J Food Sci Technol (January 2016) 53(1):132–144
DOI 10.1007/s13197-015-2022-x

ORIGINAL ARTICLE

A powerful approach to explore the potential of medicinal plants as a natural source of odor and antioxidant compounds

Rosa Perestrelo[1] · **Catarina L. Silva**[1] · **Freddy Rodrigues**[1] · **Michael Caldeira**[1] · **José S. Câmara**[1,2]

Revised: 13 July 2015 / Accepted: 4 September 2015 / Published online: 11 September 2015

Abstract In this study an efficient and reliable method based on dynamic headspace solid-phase microextraction (HS-SPME) followed by gas chromatography–mass spectrometry (GC–qMS), was developed to establish the volatile metabolomic pattern of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Ruta chalepensis* L. medicinal plants. The HS-SPME influencing parameters were investigated and the results indicated that the best extraction capability, was obtained using DVB/CAR/PDMS coating fiber at 40 °C for 45 min. Under optimal conditions, a total of 99 volatile metabolites were identified, including 53 terpenoids, 19 carbonyl compounds, 7 esters, 6 alcohols, among others. The main volatile metabolites identified in *T. vulgaris* include thymol (67 %), 3-octanone (9 %) and 1-octen-3-ol (7 %), while in *R. officinalis* the most dominant volatiles were eucalyptol (40 %), 2-decanone (20 %) and bornyl acetate (10 %). 2-Undecanone (53 %), (*E*)-2-octenal (28 %) and 2-nonanone (10 %) were the most relevant volatile metabolites identified in *R. chalepensis*. The results suggested that the HS-SPME/GC-qMS methodology is a powerful approach to establish the volatile metabolomic fingerprint of medicinal plants and providing a reliable tool for the complete characterization of these biologically active medicinal plants.

✉ José S. Câmara
jsc@uma.pt

[1] CQM/UMa, Centro de Química da Madeira, Campus Universitário da Penteada, 9000-390 Funchal, Portugal

[2] Universidade da Madeira, Centro de Ciências Exatas e de Engenharia da Universidade da Madeira, Campus Universitário da Penteada, 9000-390 Funchal, Portugal

Keywords Medicinal plants · Volatile metabolite · Solid-phase microextraction · GC-qMS · Antioxidant capacity

Introduction

Thymus vulgaris L., *Rosmarinus officinalis L.* (*Lamiaceae*) and *Ruta chalepensis L.* (*Rutaceae*) are important medicinal plants, highly recommended due to a range of biological and therapeutic properties including anti-carcinogenic, anti-inflammatory, antimicrobial, antioxidant, antiseptic, hepatoprotective, anti-HIV, diuretic and expectorant (Dorman and Deans 2000; Ruberto and Baratta 2000). The detailed chemical composition of these medicinal plants is very complex. The most important classes of its volatile compounds are characterized, namely, by monoterpenoids, sesquiterpenoids and their oxygenated derivatives, carbonyl compounds, ketones, aldehydes and higher alcohols (Cao et al. 2006; Guedes de Pinho et al. 2009). The content of volatile metabolites may vary as a function of the origin of samples. In addition to volatile metabolites, another important class of medicinal plant constituents is represented by secondary metabolites such as polyphenols, including flavonoids, phenolic acids and their esters, terpenes alkaloids and others. Their chemical complexity may explain their wide range of biological and pharmacological activities.

The biosynthetic pathways of important plant volatile metabolites have been traced back up to intermediates of primary metabolism. It has been shown that carbohydrates, fatty acids and amino acids, represent the natural carbon pool for volatile metabolites, which can also be released from their polymers (Fig. 1). In plants two biosynthetic pathways are responsible for synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), universal precursors of all terpenoids, which are localized in different subcellular

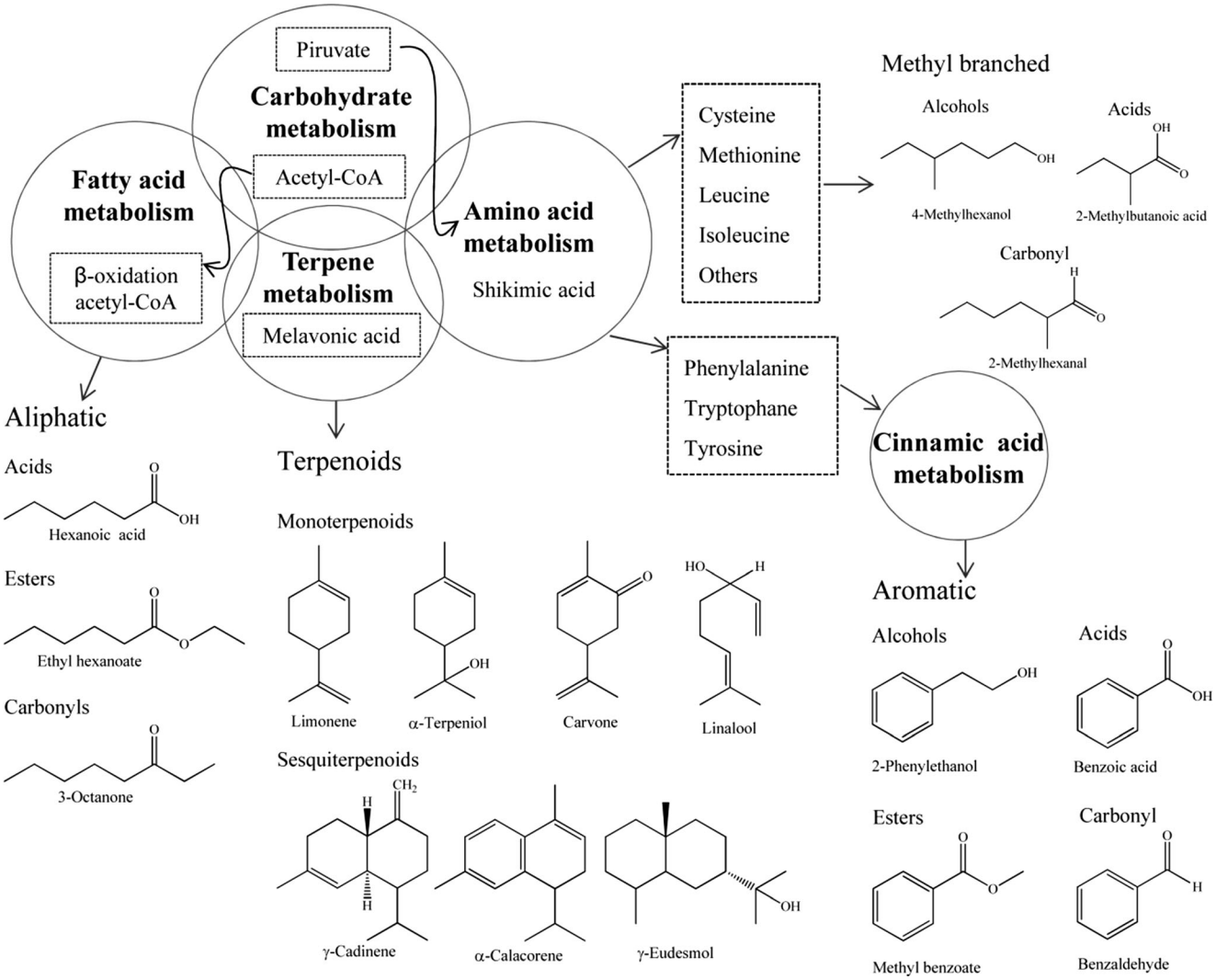

Fig. 1 Schematic of biosynthetic pathways leading to the formation of plant volatile metabolites

compartments. While DMAPP, formed in plastids, is used by isoprene synthase (ISPS) to form isoprene in some plants, IPP and DMAPP precursors are further condensed by prenyl diphosphate synthases in the respective compartments to form prenyl diphosphate intermediates serving as substrates for a large group of terpene synthases (TPS) enzymes, resulting in the final terpenoid compounds (Nagegowda 2010).

For thymol, the pathway formation proceeds from γ-terpinene via the aromatic compound, *p*-cymene, as an intermediate (Fig. 2). γ-Terpinene is most likely converted to thymol by the action of one or more cytochrome P450 oxidases, catalyzing a hydroxylation (Crocoll et al. 2010). α-Terpinyl cation is a key intermediate to form several terpenoids, such as thymol, eucalyptol, limonene, α-pinene, sabinene, among others.

Several researches reported thymol followed by carvacrol, eucalyptol, borneol and α-pinene, as the main volatiles of *T. vulgaris* (thyme, *Lamiaceae*) volatile composition. It has been reported that it possesses numerous biological activities including breaking up congestion, calming bronchial spasms, stimulating respiration, antimicrobial and antioxidant effects (Baranauskienė et al. 2003; Grigore et al. 2010), whereas *R. officinalis* (rosemary, *Lamiaceae*) is widely used in folk medicine in the treatments of variety of diseases such as external stimulant, relaxant for nervousness and muscle spasms (Barnes et al. 2007). Some volatile constituents, namely eucalyptol, camphor, borneol, bornyl acetate and α-pinene, could be responsible for these pharmacological properties. *R. chalepensis* (ruta, *Rutaceae*), is characterized mainly by aliphatic ketones being 2-decanone, 2-undecanone and 2-dodecanone the most dominant volatile constituents. It has been reported that ruta possesses numerous biological activities including emmenagogue, abortifacient, anti-helmintic, and spasmolytic (Mejri et al. 2010). The biosynthesis of methyl ketones has been hypothesized to derive from a variety of biological pathways such as fatty acid β-oxidation or aerobic alkene/alkane degradation. In plants, 3-keto fatty acids could themselves be derived from the hydrolysis of either 3-ketoacyl-

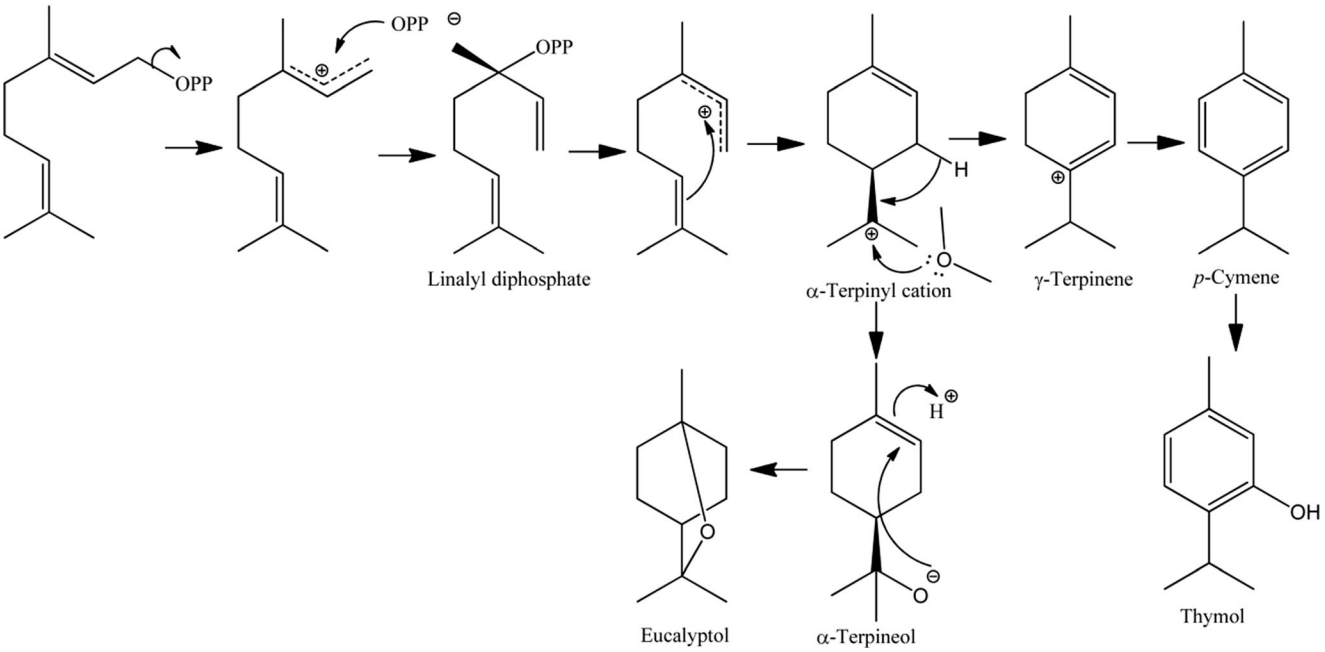

Fig. 2 Eucalyptol and thymol biosynthesis pathways

acyl carrier proteins, intermediates in the fatty acid biosynthetic pathway of chloroplasts, or could be derived from 3-ketoacyl-CoA, which are intermediates in the degradation of fatty acids in the peroxisomes (Fig. 3) (Yu et al. 2010). Decarboxylation of 3-keto fatty acids could thus give rise to straight-chain methyl ketones such as those found in the ruta plants.

Several extraction techniques, including hydrodistillation (Sourmaghi et al. 2014), microwave (Sourmaghi et al. 2014), supercritical fluid extraction (Venkatachallam et al. 2010), soxhlet extraction (Bajerová et al. 2008), and ultrasound-assisted extraction (Ince et al. 2014) have been reported adequate to extract volatile metabolites from natural products. However, it is well-known that these techniques presents some shortcomings such as long extraction time, are laborious procedures needs large volume of organic solvents and amounts of sample and present low extraction efficiency. To overcome these drawbacks, solid-phase microextraction (SPME), emerged as an attractive and simple extraction technique which offers important advantages over conventional solvent extraction procedures as it is rapid, easy-to-use, solvent free, sensitive and does not require any concentration step prior to analysis, preventing artifacts. Combined to gas chromatography (GC) and mass spectrometry analysis (MS) SPME has been successfully applied in a wide range of fields (Cao et al. 2006; Gianelli et al. 2002; Guedes de Pinho et al. 2009; Vázquez-Araújo et al. 2013).

Many of medicinal plants have been investigated through of the world due to their promising health benefits namely

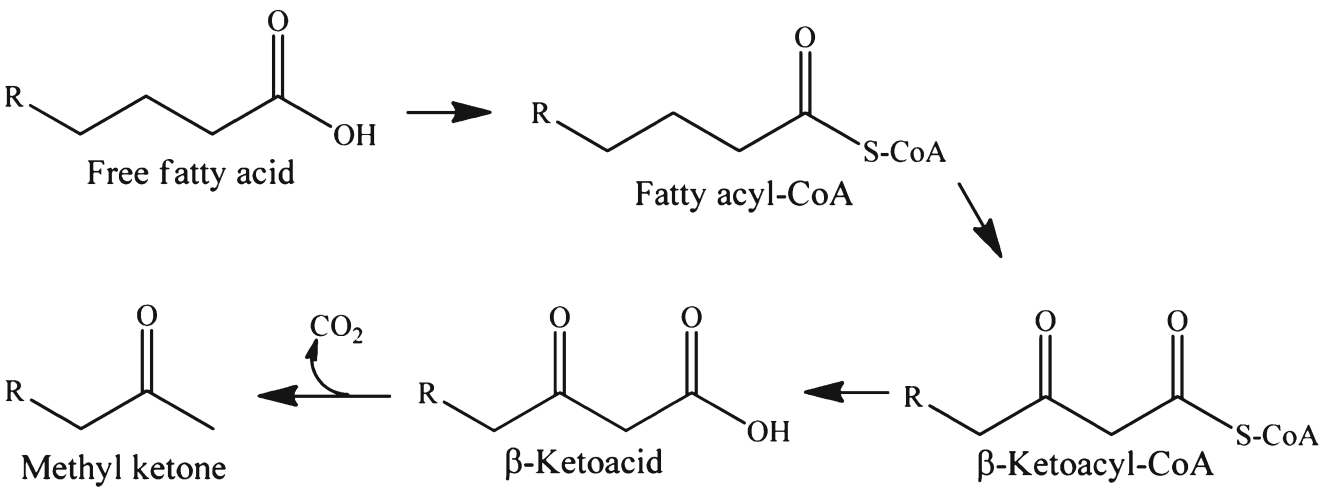

Fig. 3 Schematic biosynthesis of straight-chain methylketones

their potent antioxidant activity protecting humans from oxidative damage. As synthetic antioxidants have been reported to cause liver damage and carcinogenesis, the interest in using naturally derived antioxidants is strongly recommended avoiding the deleterious effects of synthetic antioxidants (Gouveia and Castilho 2013; Halvorsen et al. 2002). In this sense, several in vitro assays are available to measure the antioxidant activity of different medicinal plant extracts and authentic compounds. Each of these assays is based on one feature of the antioxidant activity, such as the ability to scavenge free radicals and inhibition of lipid peroxidation (LP) (Choi et al. 2002; Cvetković et al. 2015; Katalinic et al. 2006; Lin et al. 2009). The antioxidant activity of medicinal plants is a result of complex interaction between its bioactive components, such as terpenoids, phenolic compounds, flavonoids, among others (Halvorsen et al. 2002).

Aqueous herbal extracts have attracted attention since they can be consumed in a daily basis as decoctions. Many *Lamiaceae* and *Rutaceae* extracts are extensively used in traditional diet and popular medicine. Therefore, in the present work, the establishment of the volatile pattern of medicinal plants, *T. vulgaris*, *R. officinalis* and *R. chalepensis*, growing at Madeira Island (Portugal) was carried out combining the high throughput ability of HS-SPME technique with the high resolution and identification potential of GC–qMS. In order to improve the SPME performance, based on number of isolated metabolites, total peak area of identified compounds and % of RSD, the conducted research involved a previous optimization step of the most important extraction-influencing parameters, namely fiber coating, extraction time and extraction temperature. In addition, the total phenolic content (TPC) and the antioxidant activity of the target medicinal plants aqueous extracts were also evaluated and compared using previously developed assays based on Folin-Ciocalteu's assay and on scavenging activity of the stable free radical $DPPH^{\bullet}$ and thiobarbituric acid reactive substances (TBARS) assay, respectively.

Material and methods

Reagents and materials

All chemicals were of analytical quality. Sodium chloride (99.5 %) was supplied from Panreac (Spain, Barcelona). The authentic standards of volatile compounds (purity higher than 99 %) used for the identification of target compounds were purchased from Sigma-Aldrich (Madrid, Spain) and Fluka (Buchs, Switzerland). The individual stock solutions were prepared in ethanol at concentration of 1000 mg/L, and stored at 4 °C. The 2,2-diphenyl-1-picrylhydrazyl (≥ 85 %) was purchased from Fluka (Buchs, Switzerland) and the ultra-pure water from a Milli-Q system (Millipore, Bedford, USA). Helium of purity 5.0 (Air Liquid, Portugal) was used as the GC carrier gas. The glass vials, SPME fibers and SPME holder for manual sampling were purchased from Supelco (Bellenfonte, PA, USA). The retention index (RI) was calculated through the injection of a series of C_8 to C_{20} straight-chain *n*-alkanes (concentration of 40 mg/L) in *n*-hexane supplied by Fluka (Buchs, Switzerland).

Plant material

Fresh leaves, ca. 200 g, of *T. vulgaris*, *R. officinalis* (*Lamiaceae*) and *R. chalepensis* were collected randomly on September 2011, in Caniço [32°65′N, 16°85′W], Madeira Island. Medicinal plant extracts were prepared based on a standard protocol (Katalinic et al. 2006), with some modifications. 5 g of plant material were added with 200 mL of deionised water at 98 °C. The infusions were left at room temperature during 30 min. The medicinal plant extracts were filtered and their volatile profile and antioxidant activity was evaluated.

Optimization of SPME influencing parameters

Several experimental parameters affecting the SPME efficiency performance, such as fiber coating, extraction time and extraction temperature, were optimized using *R. chalepensis* aqueous extract.

A preliminary screening of six coating fibers with different polarities and retention capabilities, carboxen/poly(dimethylsiloxane) (CAR/PDMS, 75 μm), carbowax/divinylbenzene (CW/DVB, 65 μm), poly(acrylate) (PA, 85 μm), poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65 μm) and poly(-dimethylsiloxane) (PDMS, 100 μm) and divinylbenzene/carboxen/poly(dimethylsiloxane) (DVB/CAR/PDMS, 50/30 μm), were considered to select the best coating in terms of extraction efficiency, number of isolated metabolites and repeatability. All fibers were thermally conditioned, as recommended by the manufacturer. Before the first daily extraction, the fibers were conditioned during 10 min at 240 °C. A blank test was performed to check possible carry-over. At least three replicates were performed for each fiber.

The extraction time and temperature are important parameters affecting the volatility and solubility of analytes and consequently the SPME performance due to their strong influence on vapor pressure and on equilibrium of volatile compounds in the HS of the sample. The temperature also influences the distribution coefficient between the sample and the HS and between the HS and the fiber. Therefore, these parameters were tested and optimized. The HS-SPME of the *R. chalepensis* aqueous extract was carried out at different exposition times, between 15 and 60 min using DVB/CAR/PDMS fiber at 40 °C under constant stirring (800 rpm). Secondly, in order to optimize the extraction temperature,

25, 40 and 60 °C using DVB/CAR/PDMS fiber for 45 min under constant stirring (800 rpm) were tested.

HS-SPME procedure

Aliquots of 10 mL of *R. chalepensis* aqueous extract were placed into a 20 mL glass vial, which corresponds to the ratio volume of the liquid phase and headspace volume (1/β) of 0.5. After, the addition of 2 g of sodium chloride (NaCl) and a stirring bar (2 mm × 0.5 mm, stirring at 800 rpm), the vial was closed, and placed in a thermostated bath adjusted to 40.0 ± 0.1 °C. The SPME fiber was manually inserted into the sample vial headspace for 45 min. After sampling, the SPME fiber was retracted and immediately inserted into the GC injection port for thermal desorption of volatile metabolites. A desorption time of 6 min at 240 °C was used in splitless mode. Before sampling each fiber was reconditioned for 5 min at 240 °C. All measurements were performed, at least, three replicates.

GC-qMS conditions

The desorbed volatiles were separated in an Agilent Technologies 6890 N Network gas chromatograph equipped with a BP-20 (polyethylene glycol) fused silica capillary column (30 m × 0.25 mm I.D.; film thickness, 0.25 μm) from Scientific Glass Engineering (Darmstadt, Germany) connected to an Agilent 5973 N quadrupole mass selective detector (Palo Alto, CA, USA). Helium (Air Liquid, Portugal) was used as the carrier gas at a flow rate of 1.1 mL/min (column-head pressure: 89,632 Pa). The injections were performed in splitless mode (6 min). The GC oven temperature was maintained at 50 °C for 1 min, then raised at 2.5 °C/min to 100 °C (2 min), then to 180 °C (1 min) at 2 °C/min, and finally increased to 220 °C at a rate of 15 °C/min and held isothermally for further 10 min. For the MS system, the temperatures of the transfer lines, quadrupole and ionization source were 250, 150 and 230 °C, respectively; electron impact mass spectra were recorded at 70 eV and the ionization current was about 30 μA. The acquisitions were performed in full scan mode (30–300 *m*/*z*).

Volatile metabolites identification was based on (*i*) comparison of the GC retention times and mass spectra, with those, when available, of the authentic standard, (*ii*) comparison of all mass spectra with the data system library (NIST, 2005 software, Mass spectral Search Program V.2.od; NIST 2005, Washington, DC), and (*iii*) Kovats retention index (RI) value determined according to the van Den Dool (1963). For the determination of the RI, a C_8-C_{20} *n*-alkanes series was used, and the values were compared, when available with values reported in the literature for similar chromatographic columns (Culleré et al. 2004; Galindo-Cuspinera et al. 2002; Perestrelo et al. 2011; Pontes et al. 2009).

Determination of TPC

TPC of the aqueous extracts of medicinal plants were determined by the Folin-Ciocalteu's (FC) colorimetric method as described by Lim et al. (2007). Briefly, 0.6 mL of each extract and 3 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with deionised water) was added and mixed thoroughly. Then, 2.4 mL of 7.5 % (*w*/*v*) of sodium carbonate was added to the mixture and mixed gently. The reaction mixture was kept in dark during 30 min, and its absorbance measured at 725 nm against water in a Perkin Elmer Lambda 25UV/Vis spectrophotometer. The estimation of phenolic compounds in the extracts was carried out in five replications and calculated by a calibration curve (linear dynamic range from 10 to 150 mg/L) obtained with gallic acid. Total phenolics were determined according the following equation A_{765} = 0.003GAE (mg/L) - 0.010 (r^2 = 0.999) and expressed as gallic acid equivalents of aqueous extract (mg (GAE)/L). All measurements were performed in triplicate.

Antioxidant activity

DPPH free radical-scavenging activity

The DPPH free radical-scavenging activity of the investigated medicinal plant aqueous extracts was determined as previously described by Lin et al. (2009), with some modifications. Briefly, an aliquot of 100 μL solution of the extracts at different concentrations was mixed with 1.0 mL of methanolic solution of DPPH (0.2 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the reduction of DPPH radical was determined by measuring the absorbance at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The data presented are the average of five measurements given as mean ± standard deviation. The radical scavenging activity was calculated as percentage of DPPH discoloration of the extracts using the following formula and expressed as percent inhibition:

$$\text{Radical scavenging activity } (\%) = \left[\left(A_{\text{control}} - A_{\text{plant extract}}\right)/A_{\text{control}}\right] \times 100$$

where: $A_{control}$ is the absorbance of $DPPH^{\bullet}$ solution and $A_{plant\ extract}$ is the absorbance of the solution when the sample extract has been added at a particular level.

A gallic acid standard curve was obtained by using a gallic acid standard solution at various concentrations. The results obtained using following equation: ln ($\%\Delta A_{517}$) = 0.760 ln(GAE(mg/L)) + 0.937; r^2 = 0.962, were also expressed as mg(GAE)/L. All determinations were carried out three times.

TBARS

Inhibition of lipid peroxidation was determined by measuring TBARS as previous described (Gonçalves et al., 2013). Briefly, aqueous extracts of target plants were incubated at 37 °C for 1 h with 30 μL of 0.1 M tris-HCl buffer (pH 7.4) containing 250 μM of freshly prepared $FeSO_4$ and distilled water. The reaction was then stopped by adding 8.1 % SDS, acetic acid/HCl (pH 3.4) and 0.8 % TBA, and incubated at 100 °C for 1 h. The TBARS was measured by determining absorbance at 532 nm.

Results and discussion

SPME optimization

The effectiveness of SPME was optimized to obtain a broad spectrum of volatiles. In fact, fiber coatings, extraction time and extraction temperature are generally considered as the most important factors to achieve maximum extraction efficiency. The best HS-SPME/GC–qMS conditions were chosen based on intensity response (GC peak area), number of isolated and identified compounds, and relative standard deviation (RSD%). After the optimization step, the volatile pattern of *T. vulgaris*, *R. officinalis* and *R. chalepensis*, aqueous extracts was established.

Selection of the fiber coating

The selection of a suitable fiber coating is an important step in SPME optimization. The SPME efficiency depends greatly on the value of the distribution constant of analytes partitioned between the sample and fiber coating material. Thus, the performance of commercially available SPME fibers was tested and compared to evaluate the extraction efficiency of each coating towards volatile metabolites, using *R. chalepensis* aqueous extract as the matrix.

Figure 4 shows the results of the fiber selection carried out by HS-SPME/GC-qMS. Each fiber was exposed to the

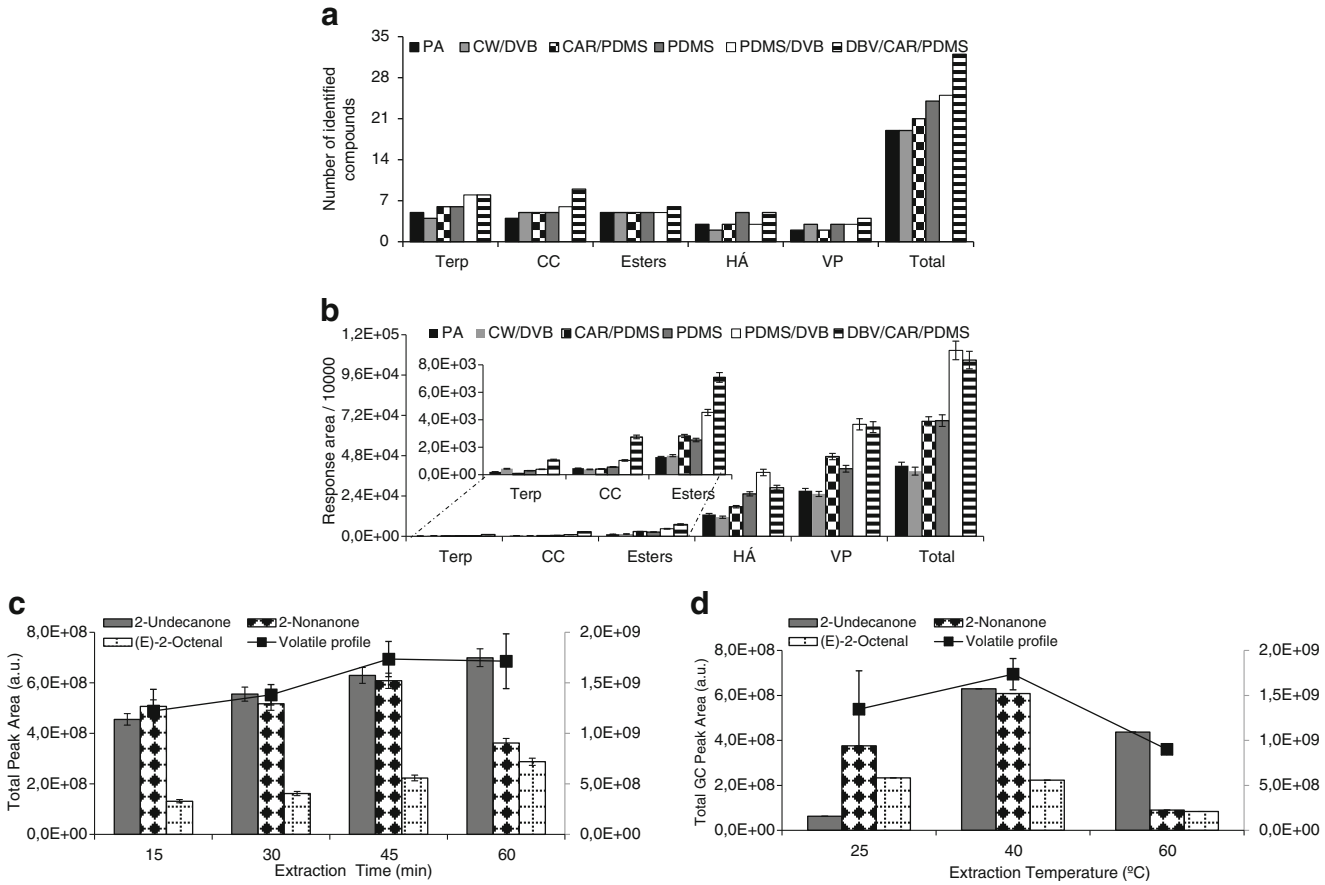

Fig. 4 Effect of the experimental conditions on the extraction performance of the HS-SPME procedure for volatile metabolites in *R. chalepensis* L. plant extract, including **a** Number of volatile compounds; **b** response areas of different terpenoids, carbonyl compounds, esters, alcohols, volatile phenols extracted with different fibers measured in the *R. chalepensis* L.; **c** Effect of extraction time (fiber: 65 μm DVB/CAR/PDMS; extraction temperature: 40 °C; stirring: 800 rpm; desorption conditions: 6 min at 240 °C); and **d** extraction temperature (fiber: 65 μm DVB/CAR/PDMS; extraction time: 45 min; stirring: 800 rpm; desorption conditions: 6 min at 240 °C). a.u. arbitrary unit. Error bars show the standard deviation of the mean ($n = 3$)

headspace for the same time at the same temperature. The results of the fiber screening revealed that DVB/CAR/PDMS coating showed a strong extraction affinity for the volatile metabolites, followed by PDMS/DVB, CAR/PDMS, PDMS, CW/DVB and PA. For all assays, the repeatability was lower than 20 %. Considering the number of identified compounds (Fig. 4a), the best performance was achieved by DVB/CAR/PDMS (32 volatiles) followed by PDMS/DVB (25), PDMS (24), CAR/PDMS (21), CW/DVB (19) and PA (19). DVB/CAR/PDMS coating combines the absorption properties of the liquid polymer with the adsorption properties of porous particles, which contains macro (> 500 Å), meso (20–500 Å) and microporous (2–20 Å). The mutually synergetic effect of adsorption and absorption of the stationary phase explains its high retention capacity. Consequently, based on the results, DVB/CAR/PDMS fiber was selected for all further optimization steps, and subsequently for the analysis of the volatile metabolites in the investigated medicinal plants (Fig. 4b).

Extraction time and extraction temperature

To optimize the extraction time, the DVB/CAR/PDMS fiber was exposed in the headspace at different times, between 15 and 60 min. The influence of the extraction time on the SPME efficiency is reported in Fig. 4c. The volatile profile, in terms of total GC peak area, shows a slight increase between 30 and 60 min, but the best extraction efficiency was obtained for 45 and 60 min. A total of 34 volatile compounds have been identified at 45 min, while using an extraction time of 60, 30 and 15 min, the number of identified volatiles are 33, 26 and 24, respectively. Repeatability (RSD%) was lower than 20 % for all extraction times tested. At 45 and 60 min, similar total GC peak areas, RSD values and number of identified volatiles were obtained. Thus, in order to implement a more expeditious methodology, the lower extraction time was selected (45 min).

The extraction temperature presents several effects on extraction efficiency. In general, increasing extraction temperature can enhance the mass transfer of the analytes from bulk sample to the headspace and diffusion coefficients and Henry's constants, while the time needed to reach equilibrium decreases (Lord and Pawliszyn 2000; Perestrelo et al. 2011). To assess the influence of temperature on SPME efficiency, different extraction temperatures ranging from 25 °C to 60 °C using DVB/CAR/PDMS fiber for 45 min were tested. To prevent analyte degradation, temperatures higher than 60 °C were not considered. Figure 4d shows the effect of the extraction temperature on the total GC peak area. The results revealed that the total GC peak areas increase with temperature

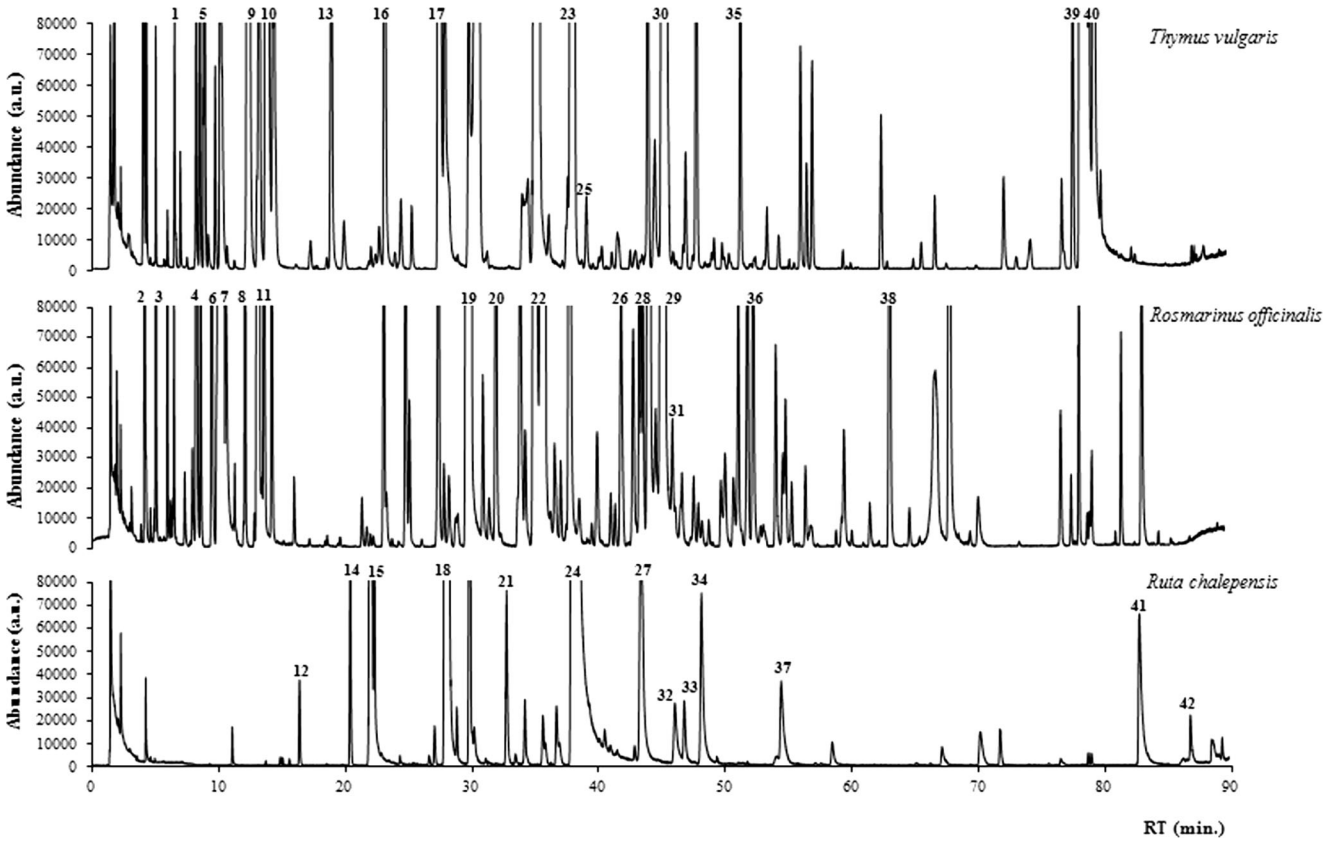

Fig. 5 TIC chromatograms of volatile metabolites from medicinal plant aqueous extracts obtained by HS-SPME/GC–qMS methodology, using DVB/CAR/PDMS fiber at 40 °C for 45 min. (Attribution of the peak number shown in Table 1). a.u. arbitrary unit

Table 1 Comparison of the volatile metabolomic pattern of *T. vulgaris*, *R. officinalis* and *R. chalepensis* using HS-SPME/GC-qMS methodology

Peak n°	R.I. Calc.[a]	R.I. Lit.[b]	Volatile metabolites	ID[e]	*T. vulgaris*		*R. officinalis*		*R. chalepensis*	
					% RPA[f] (RSD%)[g]					
1	835	796	4-Methyl-1,3-pentadiene	RI, MS	–	–	0.06	(33)	–	–
2	915	–	2-Ethoxypropane	MS	0.01	(5)	0.02	(1)	–	–
3	931	943	3-Buten-2-one	RI, MS	–	–	0.02	(5)	–	–
4	982	1007	Methyl 2-methylbutanoate	RI, MS, CO	0.64	(29)	–	–	–	–
5	987	1007	α-Pinene	RI, MS, CO	–	–	0.71	(5)	–	–
6	1008	1042	Toluene	RI, MS, CO	–	–	0.01	(9)	–	–
7	1021	1053	3-Hexanone	RI, MS	–	–	0.02	(12)	–	–
8	1026	1021	α-Thujene	RI, MS	0.07	(8)	0.06	(9)	–	–
9	1050	1067	Hexanal	RI, MS, CO	0.02	(40)	–	–	–	–
10	1060	1075	Camphene	RI, MS	0.02	(20)	0.25	(7)	–	–
11	1068	1086	Hexen-3-one	RI, MS	–	–	0.02	(13)	–	–
12	1074	–	β-Terpinene	MS	–	–	0.01	(9)	–	–
13	1076	1124	Ethylbenzene	RI, MS	–	–	0.1	(2)	–	–
14	1101	1152	4-Methyl-3-penten-2-one	RI, MS	–	–	0.04	(7)	–	–
15	1121	1166	α-Phellandrene	RI, MS	0.17	(10)	0.06	(2)	–	–
16	1123	1113	β-Pinene	RI, MS	–	–	0.13	(5)	–	–
17	1128	1161	β-Myrcene	RI, MS, CO	0.24	(13)	1.69	(7)	–	–
18	1136	1167	α-Terpinene	RI, MS, CO	–	–	0.21	(5)	–	–
19	1138	1110	2-Carene	RI, MS	0.52	(10)	–	–	–	–
20	1154	1161	Limonene	RI, MS, CO	0.1	(22)	0.62	(4)	–	–
21	1162	1190	Eucalyptol	RI, MS, CO	1	(9)	40.07	(5)	–	–
22	1177	–	2,5,5-Trimethyl-1-hexen-3-yne	MS	–	–	–	–	0.03	(23)
23	1181	1201	(*E*)-2-Hexenal	RI, MS, CO	0.01	(5)	–	–	–	–
24	1197	1225	(*Z*)-Ocimene	RI, MS	0.02	(2)	0.42	(4)	–	–
25	1200	1208	γ-Terpinene	RI, MS	2.05	(9)	0.13	(8)	–	–
26	1217	1192	3-Octanone	RI, MS, CO	8.49	(1)	8.27	(7)	–	–
27	1228	1245	(*E*)-Ocimene	RI, MS	0.2	(3)	0.47	(2)	–	–
28	1238	1242	*p*-Cymene	RI, MS, CO	0.05	(10)	0.12	(12)	–	–
29	1255	1261	Stryrene	RI, MS	–	–	–	–	0.02	(6)
30	1264	1287	1-Octen-3-one	RI, MS, CO	–	–	0.04	(10)	0.29	(12)
31	1282	1309	(*Z*)-3-Hexenyl acetate	RI, MS	–	–	0.02	(6)	–	–
32	1299	1319	Methyl heptenone	RI, MS	0.02	(27)	–	–	–	–
33	1305	1264	Hexyl acetate	RIMS	0.27	(7)	0.33	(4)	–	–
34	1326	–	Octyl acetate	MS	–	–	–	–	0.4	(6)
35	1338	1388	2-Nonanone	RI, MS	–	–	–	–	9.59	(10)
36	1347	1351	1-Hexanol	MS	–	–	0.02	(8)	0.01	(19)
37	1348	1357	(*Z*)-3-Hexen-1-ol	RI, MS, CO	0.04	(3)	0.02	(6)	–	–
38	1352	–	3-Heptene	MS	–	–	0.02	(22)	–	–
39	1357	–	4-Pentenol	MS	0.02	(20)	–	–	–	–
40	1362	1393	3-Octanol	MS, CO	0.99	(13)	0.39	(7)	–	–
41	1364	1390	2-Methylanisole	RI, MS	–	–	0.04	(14)	–	–
42	1375	–	Thujol	MS	0.1	(5)	–	–	–	–
43	1393	1419	α-Thujone	MS	–	–	–	–	0.26	(7)
44	1408	–	11-Dodecen-2-one	MS	–	–	–	–	0.07	(35)
45	1414	1423	1-Octen-3-ol	RI, MS, CO	6.93	(3)	0.58	(6)	–	–
46	1419	1415	(*E*)-2-Octenal	RI, MS	–	–	0.06	(5)	28.23	(1)
47	1433	1423	(*E*)-Linalool oxide	RI, MS	–	–	0.02	(3)	0.11	(18)

Table 1 (continued)

Peak n°	R.I. Calc.[a]	R.I. Lit.[b]	Volatile metabolites	ID[e]	*T. vulgaris*		*R. officinalis*		*R. chalepensis*	
					% RPA[f] (RSD%)[g]					
48	1446	1484	2-Decanone	RI, MS	–	–	20.32	(5)	1.95	(9)
49	1451	1447	Decanal	RI, MS, CO	–	–	–	–	0.13	(31)
50	1463	–	1,4-Bis(1-methylethyl)-benzene	MS	–	–	0.13	(8)	0.03	(22)
51	1468	1500	Benzaldehyde	RI, MS, CO	–	–	0.03	(25)	–	–
52	1474	1499	Pinan-3-one	RI, MS	–	–	0.51	(6)	–	–
53	1484	1532	2-Nonanol	RI, MS	–	–	–	–	0.35	(4)
54	1502	1550	Linalool	RI, MS, CO	–	–	0.1	(7)	–	–
55	1521	1555	Bornyl acetate	RI, MS, CO	–	–	9.83	(12)	0.18	(10)
56	1526	1556	Linalyl acetate	MS	0.04	(26)	–	–	–	–
57	1533	1560	Nonyl acetate	RI, MS	–	–	0.1	(16)	0.21	(5)
58	1539	1538	Dihydrolinalool	RI, MS	–	–	0.06	(12)	–	–
59	1546	1574	Isopulegol	RI, MS	5.69	(12)	3.18	(7)	–	–
60	1549	1543	2-Undecanone	RI, MS, CO	–	–	–	–	53.43	(1)
61	1563	1585	(*E*)-Myrcenol	RI, MS	3.94	(1)	–	–	–	–
62	1568	1583	Ho-trienol	RI, MS	–	–	0.02	(22)	–	–
63	1573	1595	β-Ciclocitral	RI, MS, CO	–	–	0.13	(6)	–	–
64	1594	1600	Dihydrocarvone	RI, MS, CO	0.03	(33)	0.28	(7)	0.03	(15)
65	1614	–	9-Decen-2-one	MS	–	–	–	–	1.11	(2)
66	1616	1639	Menthol	RI, MS, CO	–	–	0.43	(6)	–	–
67	1617	1628	(*Z*)-2-Menthenol	RI, MS	–	–	0.35	(9)	–	–
68	1623	1658	Neral	RI, MS	0.48	(20)	–	–	–	–
69	1640	1642	Borneol	RI, MS, CO	0.02	(26)	6.91	(8)	–	–
70	1646	1669	α-Terpineol	RI, MS, CO	0.05	(28)	–	–	0.22	(8)
71	1651	1635	Methyl benzoate	RI, MS	–	–	0.14	(8)	0.36	(13)
72	1664	1707	Carvone	RI, MS, CO	–	–	0.07	(14)	0.64	(5)
73	1672	1650	(*E*)-Ocimenol	RI, MS	0.02	(3)	–	–	–	–
74	1673	1697	2-Dodecanone	RI, MS	–	–	0.06	(9)	–	–
75	1681	1687	Terpinyl acetate	RI, MS	–	–	0.23	(2)	–	–
76	1701	–	3-Octyne	MS	–	–	0.07	(14)	–	–
77	1723	1727	β-Citronellol	RI, MS, CO	0.28	(16)	0.07	(5)	–	–
78	1739	1660	Isoborneol	RI, MS	–	–	0.44	(3)	–	–
79	1765	–	3,5-Dimethyl-2-cyclohexen-1-one	MS	–	–	0.21	(6)	–	–
80	1769	1782	Cumin aldehyde	RI, MS	0.05	(4)	–	–	0.47	(12)
81	1782	1808	Isoestragole	RI, MS	–	–	0.06	(15)	–	–
82	1798	1814	*p*-Cymen-8-ol	RI, MS	0.1	(10)	0.07	(30)	–	–
83	1804	1788	Geraniol	RI, MS, CO	0.17	(15)	–	–	–	–
84	1893	1890	α-Calacorene	RI, MS	–	–	0.71	(18)	–	–
85	1949	–	3-Nonyne	MS	–	–	–	–	0.05	(22)
86	1987	2007	Methyl eugenol	RI, MS	–	–	–	–	0.15	(18)
87	2110	2089	Elemol	RI, MS	–	–	–	–	0.08	(14)
88	2118	2110	Cedrenol	RI, MS	0.3	(22)	–	–	–	–
89	2137	2129	Spathulenol	RI, MS	0.02	(18)	–	–	–	–
90	2201	2192	Eugenol	RI, MS, CO	0.05	(9)	0.14	(12)	–	–
91	2206	2200	Cinnamyl alcohol	RI, MS	0.17	(14)	–	–	–	–
92	2223	2179	Thymol	RI, MS	66.61	(1)	0.13	(6)	–	–
93	2248	2252	Methyl anthranilate	RI, MS	–	–	–	–	0.03	(19)
94	2254	2248	β-Eudesmol	RI, MS	–		0.02	(5)	0.03	(15)

Table 1 (continued)

Peak n°	R.I. Calc.[a]	R.I. Lit.[b]	Volatile metabolites	ID[e]	*T. vulgaris*		*R. officinalis*		*R. chalepensis*	
					% RPA[f] (RSD%)[g]					
95	2257	2235	α-Bisabolol	RI, MS, CO	–	–	0.02	(7)	–	–
96	2320	2358	Isopropyl palmitate	RI, MS	–	–	–	–	0.68	(12)
97	2359	2372	(*E*)-Isoeugenol	RI, MS	–	–	0.07	(5)	0.13	(22)
98	2360	2329	4-Allylphenol	RI, MS	–	–	0.06	(6)	–	–
99	2376	–	*p*-Anisamide	MS	–	–	–	–	0.73	(5)

- not detected

[a] RI: Retention index calculated to BP-20 columns

[b] RI: Retention index reported in the literature for BP-20 columns or equivalents (Culleré et al., 2004; Galindo-Cuspinera et al., 2002; Perestrelo et al., 2011; Pontes et al., 2009)

[c] MW: Molecular weight

[d] IUPAC: International Union of Pure and Applied Chemistry

[e] ID: The reliability of the identification or structural proposal is indicated by the following: RI – Identified by Kovats retention index; MS – Identified based on computer matching of the mass spectra of peaks with the Wiley 275 and NIST libraries; and CO – identified by co-injection of authentic standard

[f] Relative peak area expressed as percentage of the GC peak area of corresponding volatiles over the total GC peak area of all identified volatiles

[g] RSD: Relative standard deviation

between 25 °C and 40 °C, while a slight decrease was observed at 60 °C. A total of 34 volatile compounds have been identified at 40 °C, while using an extraction temperature of 60 °C and 25 °C, the number of identified metabolites is slightly lower, 29 and 20, respectively. Therefore an extraction temperature of 40 °C was selected for the HS-SPME analysis of the target medicinal plants volatiles. Repeatability (RSD%) was lower than 20 % for all extraction temperatures tested.

Volatile metabolomic profile of aqueous plant extracts

The optimized HS-SPME/GC–qMS methodology was applied to establish the volatile profile of *T. vulgaris*, *R. officinalis* and *R. chalepensis* aqueous extracts. The typical total ion current (TIC) chromatogram profiles of the target medicinal plants are shown in Fig. 5. The percentage of relative peak area (RPA, %) of each volatile metabolite is summarized in Table 1.

As shown in Table 1, a total of 99 volatile metabolites were identified, including 53 terpenoids, 19 carbonyl compounds, 7 esters, 6 higher alcohols, and 14 miscellaneous. These volatile metabolites were identified by their mass spectrum, retention index (using C8-C20 *n*-alkanes series), and further confirmed by authentic standard, when available.

As observed, the aqueous extracts of *Lamiaceae* medicinal plants, *T. vulgaris* and *R. officinalis*, had in common 23 volatile metabolites, mainly terpenoids such as α-thujene, camphene, α-phellandrene, β-myrcene, limonene, eucalyptol, (*Z*)-ocimene, γ-terpinene, (*E*)-ocimene, p-cymene, isopulegol, dihydrocarvone, borneol, β-citronellol, *p*-cymen-8-ol, eugenol and thymol. *R. chalepensis* and *R. officinalis* plant extracts shared 12 volatile metabolites, like (*E*)-linalool oxide, dihydrocarvone, carvone, β-eudesmol, (*E*)-isoeugenol, bornyl acetate, nonyl acetate, methyl benzoate, 1,4-bis(1-methylethyl)-benzene, 2-decanone, (*E*)-2-octenal and 1-octen-3-one. Only dihydrocarvone was simultaneously identified in all three medicinal plant aqueous extracts. Figure 6 presents the distribution of chemical groups by medicinal plants investigated.

The terpenoids were the most predominant chemical group in *T. vulgaris* and *R. officinalis* aqueous extracts accounting, 83 and 69 %, respectively, of the total volatile pattern, whereas for *R. chalepensis* 95 % of total volatile profile was composed by carbonyl compounds, namely aldehydes. The main volatile metabolites identified in *T. vulgaris* aqueous extracts were thymol (67 %), isopulegol (6 %) and α-terpinene (2 %), whereas in *R. officinalis*, eucalyptol (40 %), bornyl acetate (10 %) and borneol (7 %), were the most dominant. *R. chalepensis* was characterized by the high levels of 2-undecanone (53 %), (*E*)-2-octenal (28 %) and 2-nonanone (10 %). The carbonyl compounds contribution to the *R. chalepensis* total volatile profile was almost 11 and 3 times higher, respectively, than the content found in *T. vulgaris* and *R. officinalis* aqueous extracts.

These results are in accordance with previously published data on *T. vulgaris* (Baranauskienė et al. 2003; Grigore et al. 2010) and *R. officinalis* (Barnes et al. 2007; Jiang et al. 2011) medicinal plant extracts. Terpenoids are important for plant survival and also possess medicinal properties that are beneficial to humans, such as anti-carcinogenic, antimalarial, anti-

ulcer, hepaticidal, antimicrobial or diuretic activity (Aharoni et al. 2005). Similar results were also found by Mejri et al. (2010) for *R. chalepensis* volatile profile.

TPC and antioxidant activity

The TPC and the antioxidant activity of the studied medicinal plant extracts are illustrated in Fig. 7. The TPC is significantly different among the studied medicinal plant extracts. The highest values of total phenolics were obtained for *R. chalepensis* (57 mg (GAE)/L) aqueous extract, followed by *R. officinalis* (26 mg (GAE)/L) and finally *T. vulgaris* (14.9 mg (GAE)/L)) (Fig. 7). The difference of TPC was significant, up to 4 fold, and the phenolics contribution to quality and nutritional value in terms of, flavor, aroma and also antioxidant potential providing health-beneficial effects are well-known (Vaya et al. 1997).

DPPH method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant. The antioxidant activity of *T. Vulgaris*, *R. officinalis*, and *R. chalepensis* aqueous extracts was evaluated through scavenging activity of the stable free radical DPPH$^{\bullet}$. The antioxidant activity ranged from 22 to 35 mg (GAE)/L, and the difference was not very large, up to 2 fold. The highest value was obtained for *T. vulgaris and R. officinalis* and the lowest for *R. chalepensis* plant extract (Fig. 7).

Furthermore, as the volatile profile of these medicinal plant extracts was established by HS-SPME/GC–qMS methodology, this allowed us to try a possible correlation between chemical composition and their antioxidant activity. A possible explanation for the low antioxidant activity of *R. chalepensis* plant extract could be attributed to their high content of carbonyl compounds and low levels of terpenoids. A previous study that screened the antioxidant activity of 98 pure oil components belonging to different chemical groups, observed that carbonyl compounds (e.g. 2-undecanone) exhibited a low scavenging activity, if any antioxidant effect, whereas terpenoids, such as monoterpenes hydrocarbons and phenols showed a significant protective effect with several variants

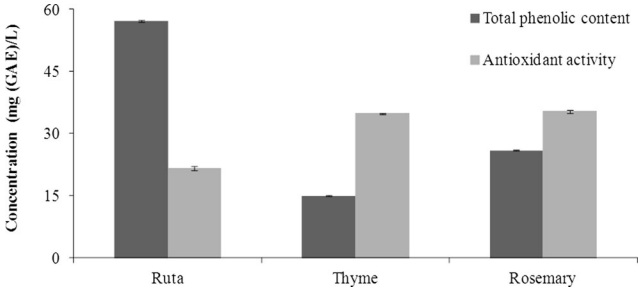

Fig. 7 TPC and DPPH radical-scavenging activity determined in investigated medicinal plants

due to their different functional groups (Ruberto and Baratta 2000). Moreover, the high antioxidant activity of *T. vulgaris* and *R. officinalis* could be explained by the presence of some oxygenated monoterpenes, such as thymol, eucalyptol, borneol, β-citronellol, geraniol and eugenol, with high antioxidant activity. From these, thymol is in fact responsible for the antioxidant activity of many essential oils (Ruberto and Baratta 2000), so these medicinal plants can be explored as a natural source of antioxidant compounds for possible applications in nutraceutical industries. The antioxidant activity of the aqueous extracts was also assessed by TBARS inhibition assay. In this assay, the greatest effectiveness was shown by *R. officinalis* (0.002 μg/mL), slightly lower for *T. vulgaris* (0.003 μg/mL), and the lowest for the *R. chalepensis* oil (0.11 μg/mL). Compared to positive control (gallic acid) the aqueous extracts exhibited quite similar antioxidant activity.

Conclusions

HS-SPME combined with GC–qMS provides a suitable tool and a reliable alternative technique to establish the volatile metabolomic pattern of medicinal plant aqueous extracts. Since SPME technique is very sensitive to experimental conditions, some parameters were optimized in order to achieve maximum extraction efficiency. According to the data obtained, DVB/CAR/PDMS fiber in addition to an extraction time

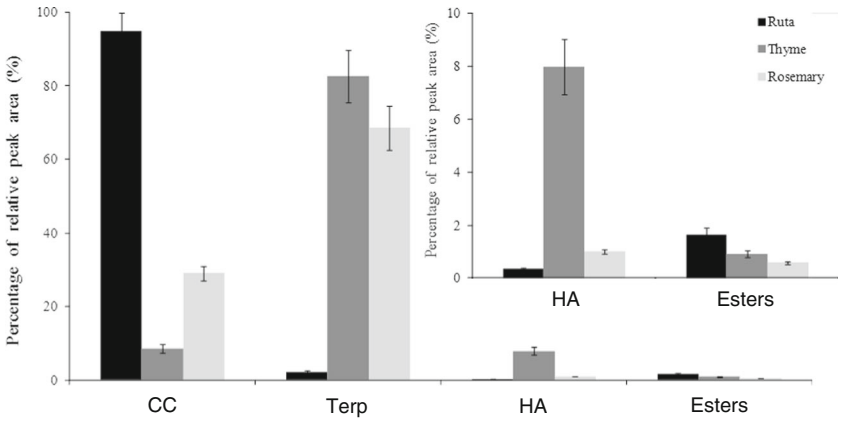

Fig. 6 Distribution of chemical groups by medicinal plant extracts. CC - carbonyl compounds

and temperature of 45 min and 40 °C, respectively, were found to be the optimum conditions to ensure the best extraction efficiency. Sixty-six, 40, and 31 volatile metabolites were identified in *T. vulgaris*, *R. officinalis*, and *R. chalepensis* aqueous extracts, belonging to different chemical families namely monoterpenes, sesquiterpens, alcohols, carbonyl compounds and esters. Terpenoids are the dominant chemical group in *T. vulgaris* and *R. officinalis* aqueous extracts, whereas in *R. chalepensis* aqueous extracts carbonyl compounds is the main chemical group. Thymol (67 %), eucalyptol (40 %) and 2-undecanone (53 %) were the main volatile metabolites detected in *T. vulgaris*, *R. officinalis* and *R. chalepensis* aqueous extract, respectively.

The obtained results indicate that among the studied medicinal plants *T. vulgaris* and *R. officinalis* possess stronger protective effects than *R. chalepensis*. These finding could be attributed to the high terpenoid content, mainly monoterpenes hydrocarbons (e.g. β-myrcene, γ-terpinene) and oxygenated monoterpenes (e.g. thymol, eucalyptol), which are responsible for several biological activitiesThe investigated medicinal plant extracts, especially thyme and rosemary, can probably be used to prevent several human diseases. In addition to their well-known traditional use in food and cosmetics, the great potential of tested medicinal plants, encourage further investigation.

Acknowledgments The support of Fundação para a Ciência e a Tecnologia (FCT) is acknowledged through the CQM pluriannual base funding/Strategic Plan: PEst-OE/QUI/UI0674/2014 and MS Portuguese Networks RNEM (REDE/1508/REM/2011).

References

Aharoni A, Jongsma MA, Bouwmeester HJ (2005) Volatile science? Metabolic engineering of terpenoids in plants. Trends Plant Sci 10: 594–602

Bajerová P, Eisner A, Ježová V, Adam M, Ventura K (2008) Comparison of supercritical fluid and soxhlet extractions for the isolation of nitro compounds from soils. J Sep Sci 31:1408–1414

Baranauskienė R, Venskutonis PR, Viškelis P, Dambrauskienė E (2003) Influence of nitrogen fertilizers on the yield and composition of thyme (*Thymus vulgaris*). J Agric Food Chem 51:7751–7758

Barnes J, Anderson LA, Phillipson JD (2007) *Rosemary* (Vol, 3rd edn. In: Herbal Medicines. Pharmaceutical Press, London

Cao J, Qi M, Fang L, Zhou S, Fu R, Zhang P (2006) Solid-phase microextraction–gas chromatographic–mass spectrometric analysis of volatile compounds from *Curcuma wenyujin* Y.H. Chen et C. Ling. J Pharm Biomed Anal 40:552–558

Choi CW, Kim SC, Hwang SS, Choi BK, Ahn HJ, Lee MY, Park SH, Kim SK (2002) Antioxidant activity and free radical scavenging capacity between korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci 163:1161–1168

Crocoll C, Asbach J, Novak J, Gershenzon J, Degenhardt J (2010) Terpene synthases of oregano (origanum vulgare L.) and their roles in the pathway and regulation of terpene biosynthesis. Plant Mol. Biol 73:587–603

Culleré L, Escudero A, Cacho J, Ferreira V (2004) Gas chromatography − olfactometry and chemical quantitative study of the aroma of six premium quality spanish aged red wines. J Agric Food Chem 52:1653–1660

Cvetković ŽS, Nikolić VD, Savić IM, Savić-Gajić IM, Nikolić LB (2015) Development and validation of an RP-HPLC method for quantification of trans-resveratrol in the plant extracts. Hemijska industrija4-4.

Dorman HJ, Deans SG (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J Appl Microbiol 88:308–316

Galindo-Cuspinera V, Lubran MB, Rankin SA (2002) Comparison of volatile compounds in water- and oil-soluble annatto (*Bixa Orellana* L.) extracts. J Agric Food Chem 50:2010–2015

Gianelli MP, Flores M, Toldrá F (2002) Optimisation of solid phase microextraction (SPME) for the analysis of volatile compounds in dry-cured ham. J Sci Food Agric 82:1703–1709

Gonçalves S, Gomes D, Costa P, Romano A (2013) The phenolic content and antioxidant activity of infusions from mediterranean medicinal plants. Ind Crop Prod 43:465–471

Gouveia SC, Castilho PC (2013) Artemisia annua L.: essential oil and acetone extract composition and antioxidant capacity. Ind Crop Prod 45:170–181

Grigore A, Paraschiv I, Colceru-Mihul S, Bubueanu C, Draghici E, Ichim M (2010) Chemical composition and antioxidant activity of *Thymus vulgaris* L. volatile oil obtained by two different methods. Rom Biotech Lett 15:5436–5443

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. J Pharm Biomed Anal 49:674–685

Halvorsen BL, Holte K, Myhrstad MCW, Barikmo I, Hvattum E, Remberg SF, Wold A-B, Haffner K, Baugerød H, Andersen LF, Moskaug Ø, Jacobs DR, Blomhoff R (2002) A systematic screening of total antioxidants in dietary plants. J Nutr 132:461–471

Ince AE, Sahin S, Sumnu G (2014) Comparison of microwave and ultrasound-assisted extraction techniques for leaching of phenolic compounds from nettle. J Food Sci Technol 51:2776–2782

Jiang Y, Wu N, Fu Y-J, Wang W, Luo M, Zhao C-J, Zu Y-G, Liu X-L (2011) Chemical composition and antimicrobial activity of the essential oil of rosemary. Environ Toxicol Phar 32:63–68

Katalinic V, Milos M, Kulisic T, Jukic M (2006) Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chem 94:550–557

Lim YY, Lim TT, Tee JJ (2007) Antioxidant properties of several tropical fruits: a comparative study. Food Chem 103:1003–1008

Lin C, Yu C, Wu S, Yih K (2009) DPPH free-radical scavenging activity, total phenolic contents and chemical composition analysis of forty-two kinds of essential oils. J Food Drug Anal 17:386–395

Lord H, Pawliszyn J (2000) Microextraction of drugs. J Chromatogr A 902:17–63

Mejri J, Abderrabba M, Mejri M (2010) Chemical composition of the essential oil of *Ruta chalepensis* L: influence of drying, hydrodistillation duration and plant parts. Ind Crop Prod 32:671–673

Nagegowda DA (2010) Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. FEBS Lett 584:2965–2973

Perestrelo R, Barros AS, Rocha SM, Câmara JS (2011) Optimisation of solid-phase microextraction combined with gas chromatography-mass spectrometry based methodology to establish the global volatile signature in pulp and skin of *Vitis vinifera* L. grape varieties. Talanta 85:1483–1493

Pontes M, Marques JC, Câmara JS (2009) Headspace solid-phase microextraction-gas chromatography-quadrupole mass spectrometric methodology for the establishment of the volatile composition of passiflora fruit species. Microchem J 93:1–11

Ruberto G, Baratta MT (2000) Antioxidant activity of selected essential oil components in two lipid model systems. Food Chem 69:167–174

Sourmaghi MHS, Kiaee G, Golfakhrabadi F, Jamalifar H, Khanavi M (2014) Comparison of essential oil composition and antimicrobial activity of Coriandrum sativum L. extracted by hydrodistillation and microwave-assisted hydrodistillation. J Food Sci Technol 1: 1–6.

VAN DEN Dool H, Dec. Kratz P (1963) A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr A 11: 463–471.

Vaya J, Belinky PA, Aviram M (1997) Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. Free Radic Biol Med 23:302–313

Vázquez-Araújo L, Rodríguez-Solana R, Cortés-Diéguez SM, Domínguez JM (2013) Use of hydrodistillation and headspace solid-phase microextraction to characterize the volatile composition of different hop cultivars. J Sci Food Agric 93:2568–2574

Venkatachallam SKT, Pattekhan H, Divakar S, Kadimi US (2010) Chemical composition of Nigella sativa L. seed extracts obtained by supercritical carbon dioxide. J Food Sci Technol 47:598–605

Yu G, Nguyen TTH, Guo Y, Schauvinhold I, Auldridge ME, Bhuiyan N, Ben-Israel I, Iijima Y, Fridman E, Noel JP, Pichersky E (2010) Enzymatic functions of wild tomato methylketone synthases 1 and $2^{1,[W][OA]}$. Plant Physiol 154:67–77