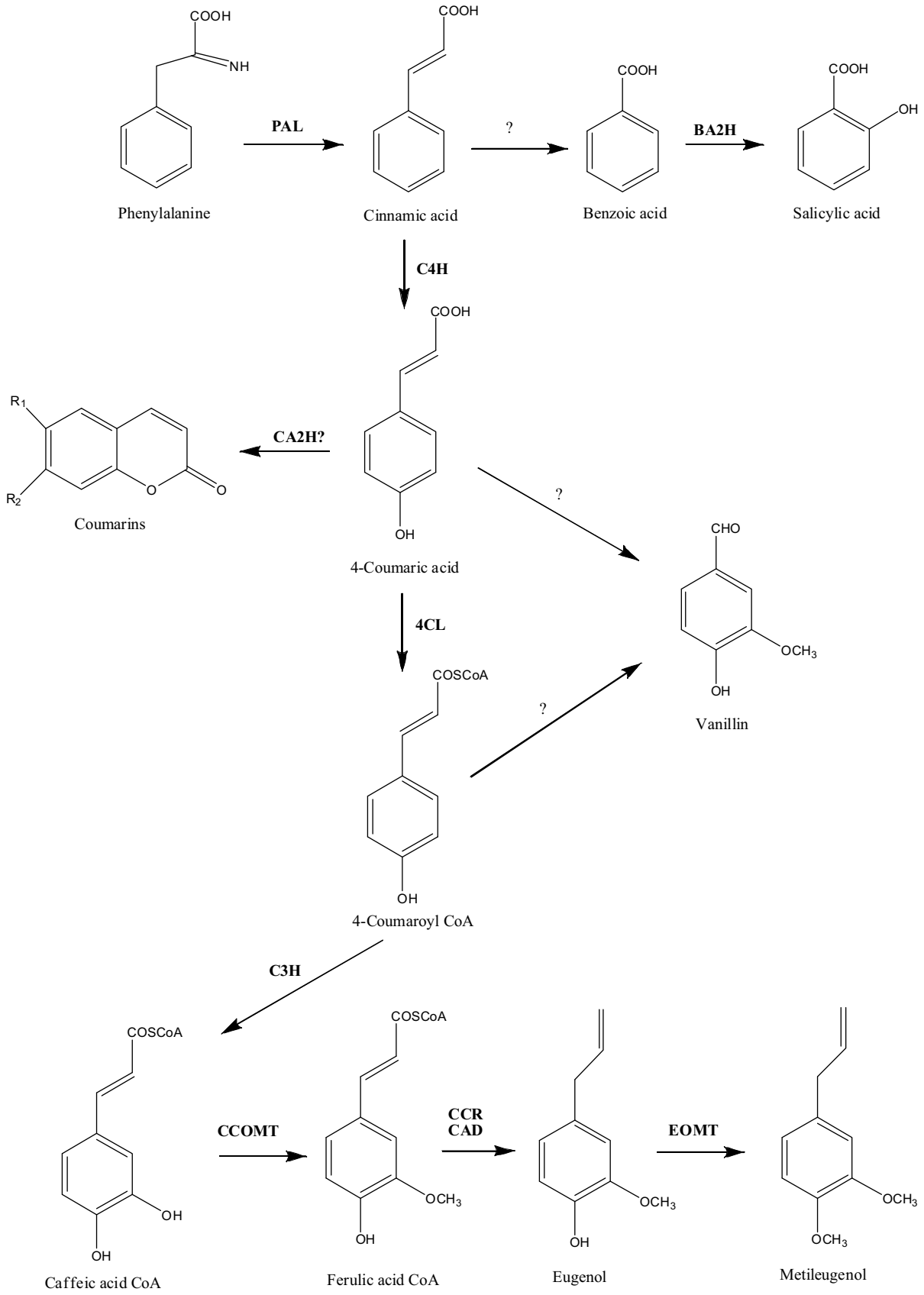

Fig. 3 Schematic view of some branches of phenylpropanoid metabolism. Solid arrows indicate enzymic reactions. Abbreviations: PAL, phenylalanine ammonia-lyase; BA2H, benzoic acid 2-hydroxylase; C4H, cinnamate 4-hydroxylase; CA2H, cinnamate 2-hydroxylase; 4CL, 4-coumarate coenzyme A ligase; C3H, *ρ*-coumarate 3-hydroxylase; CCOMT, caffeoyl coenzyme A *O*-methyl-transferase; CCR, cinnamoyl coenzyme A reductase; CAD, cinnamoyl alcohol dehydrogenase; EOMT, eugenol *O*-methyl-transferase.

Lactones

γ- and δ-lactones are fatty acid derivatives quite widespread in nature, and many of them display pronounced biological activities as attractants for pollination and seed germination stimulants. They also act as pheromones, antiseptics, allergens, or even as cardiotonic compounds. Furthermore, lactones are important flavour and aroma constituents and are extensively used as food additives and in perfumery. For example, 4-butylbutanolide occurs in the flavour of apricot,

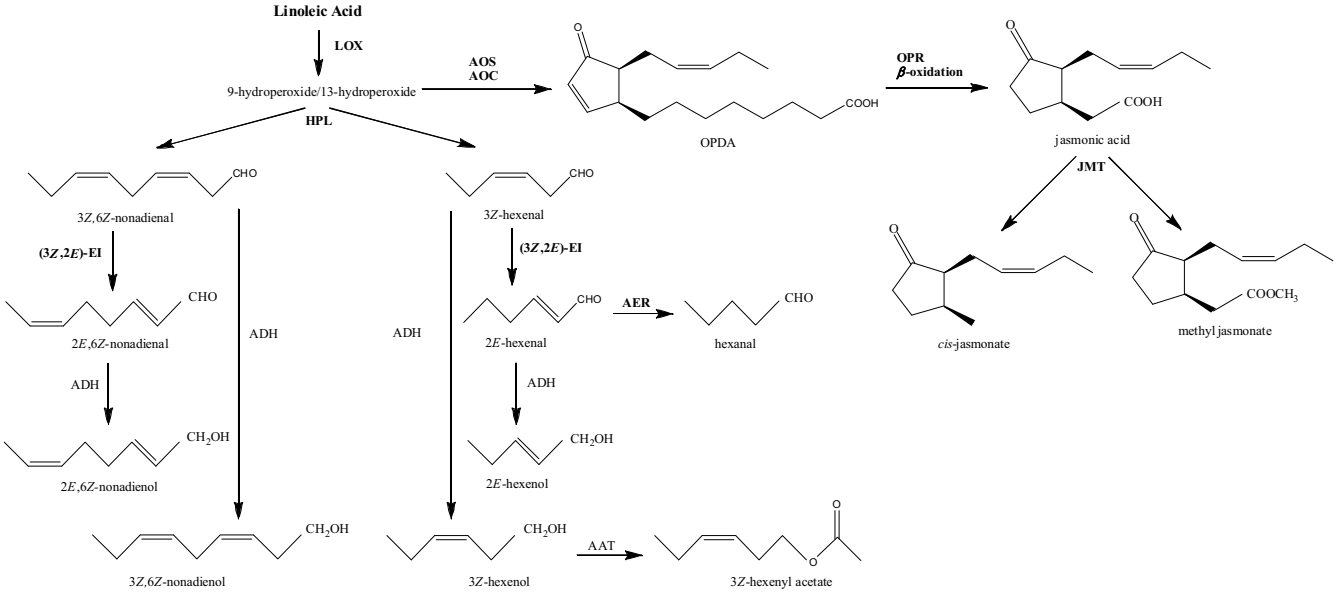

Fig. 4 Linoleic acid-derived flavour molecules. Solid arrows indicate enzymic reactions. Abbreviations: AAT, alcohol acyl transferase; ADH, alcohol dehydrogenase; AER, alkenal oxido-reductase; AOC, allene oxide cyclase; AOS, allene oxide synthase; OPDA, 12-oxo-phytodienoic acid; OPR, 12-oxophytodienoic acid reductase; HPL, hydroperoxidase lyase; JMT, jasmonate methyltransferase; LOX, lipoxygenase; 3*Z*,2*E*-EI, 3*Z*,2*E*-enal isomerase.

raspberry, hazelnut, strawberry, tea, and exhibits a sweet and creamy dairy flavour with fatty and oily coconut nuances that is recognised down to 7 ppb. A similar low threshold is displayed by 4-octylbutanolide, a characteristic flavour component of apricot, beer, peach, pineapple, rum, and strawberry (Habel and Boland 2008).

All lactones have their origin in their corresponding 4- or 5-hydroxy carboxylic acids, which in turn are formed by either of these systems: reduction of oxo acids by NAD-linked reductase, hydration of unsaturated fatty acids, epoxidation and hydrolysis of unsaturated fatty acids and reduction of hydroperoxides, from naturally occurring hydroxyl fatty acids or cleavage of hydroxylated long-chain fatty acids (Habel and Boland 2008).

EXTRACTIVE TECHNIQUES

Several extractive techniques have been developed for the analysis of the volatile profile of natural matrices. The volatile composition is usually quite complex and therefore different approaches and methods of analysis should be used to obtain a complete overview. When several different extractions take place, the sum of the obtained volatiles constitutes a good representation of the matrix's profile.

Traditional methodologies (extraction-distillation, solvent and Soxhlet extraction and solid-phase extraction) usually imply several steps, including purification and concentration of the extract (with consequent analyte losses), long preparation times and the use of large quantities of organic solvents (Prosen and Zupancic-Kralj 1999).

Despite its disadvantages, solvent extraction allows the extraction of several compounds (low, medium and high volatility) and different solvents can be used (hexane, dichloromethane, ether, acetone or pentane, among others) with different affinities for compounds and extractive capacity (Ortega-Heras *et al.* 2002). For an efficient extraction, the solvent must be able to solubilise the target molecules while leaving the sample matrix intact and the polarity of the solvent should closely match that of the target compounds. Mixing solvents of different polarities is a strategy that can be used in order to extract a wide range of compound classes.

One of the oldest and most commonly used extraction techniques is the Soxhlet extraction. This technique implies long extraction periods and uses a high volume of high purity solvents, thus being very expensive and implicating a concentration process prior to analysis. The temperature and solvents used are important to control the type of compounds extracted, however under extreme conditions thermal decomposition and reactions with the solvent may occur. Nowadays, this technique is used for the extraction of semi-volatile analytes from a solid or semi-solid sample matrix (Luque de Castro and García-Ayuso 1998).

Hydrodistillation is the most common extraction technique employed to obtain essential oils from different plant materials (Guenter 1948). There are three types of hydrodistillation: with water immersion, with water immersion and vapor injection, and with direct vapor injection (Silva *et al.* 2005). Water gives better extraction of polar components and less favourable extraction of non-polar compounds (Marriot *et al.* 2001). However, this technique presents some shortcomings, namely losses of compounds, low extraction efficiency, long extraction time, laborious process and the need of large amounts of sample (Wang *et al.* 2008a). Essential oils can undergo chemical alterations and thermal decomposition that can easily destroy some compounds, thus changing the volatile profile.

Solid-phase extraction (SPE) allows obtaining compounds without applying heat. This technique is performed by using an adsorbent surface to extract organic compounds from a liquid phase and, for this purpose, different resins can be applied. Although SPE is limited to semi-volatile and non-volatile compounds (Masqué *et al.* 1998), it appears to be applicable to both polar and non-polar compounds. Compared to the other techniques referred above, SPE offers a reduction of processing time and quantity of solvent used. Nevertheless, it still requires multiple steps, long extraction times, losses in the evaporation step and risk of contamination (Alpendurada 2000).

The disadvantages of traditional extraction methodologies, previously referred, and especially the use of large quantities of solvents led to the development of new methods, which main advantages are the small volume of solvent used and the relatively fast extraction step.

Supercritical fluid extraction is an interesting technique for the extraction of volatile compounds from vegetable matrices. This technique has several advantages over traditional extraction techniques, such as the possibility to adjust a number of parameters (solvent, extraction temperature and pressure) to obtain better selectivity and extractive efficiency, also allowing the reduction of the use of polluting organic solvents. As it is performed at low temperatures, this technique can prevent the thermal degradation and hydrolysis of the matrix. Nevertheless, the complex and expen-

Table 1 Characteristics of SPME fibres commercially available.

Stationary phase	Recommended use
Polydimethylsiloxane (PDMS)	
100 μm / non-bonded	Low molecular weight or volatile compounds
30 μm / non-bonded	Non-polar semivolatiles
7 μm / bonded	Large molecular weight compounds
Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)	
65 μm / partially crosslinked	Polar volatile analytes, such as amines and alcohols
60 μm / partially crosslinked	General purpose SPME fiber for HPLC
Carboxen/Polydimethylsiloxane (CAR/PDMS)	
75 μm / partially crosslinked	Trace-level volatiles
Carbowax/Templated Resin (CW/TPR)	
50 μm / partially crosslinked	Surfactants (for HPLC only)
Carbowax/Divinylbenzene (CW/DVB)	
65 μm / partially crosslinked	Polar analytes
Polyacrylate	
85 μm / partially crosslinked	Polar semi-volatiles
Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS)	
50/30 μm divinylbenzene/Carboxen on PDMS fiber	Trace compounds (MW 40-275)
Carbowax-Polyethylene Glycol (PEG)	
60 μm/ partially crosslinked	Alcohols and polar compounds (MW 40-275)

sive equipment necessary, not available in all laboratories, constitutes its main disadvantage (Pourmortazavi and Hajimirsadeghi 2007; Wang *et al.* 2008b).

Pawlisyn (Pawlisyn 1997) developed an adsorptive technique called solid-phase microextraction (SPME), which can be regarded as replacement and improvement of classical sample preparation methods (Ulrich 2000). SPME was originally developed for environmental analysis (Pawliszyn 1997), although today it is widely used for analysing volatiles in a wide range of areas, such as food, environmental, botanical and clinical analysis, as well as in forensic applications (Alpendurada 2000; Kataoka *et al.* 2000).

SPME is a well-established sample preparation technique for the analysis of volatile and semi-volatile compounds, presenting many advantages such as high sensitivity and reproducibility, simplicity, absence of solvents, cost and combination of extraction and pre-concentration into a single step (Kataoka *et al.* 2000). This technique, based on absorption and/or adsorption mechanisms, can be performed in three different sampling modes: direct injection (immersion), HS extraction and extraction with membrane protection (Prosen and Zupancic-Kralj 1999). HS-SPME is the most used for several reasons, being the main one the lack of contact with the sample that reduces the matrix influence and prevents decomposition or contamination of the fibre coating (Alpendurada 2000). Moreover, the time necessary to reach the equilibrium between the analyte in the gaseous phase of the sample and the stationary phase is shorter than for aqueous samples, and so HS-SPME is recommended for compounds with high volatility (Prosen and Zupancic-Kralj 1999). Several coatings are available, the particular choice of a coating being highly influenced by the chemical structure of the target compounds. The careful selection of the polarity and thickness of coating allows the extraction of different compounds, with other variables being equally important, namely temperature, agitation, pH and the addition of a salt to help the release of some compounds (Ulrich 2000).

Solid Phase Microextraction – Optimization

Achieving good reproducibility, selectivity and sensitivity with SPME requires a careful optimization of the extractive process. The performance of this method is affected by the combination of several parameters, which are all interrelated.

Several fibres are commercially available (**Table 1**). The selection of the adequate fibre is very important because compounds with different polarity and volatility can be sampled simultaneously by the careful selection of the polarity and thickness of the fibre coating. In general, the "like dissolves like" rule can be applied and by changing the fibre coating we can modulate the compounds extracted (Kataoka *et al.* 2000). The different fibres are available in various thicknesses. A thin coating has a faster diffusion rate and allows preferentially the retention of semivolatile compounds. On the other hand, the thick coatings permit the retention of highly volatile compounds with an increased sensitivity; however, they require a longer equilibrium time (Prosen and Zupancic-Kralj 1999). By constructing an extraction-time profile curve, we can observe that the amount of the analyte extracted is a function of time. In order to extract the maximum amount of analyte, the equilibrium time has to be reached, and this is dependent on the partition coefficient of the analyte. Consequently, the shorter exposure time is chosen according to the extraction-time profile curve and the analyte detection limit (Ulrich 2000). **Fig. 5** shows a schematic representation of the extraction and desorption procedure using SPME. To achieve the optimal stage of equilibrium several parameters can be changed, such as temperature, agitation, pH, ionic strength, and derivatization.

Agitation is normally used to achieve faster equilibrium, because it enhances the diffusion of analytes to the fibre. The rate of stirring should be constant for all samples and has to be experimentally determined. The increase of extraction temperature also raises the diffusion coefficient of analytes, although it reduces the distribution constant (Ulrich 2000). The pH of the sample is important for weak acid or basic compounds, mainly because the extraction of these compounds is more effective when they are in the undissociated form (Alpendurada 2000). The addition of a soluble salt, usually sodium chloride or sodium sulphate, increases the ionic strength of the solution, thus making organic compounds less soluble and increasing the partition coefficients (Steffen and Pawliszyn 1996; Ulrich 2000). Derivatization can be used when aiming the extraction of very polar compounds. The derivatization agent may be either added into the matrix, or bonded to the SPME fibre, where analytes are adsorbed and derivatized simultaneously (Prosen and Zupancic-Kralj 1999).

Gas Chromatography and Mass Spectrometry (GC-MS)

Gas chromatography is a powerful tool to analyse volatile compounds. Mixtures to be analysed are injected into an inert gas stream and swept into a tube, which is packed with a solid support coated with a resolving liquid phase. Absorptive interactions between the components in the gas stream and the coating lead to a differential separation of the components of the mixture, which are then swept in

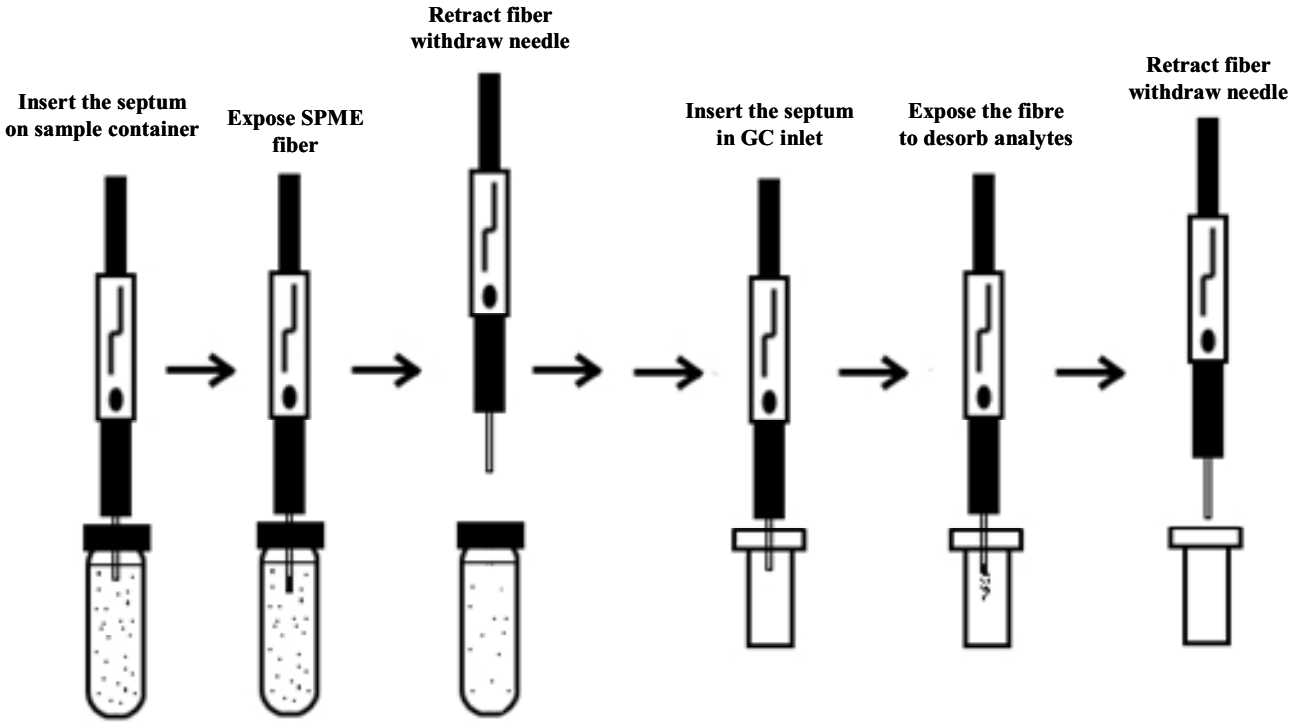

Fig. 5 Solid phase microextraction procedure.

order through a detector flow cell.

MS is based on the production of ions from the analyte. This is ionized in high vacuum and its ions and fragmentation products are impelled and focused through a magnetic mass analyzer, to be further collected and the amounts of each selected ion measured in a detector.

The combination of GC and MS yields an instrument capable of separating mixtures into their individual components, identifying and providing quantitative and qualitative information on the amounts and chemical structure of each compound.

From an instrumental point of view, three components are essential to perform mass spectrometric experiment: the first is the ion source, the second is the mass analyser and the last one is the detector. These three compartments are submitted to a very high vacuum to prevent the undesirable movement of particles (Karasek and Clement 1991).

Ion sources

The choice of the ionization method to be employed is dependent of the physico-chemical properties of the analyte(s) of interest (volatility, molecular weight, thermolability, complexity of the matrix in which the analyte is contained, among others).

The ion sources can be divided in two main classes: those requiring sample in the gas phase prior to ionization, and those able to manage low volatility and high molecular weight samples.

The first class includes electron ionization (EI) and chemical ionization (CI) sources, which are the most diffused nowadays due to their multiple uses in GC-MS. The other ones can be further divided into those operating with solutions, such as Electrospay Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) or Atmospheric Pressure Photoionization (APPI), and those based on sample desorption and ionization from a solid substrate: Matrix-Assisted Laser Desorption/Ionization (MALDI) and Thermospray Ionisation (TSI). These last two are mainly applied in liquid chromatography. As our work deals with the identification of volatile compounds, we will focus only on EI and CI ion sources.

EI is based on the interaction of an energetic electron beam, generally 70 eV, with the sample vapour (at a pressure in the range 10^{-7}-10^{-5} Torr). This interaction leads to the production of a series of ions related to the chemical properties of the compounds under study. CI methods generally apply lower levels of electronic impact (12 eV.). This method is employed to select species of interest in complex matrices (Karasek and Clement 1991).

Analysers

There are several types of analysers: Magnetic Sector, Time of Flight (TOF), Ion Mobility, Fourier-Transform Ion Cyclotron Resonance (FT-ICR), Quadrupole and Ion Trap.

Quadrupole and ion trap are currently the mass analysers most widely employed in GC-MS. Both systems start from the same considerations: ions of different *m*/*z* values will interact in a different manner with alternated electrical fields (radio frequency (RF)).

Four hyperbolic and equidistant rods constitute the Quadrupole analyser. Ions “travel” through the rods and are separated along them according to their m/z values.

The Ion Trap analyser is constituted by a central hyperbolic electrode and two other electrodes called the “end caps”. An RF is applied in the central electrode creating a magnetic field in the trap. Molecules coming from the gas chromatographic column go in to the trap. After changing the potential, the electrons also go into the trap. The impact of electrons with the molecule causes its ionization. The formed ions describe then a characteristic orbital in the shape of an eight. The orbital described by the ions increase in its amplitude being sequentially ejected from the trap (first those with lower *m/z* values) directly to the electron multiplier.

There are some advantages and disadvantages for using the quadrupole and/or the ion trap. Briefly, quadrupole is more tolerant to weak vacuum, though it is adapted to electrospray ionization. In addition, it can reach m/z values of 3000, which is of great interest for electrospray ionization of bio-molecules The Ion trap has the great advantage of being much more sensitive in full scan mode acquisition (monitoring a range of m/z). The most important difference between ion trap and quadrupole analysers concerns the separation of the m/z produced into the analyser. In quadrupole, this separation is performed along the rods while in the ion trap the separation is done into the trap. Consequently, ion trap is able to produce full scan spectra with high detection limits; however, it cannot work in SIM

(Selected Monitoring Ion) mode, which is a characteristic of quadrupole analysers. Ion trap has the possibility to do several fragmentations of each m/z (MS/MS until *n* levels) in opposition to quadrupole, which works only with one level of MS/MS. Finally, Ion trap analyser is able to separate very similar ranges of m/z, which means that its resolution is better. Depending on the objectives of the work, the option for one or another can be made (Watson and Sparkman 2007).

Detectors

The most used detector for GC-MS purposes is the electron multiplier. This component is the part of the mass spectrometer, which is responsible for increasing the signals coming from the analyser. A semi-conductor material (SnO, PbO) in the internal surface plates the electron multiplier and the top of the multiplier is maintained with a negative voltage. Each ion entering in the electron multiplier collides with the surface material and releases two electrons. These two electrons collide with the opposite wall of the electron multiplier releasing two more electrons. This process is repeated successively: for one ion entering in the electron multiplier 100 000 electrons are generated, which corresponds to a gain of 10^5. The electric signal is then processed and the data is treated with the acquisition software (Lavagnini *et al.* 2006; Watson and Sparkman 2007; MacMaster 2008).

APPLICATIONS

A complete identification and ulterior quantification of volatiles, in complex matrices such as natural products, needs some previous proceedings due to volatiles being present in a wide range of concentrations. Some volatiles can be present in high levels (mg/L or mg/Kg) while others occur in very low ones (ng/L or ng/Kg). As was discussed previously, an ion trap analyser stores all *m/z* particles for a small period. When a molecule is present in high amounts, the consequence is an "overloading" in the trap, which causes a distortion of mass spectrum of the molecule that may prevent a correct identification. In opposition, when volatile molecules are present in low levels (ng or even pg levels) a very accurate spectrum can be obtained, which is usually coincident with those of most libraries. Consequently, and due to this specificity/limitation, quantification can be analytically difficult. Linearity studies must be performed in certain ranges of concentrations and, when compounds are present in high concentration, dilution of samples is recommended; when SPME is used the time of fibre exposition to the headspace has to be reduced.

Our research group has applied HS-SPME allied to GC-IT-MS to several natural matrices, namely mushrooms, *Catharanthus roseus* (L.) G. Don, formerly *Vinca rosea* L. (Apocynaceae), *Brassica oleracea* L. var. *acephala* leaves and sprouts, *Rumex induratus* and algae, among others matrices.

Mushrooms

Wild edible mushrooms are a source of interesting organoleptic properties such as flavour, texture and colour. The taste of edible mushrooms is primarily attributed to several water-soluble substances, including 5'-nucleotides, free amino acids and soluble carbohydrates. Among the diverse volatile compounds, a series of aliphatic eight carbon (C8) components, such as 1-octen-3-ol, 2-octen-1-ol, 3-octanol, 1-octanol, 1-octen-3-one and 3-octanone, have been reported to be the major contributors to the characteristic mushroom flavour (Cho *et al.* 2006). Besides that, other compounds, such as esters, terpenes and indoles have been identified and can also be related to mushroom flavour.

The objective of the work of Guedes de Pinho *et al.* (2008) was to obtain a complete screening of the volatiles and semi volatiles of eleven edible mushrooms (*Suillus bellini*, *Suillus luteus*, *Suillus granulatus*, *Tricholomopsis rutilans*, *Hygrophorus agathosmus*, *Amanita rubescens*, *Russula cyanoxantha*, *Boletus edulis*, *Tricholoma equestre*, *Fistulina hepatica*, and *Cantharellus cibarius*). Additionally, the contents of the identified volatiles were correlated with sensorial descriptors. For this propose, HS-SPME and liquid extraction with organic solvents combined to GC-IT-MS was used.

A comparison between three fibres (CAR/PDMS, CW/DVB and DVB/CAR/PDMS) was performed and DVB/CAR/PDMS fibre was selected for the analysis of all mushrooms species, as it was the one that could give the most complete profile of the compounds present in the analysed species. In addition, it revealed to be the best and more selective fibre for the identification of aldehydes like methional and phenylacetaldehyde, regarded as important compounds to the mushroom flavour (Barker *et al.* 1983; Silva Ferreira and Guedes de Pinho 2003).

A sensorial analysis was performed and some descriptors, such as "mushroom-like", "farmfeed-like", "floral", "honey-like", "hay-herb" and "nutty" were obtained. Applying multivariate analysis (principal component analysis (PCA) and agglomerative hierarchic cluster analysis (HCA)) to the sensorial and chemical data, it was possible to relate sensory descriptors and volatiles. The descriptors selected were "farm-feed" (28%), "mushroom-like" (24%), "floral" (18%), "honey-like" (8%), "nutty" (8%), "hay-herb" (7%) and 7% corresponded to other descriptors, which were discarded. The eleven species were divided in three groups (**Fig. 6**). The one with "floral" and "honey" descriptors included *S. granulatus* and *S. luteus*; the second group, presenting "hay-herb", "nutty" and "mushroom-like" notes was composed by *A. rubescens*, *C. cibarius*, *S. bellini* and *T. equestre*; and finally the species characterized by "farm-feed" were *H. agathosmus*, *T. rutilans*, *R. cyanoxantha*, *B. edulis* and *F. hepatica*.

HS-SPME and dichloromethane extractions allowed the identification of 64 volatile and non-volatile compounds in the analyzed mushroom species. These included 5 volatile acids, 8 non-volatile acids, 7 esters, 9 alcohols, 7 aldehydes, 7 ketones, 11 terpenes, 1 volatile phenol, 2 lactones and 7 other compounds. Thirteen compounds were tentatively identified and fifty-one were identified by comparison of the kovats index and the MS spectrum of the pure standard. Among the volatile acids, we identified five fatty acids (myristoleic, palmitoleic, stearic, linoleic and oleic acids) and three others: benzoic, cinnamic and phenylacetic acids. *H. agathosmus* presented the highest percentage of myristoleic, palmitoleic, oleic and cinnamic acids. *S. bellini* was identified as the richest species in alcohols, which are considered to be the main odorants of the "mushroom-like" aroma (Cho *et al.* 2006). Among these compounds, *C. cibarius* presented the highest percentage of 1-octen-3-ol, while *A. rubescens* was the one with the highest amount of 3-octanol. Statistical results showed that these alcohols have higher correlations with the "nutty" descriptor than with the "mushroom-like" aroma. *T. equestre* and *S. luteus* presented the highest levels of aldehydes and benzaldehyde and phenylacetaldehyde were identified in all of the species. Phenylacetaldehyde is considered responsible for "honey" notes (Cho *et al.* 2006), with *S. luteus* being the species presenting the highest levels of this compound. *B. edulis* was the richest specie in methional. This compound has a very low olfactive perception limit and its descriptor is "boiled potato" (Soares da Costa *et al.* 2004). The panel for these mushroom species did not use this descriptor; however, this species was described with notes of "farm-feed". A very high correlation between methional and "farm-feed" descriptor has been found, and the presence of this compound could explain its aroma characteristics (Soares da Costa *et al.* 2004; Cho *et al*. 2006). Among the identified ketones, two different groups emerged: one was constituted by 3-octanone and 1-octen-3-one, while the other one was composed by volatile norisoprenoids, such as β-ionone, 6-methyl-5-hepten-2-one, *trans*-geranylcetone and (*E,E*)-far-

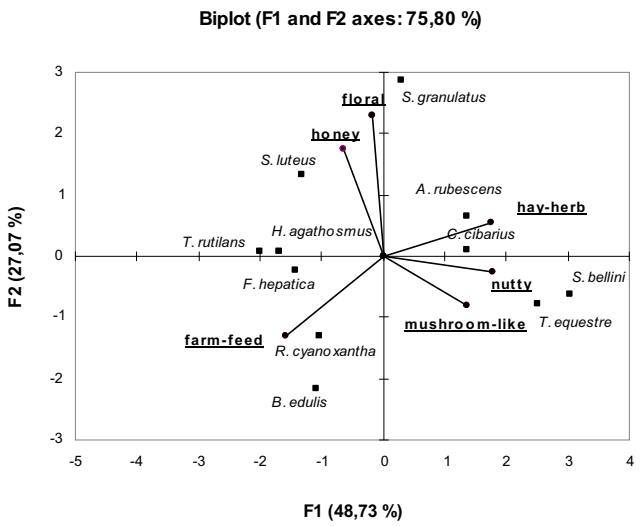

Fig. 6 Projection of sensory variables ("floral", "honey-like", "farm-feed" "mushroom-like", "nutty" and "hay-herb") and observation – mushroom samples into the plan composed by the two principle axes F1 and F2. The two planes contain 75.8% of the total variance. Reprinted from **Guedes de Pinho P, Ribeiro B, Gonçalves RF, Baptista P, Valentão P, Seabra RM, Andrade PB** (2008) Correlation between the pattern volatiles and the overall aroma of wild edible mushrooms. *Journal of Agricultural and Food Chemistry* **56**, 1704-1712, ©2008, with kind permission of the American Chemical Society.

nesylacetone. These four compounds had never been identified in mushrooms before this work. *trans*-Geranylcetone and (*E,E*)-farnesylacetone were present in higher levels in *S. bellini*, *S. granulatus* and *S. luteus* mushroom species and it is possible that these compounds constitute markers of this mushroom genus. *A. rubescens* was the species that presented the highest contents of 3-octanone, while *C. cibarius* contained the highest amount of 1-octen-3-one. *S. bellini*, *S. granulatus* and *S. luteus*, were the richest species in norisoprenoid compounds. Several terpene compounds have been identified in fresh wild mushrooms before (Breheret and Talou 1997), however *trans*-nerolidol, eucalyptol, menthol and 1,4-cineole have not been found before in mushroom species.

Using HCA, the eleven studied species were divided in three groups: group 1 was composed by *S. bellini*, *A. rubescens*, *T. equestre* and *C. cibarius*; group 2 comprised *T. rutilans*, *H. agathosmus* and *B. edulis*; and group 3 included *S. luteus*, *S. granulatus*, *R. cyanoxantha* and *F. hepatica* (**Fig. 7**).

Catharanthus roseus

Catharanthus roseus (L.) G. Don is commonly known as the Madagascar periwinkle. This plant is characterized by the presence of several compounds with important pharmacological activities, namely indolic alkaloids (van der Heijden *et al.* 2004; Sottomayor and Barceló 2005). The water extracts and leaves from *C. roseus* are traditionally used in folk medicine for preventing some diseases and suppress the sensation of hunger and fatigue (Ross 2003; van der Heijden *et al.* 2004).

In our work (Guedes de Pinho *et al.* 2009), 88 compounds were identified in flowers, leaves and stems, by HS-SPME and by dichloromethane extraction, combined with GC-MS. These two methodologies were used concerning the determination of the total amount of volatile and semi-volatile, and other non-volatile compounds, respectively. HS-SPME was applied to fresh plant (to identify the most volatile compounds) and to lyophilized extract (to determine the less volatile compounds). The dichloromethane extraction was performed to characterize the non-volatile compounds.

The most effective fibres for HS-SPME were those characterized by two components: a liquid (PDMS) for the less polar compounds and a solid (DVB, CAR or both) polymeric coating for the more polar constituents. In this work three fibres were evaluated with the following phases: CAR/PDMS, CW/DVB and DVB/PDMS, with the last one having the particularity of being selective to nitrogen-containing compounds.

Using the CAR/PDMS fibre, 25 compounds were identified: *n*-hexanal, 2-hexen-1-ol, *cis*-3-hexen-ol, hexanol, benzaldehyde, 1,4-cineole, limonene, benzylic alcohol, eucalyptol, 3-methoxy-2,5-dimethylpyrazine, phenylacetaldehyde, linalool, 2-nonen-1-ol, phenylethanol, camphor, isobutylmethoxypyrazine, *trans*-2-decenol, verbenone, *β*-cyclocitral, bornilacetate, eugenol, valencene, *β*-ionone, epoxy-*β*-ionone and *(E)*-geranylacetone. When the CW/DVB fibre was applied, some diterpenic compounds, such as manool and their oxides and *α*-bisabolol were detected, in addition to the above-mentioned compounds. Finally, using the DVB/PDMS, 10 aldheydes, 14 alcohols, 8 esters, 10 nitrogen-bearing compounds, 12 terpenes, 8 carotenoid

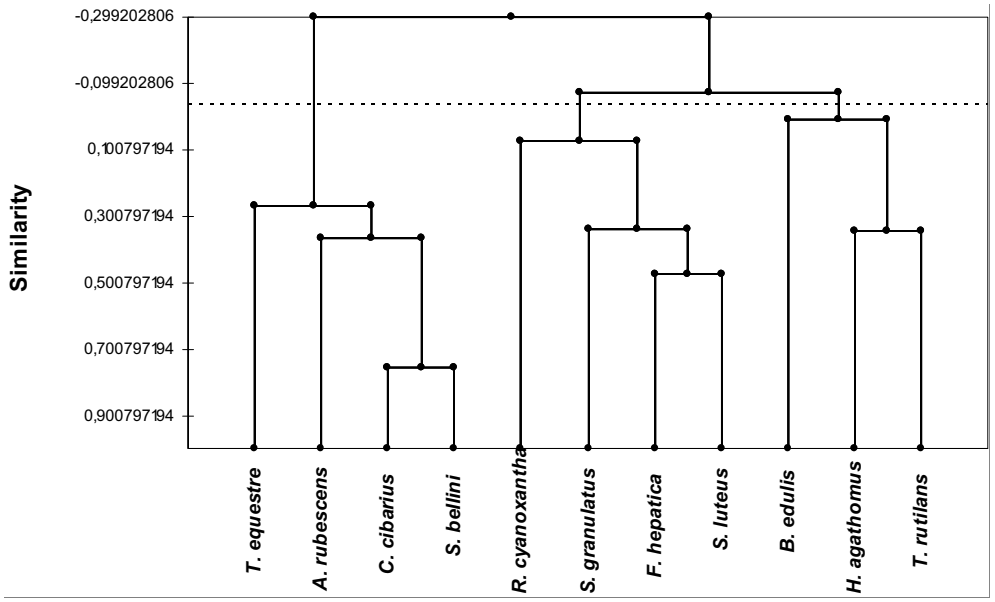

Fig. 7 Dendogram of edible mushroom species with volatile compounds contents and sensory analysis values. Reprinted from **Guedes de Pinho P, Ribeiro B, Gonçalves RF, Baptista P, Valentão P, Seabra RM, Andrade PB** (2008) Correlation between the pattern volatiles and the overall aroma of wild edible mushrooms. *Journal of Agricultural and Food Chemistry* **56**, 1704-1712, ©2008, with kind permission of the American Chemical Society.

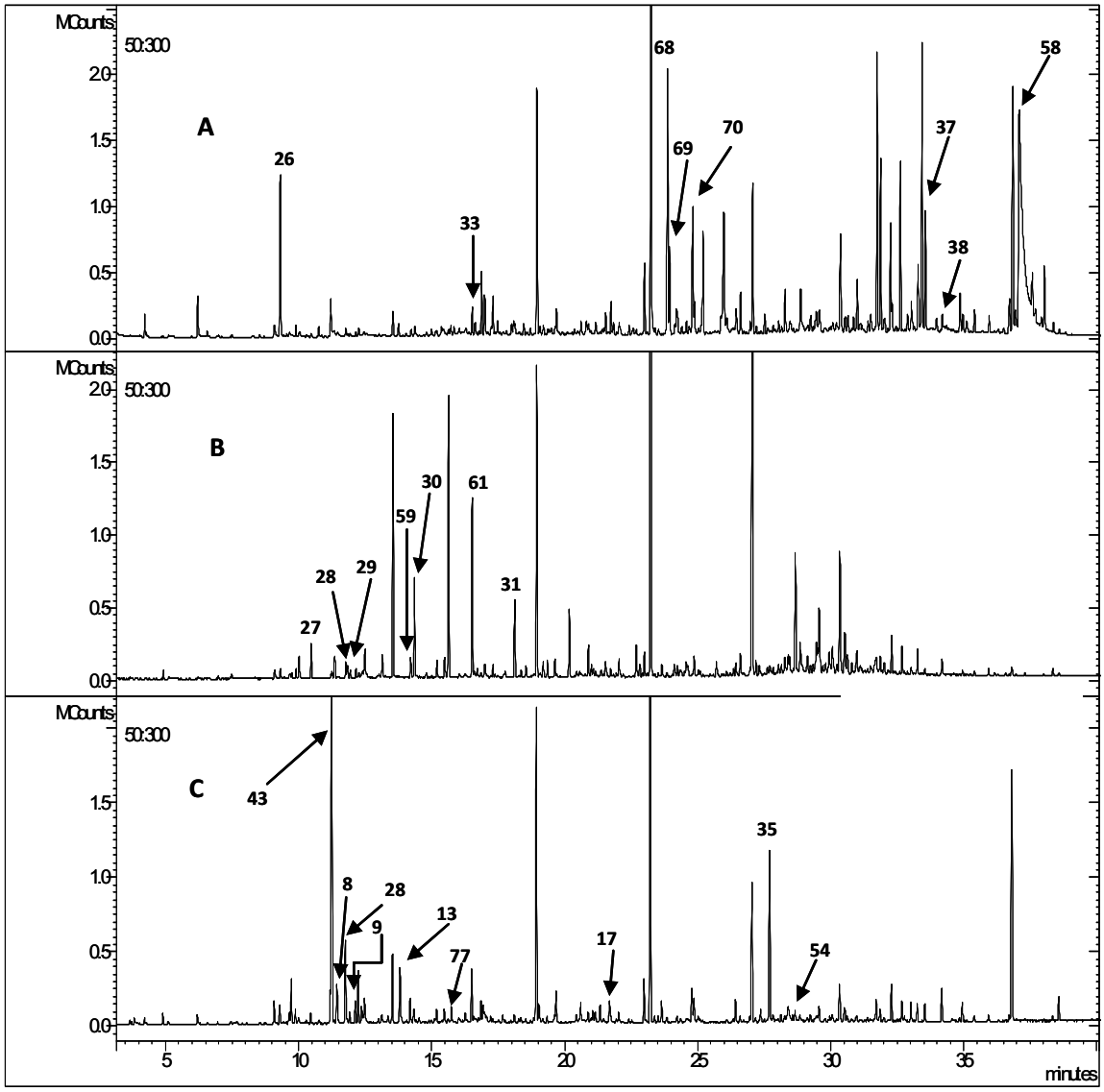

Fig. 8 Chromatograms of the SPME using DVB/PDMS fibre analysis in leaves (A), stems (B) and flowers (C) of *C. roseus.* Identity of compounds: 8 – benzyl alcohol, 9 – 1-phenylethanol, 13 – 2-phenylethanol, 17 – (*Z*)-jasmone, 26 – benzaldehyde, 27 – octanal, 28 – phenylacetaldehyde, 29 – (*E*)-2-octenal, 30 – (*E*)-2-nonenal, 31 – (*E*)-2-decenal, 33 – ethylhexanoate, 35 – methyljasmonate, 37 – palmitic acid methyl ester, 28 – palmitic acid ethyl ester, 43 – limonene, 54 – α-bisabolol, 58 – (*E*)-phytol, 59 – (*Z,E*)-2,6-nonadienal, 61 –(*Z,E*)-2,4-decadienal, 68 – β-ionone, 69 – 2,3-epoxy-α-ionone, 70 – dihydroactinolide, 77 – 2-isobutyl-3-methoxypyrazine. Chromatographic conditions: Oven temperature - 40 °C (for 1 min), 2 °C/min to 220 °C and held for 30 min. Injector port was heated to 220 °C, in splitless mode. Carrier gas - Helium C-60, at 1 mL/min, constant flow. Chromatographic column - VF-5ms 30m x 0.25mm x 0.25µm (FactorFour) from VARIAN. Reprinted from **Guedes de Pinho P, Gonçalves RF, Valentão P, Pereira DM, Seabra RM, Andrade PB, Sottomayor M** (2009) Volatile composition of *Catharanthus roseus* (L.) G. Don using solid-phase microextraction and gas chromatography/mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **49**, 674-685, ©2009, with kind permission of Elsevier.

derivatives, 4 ketones plus 3 other compounds were identified for the first time in fresh flowers, stems and leaves of *C. roseus*. In general, SPME allowed the determination of 12 aldehydes, 14 alcohols, 9 esters, 10 nitrogen containing compounds, 17 terpenic compounds (including aliphatic mono and diterpenes), 12 carotenoid derivatives, 6 ketones, 1 hydroxycinnamic acid and 3 phenol compounds (**Fig. 8**).

Dichloromethane extraction allowed the identification of 14 other compounds. Among these compounds, some structures like alkaloid molecules could be identified, as well as 2,2,7,7-tetramethyltricyclo(6.2.1.0(1,6)undec-4-en-3-one, *α*-farnesene, phytol, (3,7,11,15-tetranethyl-2-hexadecen-1-ol). In this way, the SPME showed to be a more effective technique.

Moreover, statistical analysis (principal component analysis) allowed the distinction of the different organs of the plant (leaves, stems and flowers) in what concerns their volatile composition. Succinctly, flowers were richer in terpene molecules (including limonene), aldehyde compounds, esters compounds, namely methyljasmonate, and phenols (due to the high amounts of eugenol). Leaves were correlated to carotenoid derivative compounds, sum of ketones and ester compounds. Finally, stems were in good correlation with nitrogen containing compounds, alcohols and miscellaneous compounds (**Fig. 9**).

A deeper knowledge of *C. roseus* volatile constituents was achieved by the use of HS-SPME fibre. Some of the compounds identified have important bioactivities in the human body.

Brassica oleracea L.

Plants from the Brassicaceae family play a major role in worldwide vegetable production and consumption, ranking second after Solanaceae. *Brassica* vegetables, including all cabbage-like ones, are consumed in great quantities all over the world. Concerning the determination of volatiles, several studies have reported the volatile composition of different species of *Brassica*, namely *Brassica rapa* L. var. *perviridis* Bailey (Miyazawa *et al.* 2005) *and Brassica oleracea* L. var. *botrytis* L. (Valette *et al.* 2003). These two previous works used hydrodistillation and organic solvents to

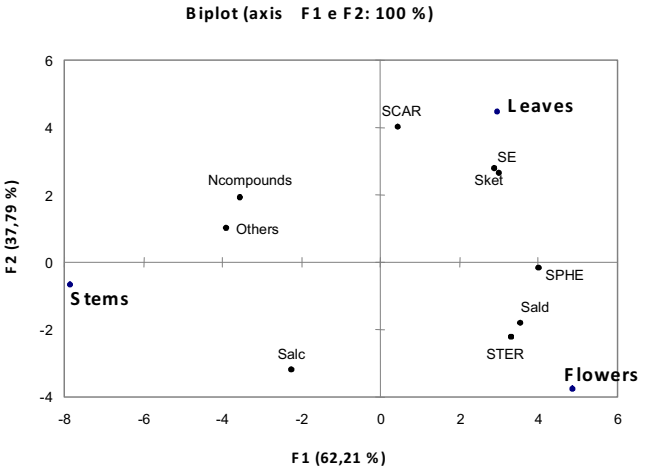

Fig. 9. **Principal component analysis of all volatile compounds analysed by HS-SPME-GC-MS grouped by family classes in flowers, stems and leaves of *C. roseus*.** Abbreviations: SCAR, sum of carotenoid molecules; Sket, Sum of ketones; SPHE, sum of phenols; SE, Sum of esters compounds; Salc, Sum of alcohols; Sald, Sum of aldehydes; STER, Sum of terpenes; Ncompounds, nitrogen-containing compounds. Reprinted from **Guedes de Pinho P, Gonçalves RF, Valentão P, Pereira DM, Seabra RM, Andrade PB, Sottomayor M** (2009) Volatile composition of *Catharanthus roseus* (L.) G. Don using solid-phase microextraction and gas chromatography/mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **49**, 674-685, ©2009, with kind permission of Elsevier.

extract volatiles and had screened the leaves for this kind of compounds. The aim of our work (de Pinho *et al.* 2009) was to determine the volatile profile of internal and external leaves of tronchuda cabbage grown under different fertilization regimens, using HS-SPME combined with GC-MS-IT. The DVB/PDMS fiber was selected for the analyzes of all samples, as it revealed to be the best and more selective fiber for the identification of sulfur compounds, the breakdown products of glucosinolates, considered as important chemical classes for kale characterization. Hence, 34 volatile and non-volatiles components were formally identified and 38 others were tentatively identified. Qualitative and quantitative differences were noticed between internal and external leaves. In general, internal and external leaves exhibited aldehydes, sulphur-bearing volatile compounds, ketones, terpenes and norisoprenoids, and it was possible to denote interesting differences between the leaves.

A parallel study was performed in order to evaluate the variation of volatiles along the seedling development of *Brassica oleracea* L. var. *acephala* (kale). In this work, 66 volatile compounds, distributed by several chemical classes were determined: alcohols, carbonyl compounds (ketones, aldehydes and esters), norisoprenoids, terpenes, sulphur-bearing compounds, among others (Fernandes *et al.* 2009). This is the first work on the volatiles profiling of kale during seeds sprouting, as well as on the leaves of mature plant. According to the results obtained, it may be anticipated that, in agreement with the state of development of the plant, it is possible to find different chemical classes of volatiles.

These results suggest that *Brassica* spp., namely its seeds and sprouts, can have a large potential for biological activity, which include both negative (outright toxicity) and positive (chemoprotective effect against certain cancers) nutritional attributes.

Rumex induratus

Rumex induratus is an endemic Iberic herb, growing spontaneously in Northeast Portugal, where leaves are highly appreciated and consumed, specially in salads. Despite the high consumption, its phytochemical characterization is quite scarce. Previous works were performed concerning organic acids and phenolic composition (Ferreres *et al.*

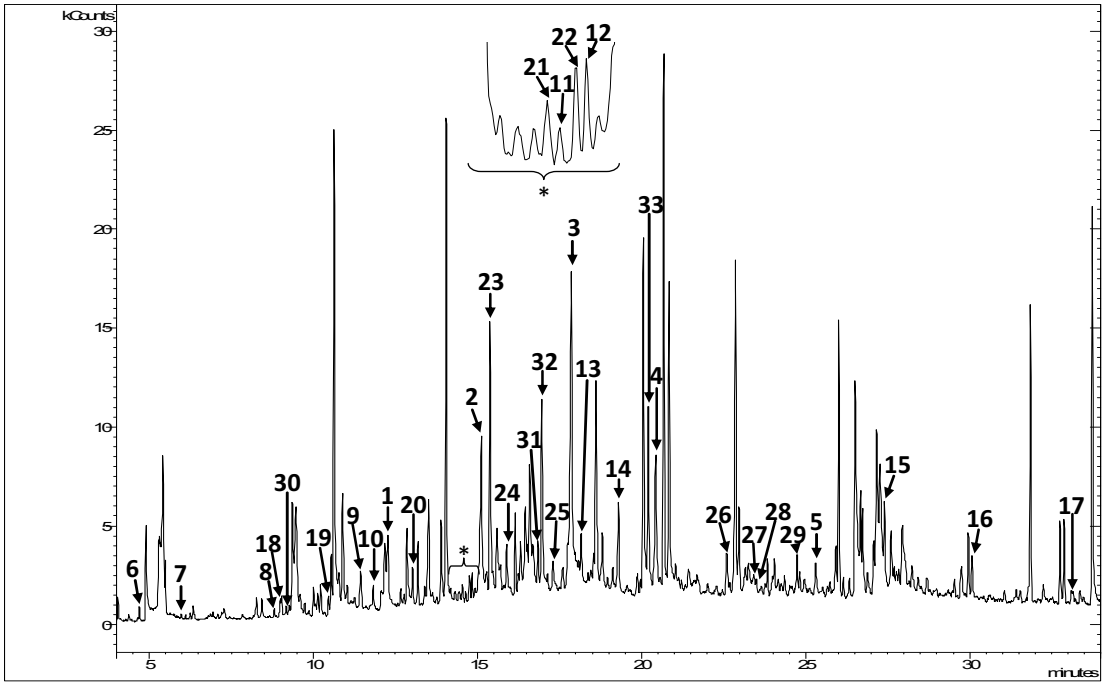

Fig. 10 Chromatogram in FullScan mode of HS-SPME combined with GC/ MS, using Divinylbenzene/PDMS fibre, of the brown algae *F. spiralis*. Identity of compounds: 1 – heptanoic acid, 2 – octanoic acid, 3 – nonanoic acid, 4 – undecanoic acid, 5 – dodecanoic acid, 6 – (*Z*)-4-hexenol, 7 – (*E*)-2-hexenal, 8 – (*E*)-2-heptenal, 9 – phenylacetaldehyde, 10 – (*E*)-2-octenal, 11 – (*E,Z*) – 2,6-nonadienal, 12 – (*E*)-2-nonenal, 13 – (*E*)-cinnamaldehyde, 14 – (*E,E*)-2,4-decadienal, 15 – methyl dihydrojasmonate, 16 – benzyl benzoate, 17 – methyl palmitate, 18 – benzoyl bromide, 19 – 4-bromo-1-cyclohexene, 20 – linalool, 21 – (+)-camphor, 22 – menthone, 23 – menthol, 24 – α-terpineol, 25 – carvone, 26 – geranyl acetone, 27 – β-ionone, 28 – 5,6-epoxy-β-ionone, 29 – dihydroactinidiolide, 30 – dimethyl trisulfide, 31 – benzothiazole, 32 – methylethylmaleimide, 33 – eugenol. Chromatographic conditions: Oven temperature - 40°C (for 1 min), 2°C/min to 220°C and held for 30 min. Injector port was heated to 220°C, in splitless mode. Carrier gas - Helium C-60, at 1 mL/min, constant flow. Chromatographic column - VF-5 ms 30 m × 0.25 mm × 0.25 μm (FactorFour) from VARIAN.

2006; Guerra *et al.* 2008).

To establish the volatile composition of *R. induratus* leaves different extractive methods (hydrodistillation, SPME, Soxhlet and solvent extraction) were performed, followed by GC-MS-IT analysis (Taveira *et al.* 2009). Incidentally, it was possible to identify 81 volatile compounds, distributed by several chemical classes: esters, terpenes, aldehydes, acids, norisoprenoids, ketones, naphtalene derivatives, steroids derivatives, alcohols, among others. The liquid solvent allowed the extraction of molecules with high molecular weights, while HS-SPME with a DVB/PDMS fibre, extracted the most volatile compounds.

Algae

Marine algae have been regarded as an attractive source of pharmaceutical agents in several biochemical and pharmacological investigations (Kamenarska *et al*. 2009). *Fucus spiralis* L. is an intertidal marine seaweed that lives in a harsh environment, where it is subjected to repeated immersion and emersion due to tidal fluctuations (Dring 2006). As result, it is exposed to a wide range of environmental stress, having, therefore, a protective antioxidant defense system (Dring 2006). In fact, powerful antioxidant molecules (phlorotannins, ascorbic acid, tocopherols, carotenoids, phospholipids, chlorophyll related compounds, bromophenols, catechins, mycosporine-like amino-acids, among others), similar to those of vascular plants, are reported in algae (Dring 2006).

Volatiles play important roles in chemical communication in marine ecosystems, acting as pheromones or allelochemicals, chemical defenses such as deterrence against predators, inhibition of bacterial and fungal fouling, and suppression of competing neighbours (Akakabe and Kajiwara 2008).

The determination of the volatiles in the aqueous extract of *F. spiralis*, was achieved by GC-MS-IT, using a DVB/PDMS fibre.

Thirty-three compounds were identified (**Fig. 10, Table 2**) and, as far as we know, with the exception of geranyl acetone (**26**) (Sartin *et al*. 2001), all of them are described for the first time in *F. spiralis*. These compounds belong to different classes: five acids (**1-5**), one alcohol (**6**), eight aldehydes (**7-14**), three esters (**15-17**), two halogenated compounds (**18-19**), six monoterpenes (**20-25**), four norisoprenoid derivatives (**26-29**), two sulphated compounds (**30-31**) and two other volatiles (**32-33**). Acids were predominant (*ca*. 47.6%), with nonanoic acid (**3**) being the main compound (*ca*. 21.5%) (**Table 2**) (unpublished data).

In parallel with chemical analysis, studies regarding biological activity of this and other species are required, as oceans remain as one of the most promising sources for bioactive molecules in the nearer future.

In vivo analysis

Being GC-MS an increasingly powerful and popular analytical approach, its application has been extended to other areas, such as chemical ecology. Volatiles play a very important role in the relations between organisms of different trophic levels, such as insects and plants. As so, GC-MS has been used in the study of the influence of herbivory upon host plants, which responds by synthesizing and releasing a set of compounds with the objective of warning neighbour plants of the presence of a predator or attraction of insect parasitoids. The work of Vercammen *et al.* (2001) is a good example of a versatile GC-MS system that was used for the *in vivo* monitorization of plant responses when challenged by biotic and abiotic stress. In this work, feeding of the cotton leafworm (*Spodoptera litorallis*) on ivy (*Hedera helix*) and tomato plants (*Lycopersicon esculentum* Mill) was used as a model of insect plant interactions (per. obs.). In the near future, the number of studies applying GC-MS to living organisms is expected to rise.

Table 2 Average content (in percentage) of volatile compounds identified in lyophilized aqueous extract of *F. spiralis*.

Number	Compound	RT[a] (min)	QI[b] (*m/z*)	RA[c] (%) *F. spiralis*
Acids				
1	Heptanoic acid[d]	12.276	60/73/87	6.015
2	Octanoic acid[d]	15.122	60/73/85	14.765
3	Nonanoic acid[d]	17.863	60/73	21.508
4	Undecanoic acid[d]	20.438	60/73/87	4.371
5	Dodecanoic acid[d]	25.294	60/73/85	0.966
Alcohols				
6	(*Z*)-4-Hexenol[d]	4.695	55/67/82	0.538
Aldehydes				
7	(*E*)-2-Hexenal[d,e]	5.960	55/69/83	0.220
8	(*E*)-2-Heptenal[d]	8.795	57/70/83	0.470
9	Phenylacetaldehyde[d,e]	11.411	91	3.990
10	(*E*)-2-Octenal[d,e]	11.807	70/93	0.543
11	(*E,Z*)-2,6-Nonadienal[d,e]	14.616	41/67/70	0.688
12	(E)-2-Nonenal[d,e]	14.824	55/70/93	0.918
13	(*E*)-Cinnamaldehyde[d]	18.129	77/103/131	4.488
14	(*E,E)*-2,4-Decadienal[d,e]	19.286	81/152	4.264
Esters				
15	Methyl dihydrojasmonate[d,e]	27.397	83/93/151	3.254
16	Benzyl benzoate[d]	30.068	77/91/105	2.847
17	Methyl palmitate[d]	33.102	87/143/270	0.386
Halogenated compounds				
18	Benzoyl bromide[d]	8.983	77/195	1.268
19	4-Bromo-1-cyclohexene[d]	10.431	81/93	3.272
Monoterpenes				
20	Linalool[d,e]	13.016	93/121	0.869
21	(+)-Camphor[d]	14.532	81/95/108	0.739
22	Menthone[d]	14.741	112/139/154	0.322
23	Menthol[d]	15.387	81/95/123	8.251
24	α-Terpineol[d,e]	15.888	93/121/136	1.220
25	Carvone[d]	17.285	82/108/150	0.906
Norisoprenoid derivatives				
26	Geranyl acetone[d,e]	22.581	107	0.274
27	β-Ionone[d,e]	23.426	177	0.523
28	5,6-Epoxy-β-ionone[d]	23.510	123/177	0.446
29	Dihydroactinidiolide[d]	24.740	111/137/180	1.287
Sulphated compounds				
30	Dimethyl trissulfide[d,e]	9.170	45/79/126	0.566
31	Benzothiazole[d]	16.826	69/108/135	2.944
Others				
32	Methylethylmaleimide[d]	16.951	96/124/139	4.180
33	Eugenol[d,e]	20.193	164	2.763
Identified compounds				33
Acids				5 (47.625)
Alcohols				1 (0.538)
Aldehydes				8 (15.581)
Esters				3 (6.487)
Halogenated compounds				2 (4.540)
Monoterpenes				6 (12.307)
Norisoprenoid derivatives				4 (2.530)
Sulphated compounds				2 (3.510)
Others				2 (6.943)

[a]RT = retention time. [b] QI = quantification ions. [c] RA (%) = relative area in percentage. [d] MS = tentatively identified by NIST05. [e] identified by comparison with reference compound.

CONCLUSION

The study of the volatile composition of natural products is important due to the organoleptic characteristics and bioactivity that these compounds can exhibit. Flavours and scents are directly tied to food preference and palatability and while some volatile metabolites emitted from plants or fungi are pleasant to mammals or insects, other compounds are mainly unpleasant.

The research on these flavour components from the food and beverage, as well as perfumery and cosmetics industries, draws closer our understanding about why and how these compounds are metabolized, leading to several discoveries about the biological activities of some volatiles.

In fact, some of them have protective effect against different kinds of cancer (Singletary 2000), while others play important roles in reproduction and metabolic regulation (Liechti and Farmer 2002). Essential oils, that contain a variety of volatile molecules, are widely used for antiseptic and insecticidal applications (Bakkali *et al*. 2008). Due to their properties, these metabolites earned increasing attention in pharmaceutics, therapeutics or plague control researches.

The recent adoption of simple and sensitive methods using headspace sampling coupled with GC-MS, allowed a substantial increase in the number of identified volatile compounds in the last two decades. The main advantages of HS-SPME, in comparison to other classical extraction techniques, are simplicity, elimination of several steps, non-usage of solvents, high sensitivity, requirement of small amounts of sample and low cost. This technique applied to GC-MS has proved to be an important tool to researchers, as a general volatile profile of the matrix in study can be rapidly obtained. For the reasons described above, HS-SPME has become the first choice in quality control laboratories.

REFERENCES

Akakabe Y, Matsui K, Kajiwara T (2005) Stereochemical correlation between 10-hydroperoxyoctadecadienoic acid and 1-octen-3-ol in *Lentinula edodes* and *Tricholoma matsutake* mushrooms. *Bioscience, Biotechnology, and Biochemistry* **69**, 1539-1544

Akakabe Y, Kajiwara T (2008) Bioactive volatile compounds from marine algae: feeding attractants. *Journal of Applied Phycology* **20**, 661-664

Alpendurada MF (2000) Solid-phase microextraction: a promising technique for sample preparation in environmental analysis. *Journal of Chromatography A* **889**, 3-14

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effect of essential oils. *Food Chemical Toxicology* **46**, 446-475

Barker RL, Gracey DE, Irwin AJ, Pipasts P, Leiska E (1983) Liberation of staling aldehydes during storage of beer. *Journal of the Institute of Brewing. Institute of Brewing (Great Britain)* **89**, 411-415

Beck HC, Hansen AM, Lauritsen FR (2003) Novel pyrazine metabolites found in polymyxin biosynthesis by *Paenibacillus polymyxa*. *FEMS Microbiology Letters* **220**, 67-73

Boatright J, Negre F, Chen X, Kish CM, Wood B, Peel G, Orlova I, Gang D, Rhodes D, Dudareva N (2004) Understanding *in vivo* benzenoid metabolism in petunia petal tissue. *Plant Physiology* **135**, 1993-2011

Breheret S, Talou T (1997) Monoterpenes in the aromas of fresh wild mushrooms (Basidiomycetes). *Journal of Agricultural and Food Chemistry* **45**, 831-836

Chauser-Volfson E, Gutterman Y (1997) Content and distribution of the secondary phenolic compound homonatalion in *Aloe hereroensis* leaves according to leaf part, position and monthly changes. *Journal of Arid Environments* **37**, 115-122

Cho IH, Kim S-Y, Choi, H-K, Kim Y-S (2006) Characterization of aroma-active compounds in raw and cooked pine-mushrooms (*Tricholoma matsutake* Sing.). *Journal of Agricultural and Food Chemistry* **54**, 6332-6335

Cho IH, Namgung H-K, Kim Y-S (2007) Volatiles and key odorants in the pileus and stipe of pine-mushroom (*Tricholoma matsutake* Sing.). *Food Chemistry* **106**, 71-76

Dring MJ (2006) Stress resistance and disease resistance in seaweeds: the role of reactive oxygen metabolism. In: Callow JA (Ed) *Advances in Botanical Research*, Academic Press, United Kingdom, pp 175-207

Dudareva N, Pichersky E, Gershenzon J (2004) Biochemistry of plant volatiles. *Plant Physiology* **135**, 1893-1902

Edris AE (2007) Pharmaceutical and therapeutic potencials of essential oils and their individual volatile constituents: a review. *Phytotherapy Research* **21**, 308-323

Eisenreich W, Schwarz M, Cartayrade A, Arigoni D, Zenk MH, Bacher A (1998) The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. *Chemistry and Biology* **5**, 221-233

Fernandes F, Guedes de Pinho P, Valentão P, Pereira JA, Andrade PB (2009) Volatile Constituents throughout *Brassica oleracea* L. var. *acephala* germination. *Journal of Agricultural and Food Chemistry* **57**, 6795-6802

Ferreres F, Pereira DM, Valentão P, Andrade PB, Seabra RM, Sottomayor M (2008) New phenolic compounds and antioxidant potential of *Catharanthus roseus*. *Journal of Agricultural and Food Chemistry* **56**, 9967-9974

Ferreres F, Ribeiro V, Izquierdo AG, Rodriguez MA, Seabra RM, Andrade PB, Valentão P (2006) *Rumex induratus* leaves: interesting dietary source of potential bioactive compounds. *Journal of Agricultural and Food Chemistry* **54**, 5782-5789

Fujimura T, Kaway T, Kajiwara T, Ishida Y (1994) Volatile components in protoplasts isolated from the marine brown alga *Dictyopteris prolifera* (Dictyotales). *Plant Tissue Culture Letters* **11**, 34-39

Goepfert S, Poirier Y (2007) β-Oxidation in fatty acid degradation and beyond. *Current Opinion in Plant Biology* **10**, 245-251

Goff SA, Klee HJ (2006) Plant volatile compounds: sensory cues for health and nutritional value? *Science* **311**, 815-819

Guedes de Pinho P, Gonçalves RF, Valentão P, Pereira DM, Seabra RM, Andrade PB, Sottomayor M (2009) Volatile composition of *Catharanthus roseus* (L.) G. Don using solid-phase microextraction and gas chromatography/mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **49**, 674-685

Guedes de Pinho P, Ribeiro B, Gonçalves RF, Baptista P, Valentão P, Seabra RM, Andrade PB (2008) Correlation between the pattern volatiles and the overall aroma of wild edible mushrooms. *Journal of Agricultural and Food Chemistry* **56**, 1704-1712

Guedes de Pinho G, Valentão P, Gonçalves RF, Sousa C, Andrade PB (2009) Volatile composition of *Brassica oleracea* L. var. *costata* DC leaves using solid-phase microextraction and gas chromatography/ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry* **23**, 2292-2300

Guenther E (1948) *The Essential Oils* (Vol 1), D. Van Nostrand Company, New York, pp 111-122

Guerra L, Pereira C, Andrade PB, Rodrigues MA, Ferreres F, Guedes de Pinho P, Seabra RM, Valentão P (2008) Targeted metabolite analysis and antioxidant potential of *Rumex induratus*. *Journal of Agricultural and Food Chemistry* **56**, 8184-8194

Habel A, Boland W (2008) Efficient and flexible synthesis of chiral γ- and δ-lactones. *Organic and Biomolecular Chemistry* **6**, 1601-1604

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology* **57**, 303-333

Hamberg M, Sanz A, Castresana C (1999) α-Oxidation of fatty acids in higher plants. *The Journal of Biological Chemistry* **274**, 24503-24513

Harborne JB (1997) Recent advances in chemical ecology. *Natural Product Reports* **16**, 509-523

Josephson DB, Lindsay RC, Stuiber DA (2006) Volatile compounds characterizing the aroma of fresh Atlantic and Pacific oysters. *Journal of Food Science* **50**, 5-9

Kamenarska Z, Serkedjieva J, Najdenski H, Stefanov K, Tsvetkova I, Dimitrova-Konaklieva S, Popov S (2009) Antibacterial, antiviral, and cytotoxic activities of some red and brown seaweeds from the Black Sea. *Botanica Marina* **52**, 80-86

Karasek FW, Clement RE (1991) Basic gas chromatography - mass spectrometry: principles and techniques (2nd Ed), Elsevier, Netherlands, total pp

Kataoka H, Lord HL, Pawliszyn J (2000) Applications of solid-phase microextraction in food analysis. *Journal of Chromatography A* **880**, 35-62

Kloer DP, Schulz GE (2006) Structural and biological aspects of carotenoid cleavage. *Cellular and Molecular Life Sciences* **63**, 2291-303

Lavagnini I, Magno F, Seraglia R, Traldi P (Eds) (2006) *Quantitative Applications of Mass Spectrometry*. John Wiley & Sons Ltd, England, pp 2-53

Liechti R, Farmer EE (2002) The jasmonate pathway. *Science* **31**, 1649-1650

Luque de Castro MD, García-Ayuso LE (1998) Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. *Analytica Chimica Acta* **369**, 1-10

MacMaster MC (2008) *GC-MS: A Practical User Guide Authors* (2nd Edn), John Wiley & Sons Ltd., England, total pp

Marriott PJ, Shellie R, Cornwell C (2001) Gas chromatographic technologies for the analysis of essential oils. *Journal of Chromatography A* **936**, 1-22

Masqueè N, Marceè RM, Borrull F (1998) New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from environmental water. *Trends in Analytical Chemistry* **17**, 384-394

Miyazawa M, Nishiguchi T, Yamafugi C (2005) Volatile components of the leaves of *Brassica rapa* L. var. *perviridis* Bailey. *Flavour and Fragrance Journal* **20**, 158-160

Ortega-Heras M, González-SanJosé ML, Beltrán S (2002) Aroma composition of wine studied by different extraction methods. *Analytica Chimica Acta* **458**, 85-93

Pawliszyn J (1997) *Solid Phase Microextraction: Theory and Practice*, Wiley, New York, total pp

Pichersky E, Noel JP, Dudareva N (2006) Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science* **311**, 808-811

Pourmortazavi SM, Hajimirsadeghi SS (2007) Supercritical fluid extraction in plant essential and volatile oil analysis. *Journal of Chromatography A* **1163**, 2-24

Prosen H, Zupancic-Kralj L (1999) Solid-phase microextraction. *Trends in Analytical Chemistry* **18**, 272-282

Rodríguez-Bustamante E, Sánchez S (2007) Microbial production of C_{13}-Norisoprenoids and other aroma compounds via carotenoid cleavage. *Critical Reviews in Microbiology* **33**, 211-230

Rohdich F, Eisenreich W, Wungsintaweekul J, Hecht S, Schuhr CA, Bacher A (2001) Biosynthesis of terpenoids. *European Journal of Biochemistry* **268**, 3190-3197

Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochemical Journal* **15**, 17-524

Ross IA (2003) *Catharanthus roseus*. In: Ross IA (Ed) *Medicinal Plants of the World* (Vol I, 2nd Edn) Humana Press, New Jersey, USA, pp 175-196

Sartin JH, Halsall CJ, Davison B, Owen S, Hewitt CN (2001) Determination of biogenic volatile organic compounds (C_8-C_{16}) in the coastal atmosphere at Mace Head, Ireland. *Analytica Chimica Acta* **428**, 61-72

Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Biosynthesis of plant-derived flavor compounds. *The Plant Journal* **54**, 712-732

Silva Ferreira AC, Guedes de Pinho P (2003) Analytical method for determination of some aroma compounds on white wines by solid phase microextraction and gas chromatography. *Journal of Food Science* **68**, 2817-2820

Silva LV, Nelson DL, Drummond MFB, Dufossé L, Glória MBA (2005) Comparation of hydrodistillation methods for the deodorization of turmeric. *Food Research International* **38**, 1087-1096

Singhara A, Macku C, Shibamoto T (1998) Antioxidative activity of brewed coffee extracts. In: Shibamoto T, Terao J, Osawa T (Ed) *Functional Foods for Disease Prevention II: Medicinal Plants and other Foods*, ACS Symposium Series 701, American Chemical Society, Washington, DC, pp 101-109

Singletary K (2000) Diet, natural products and cancer chemoprevention. *The Journal of Nutrition* **130**, 465-466

Soares da Costa M, Gonçalves C, Ferreira A, Ibsen C, Guedes de Pinho P, Silva Ferreira AC (2004) Further insights into the role of methional and phenylacetaldehyde in lager beer flavor stability. *Journal of Agricultural and Food Chemistry* **52**, 7911-7917

Sottomayor M, Ros Barceló A (2005) The *Vinca* alkaloids: From biosynthesis and accumulation in plants cells, to uptake, activity and metabolism in animal cells. In: Atta-ur-Rahman (Ed) *Studies in Natural Products Chemistry (Bioactive Natural Products*), Elsevier Science Publishers, The Netherlands, pp 813-857

Steffen A, Pawliszyn J (1996) Analysis of flavor volatiles using headspace solid-phase microextraction. *Journal of Agricultural and Food Chemistry* **44**, 2187-2193

Taveira M, Guedes de Pinho G, Gonçalves RF, Andrade PB, Valentão P (2009) Determination of eighty-one volatile organic compounds in dietary *Rumex induratus* leaves by GC/IT-MS, using different extractive techniques. *Microchemical Journal* **93**, 67-72

Takagi M, Kuzuyama T, Takahashi S, Seto H (2000) A gene cluster for the mevalonate pathway from *Streptomyces* sp. strain CL190. *Journal of Bacteriology* **182**, 4153-4157

Ulrich S (2000) Solid-phase microextraction in biomedical analysis. *Journal of Chromatography A* **902**, 167-194

Valette L, Fernandez X, Poulain S, Loiseau A–M, Lizzani-Cuvelier L, Levieil R, Restier L (2003) Volatile constituents from Romanesco cauliflower. *Food Chemistry* **80**, 353-358

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: Pharmacognosy and biotechnology. *Current Medicinal Chemistry* **11**, 607-628

van Schie CCN, Haring MA, Schuurink RC (2006) Regulation of terpenoid and benzenoid production in flowers. *Current Opinion in Plant Biology* **9**, 203-208

Vercammen J, Pham-Tuan H, Sandra P (2001) Automated dynamic sampling system for the on-line monitoring of biogenic emissions from living organisms. *Journal of Chromatography A* **930**, 39-51

Vichi S, Riu-Aumatell M, Mora-Pons M, Buxaderas S, Lopéz-Tamames E (2005) Characterization of volatiles in different dry gins. *Journal of Agricultural and Food Chemistry* **53**, 10154-10160

Wang L, Chen Y, Song Y, Chen Y, Liu X (2008a) GC-MS of volatile components of *Schisandra chinensis* obtained by supercritical fluid and conventional extraction. *Journal of Separation Science* **31**, 3238-3245

Wang J, Zhang Q, Zhang Z, Li Z (2008b) Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules* **42**, 127-132

Watson JT, Sparkman OD (2007) *Introduction to Mass Spectrometry: Instrumentation, Applications and Strategies for Data Interpretation* (4rd Edn), John Wiley and Sons Ltd, England, 818 pp

Yan ZG, Wang CZ (2006) Wound-induced green leaf volatiles cause the release of acetylated derivatives and a terpenoid in maize. *Phytochemistry* **67**, 34-42