Stir bar sorptive extraction coupled with GC/MS applied to honey: optimization of method and comparative study with headspace extraction techniques

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Abstract The stir bar sorptive extraction (SBSE) tech-niques, coupled with gas chromatography–mass spectrom-etry, were successfully applied to the study of Eucalyptus honey's for the determination of volatile organic com-pounds (VOCs). An optimization of the extraction method was carried out and the variables, NaCl concentration (used as matrix modifier), and the concentration of honey solu-tion were studied targeting the whole VOCs composition. After the evaluation of the experiments, the best condi-tion for the extraction of honey volatile components was 2 mol/L of NaCl and the more concentrated honey solution (0.5 g of honey per mL of water). Additionally, the results were compared with those obtained by two headspace (HS) techniques, namely solid-phase microextraction (SPME) and dynamic headspace (DHS). SBSE volatiles differ qualitatively and quantitatively from those obtained by the SPME and DHS methods. In any event, the chemical composition of Eucalyptus honey volatiles extracted by all three techniques shows the presence of some typical foral markers. Our results confirm a general trend reported in the literature, which show the higher sensitivity of SBSE in the extraction of less volatile compounds in comparison with HS methods.

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Introduction

The analysis of organic compounds in honey has stimulated a lot of interest in the last decade [1]. Honey is usually considered as an animal source food, since it is produced by bees; although its origin is also closely linked to plants. The main tool used to obtain information about its botanical origin is the study of microscopic particles (pollen grain and spores) present in honey. This powerful method, known as melissopalynological analysis, sometimes does not allow an unambiguous identification of the botanical origin of a sample. In fact, the pollen in the sediment of honey from some botanical species is known to be underrepresented (class I, <20,000 pollen grains per 10 g of honey) limiting the applicability of the technique. The concentration of pollen is linked to several parameters such as the morphology of the fowers as well as to the size of pollen grains [2]. Typical underrepresented examples include *Asphodelus microcarpus* Salzm. et Viv. honey, *Arbutus unedo* L. honey or *Thystle* honey whose botanical classification based on the melissopalynology is quite difficult [3].

A great number of components in honey derive directly from the foral source extracted by honey bees; therefore, the investigation of the chemical composition of honey is an important tool to understand the botanical origin of honey, since several compounds are markers of the nectar collected by bees [4]. Out of all secondary metabolites, volatile organic compounds (VOCs) play a key role in the investigation of the foral markers able to allocate honey to a specific botanical origin [5, 6].

Besides the contribution to the aroma, one of the most sensory properties that determine the selection of this food

by consumers, VOCs are also an important marker linked to the freshness of honey: for example the presence of furanic aldehydes as well as some terpenoid compounds has to be related to the freshness of honey [7].

Several extraction methods, coupled with gas chromatographic (GC) analysis, have been employed to study the volatile fraction of honey [6]. Particular attention has been given to solid-phase microextraction (SPME) which is able to limit the introduction of artifacts in the sample preparation or the loss of compounds during the evaporation step, problems that are typically found with solvent extraction methods.

In addition, the solvent-free fractionation of volatiles in honey has been successfully carried out by several authors using dynamic headspace (DHS) extraction [8]. This technique shows a high sensitivity for fractionation of highly volatile compounds, however extraction conditions need to be further optimized in order to better extract the less vola-tile components, such as medium–low volatile terpenes [8].

Stir bar sorptive extraction (SBSE) is a relatively novel technique which involves a magnetic stir bar coated with a film of stationary extraction phase, mainly polidimethylsyloxane (PDMS), commonly commercialized under the name "Twister®." The extraction can be performed both by stirring the Twister® into the liquid samples or also by suspending the Twister® on the headspace (HS-SBSE or HSSE). The main advantage of HSSE, over HS-SPME extraction, is the larger amount of extracting phase, which consequently allows a higher recovery of volatiles and thus greater sensitivities [9], as well as lower risk of saturation or competition phenomena [10].

The SBSE technique has been widely used for several applications [11], though it is still a rather unexploited method in food analysis, with relatively few papers published [11].

The majority of the studies on volatiles by SBSE in food are targeted on the investigation of pollutants and toxins [12] and, to the best of our knowledge, no study has been targeted on the analysis of honey's VOCs. Thus, we focused our study on the application of SBSE followed by GC–MS analysis on the extraction of volatiles from Eucalyptus honey. Furthermore, the results were compared with those obtained by the more common solvent-free SPME and DHS techniques on the same Eucalyptus honey. The concentra-tion of aqueous honey solution and matrix modifiers was screened in order to obtain the best SBSE-GC/MS response.

Materials and methods

Chemicals and reagent

Unless stated otherwise, all chemicals and reagents were supplied by Sigma (Dorset, UK). n-Alkanes (C8–C23) were purchased by Lancaster Synthesis, Eastgate, White Lund, Morecambe, England; methyl salicilate was purchased by Carlo Erba Reagents s.r.l.

Honey sample

For this study, a monoforal sample of Eucalyptus honey (*Eucalyptus camaldulensis* Dehnh.) was obtained from a professional beekeeper from Sardinia who declared the botanical origin of the sample. After acquisition, the honey was stored at 4 °C in the dark and analyzed within 3 months.

Gas chromatography–mass spectrometry (GC–MS) analysis

The GC–MS analysis was carried out using an Agi-lent 7890 GC equipped with a Gerstel MPS autosam-pler, coupled with an Agilent 7000C MSD detector. The chromatographic separation was performed on a VF-Wax 60 m × 0.25 mm i.d., 0.5 µm film thickness col-umn (Agilent), as well as on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.17 µm), the following temperature program was used for the VF-Wax column: 40 °C hold for 4 min, then increased to 150 °C at a rate of 5.0 °C/min, held for 3 min then increased to 240 °C at a rate of 10 °C/min, and finally held for 12 min. For the HP-5MS column, the following temperature program was used: 60 °C hold for 3 min, then increased to 210 °C at a rate of 4 °C/min, then held at 210 °C for 15 min, then increased to 280 °C at a rate of 10 °C/min. Helium was used as the carrier gas at a constant fow of 1 mL/min for both columns. The data were analyzed using a MassHunter Workstation B.06.00 SP1, with identification/tentatively identification of the individual components performed by comparison with the co-injected pure compounds and by matching the MS fragmentation patterns and retention indices with the built in the libraries or the literature data or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 pur-chased from Agilent Technologies).

SPME conditions

SPME analysis was performed following the optimized method proposed by Kus et al. [13] with only minor modifications.

The isolation of headspace volatile compounds was carried out from a honey/aqueous saturated NaCl solution (10 mL, 1:1 v/v). into a 20 mL SPME vial, 75.5 × 22.5 mm, which was tightly closed with a septum and allowed to equilibrate under agitation for 60 min at 60 °C. A 1 cm, PDMS 50/30 Stablefex (Supelco, Milano, Italy) SPME fiber was preconditioned at 250 °C for 0.5 h

in a Gerstel MPS bake-out station, according to the manufacturer's instructions, before being introduced to the headspace for extraction. Prior to and after each analysis, the fiber underwent a further bake-out step for 5 min at 250 °C. The extraction time was fixed at 40 min, after which it was desorbed for 2 min into a Gerstel CIS6 PTV injector oper-ating at 250 °C in a splitless mode.

SBSE conditions

Three concentrations of NaCl (0, 2.0 and 4.0 mol/L) and three honey concentrations (dilution ratio: 1/2, 1/4, 1/6 w/v) were used, for a total of nine experiments. The experi-ments were carried out randomly and in triplicate. The sorption was carried out for 6 h while stirring at 900 rpm at 25 °C. After extraction, the twister® stir bar was properly cleaned with ultrapure water.

The desorption of stir bars was performed into a Gerstel thermal desorption unit (TDU) operating in splitless mode, directly connected with a Cooling Injection System (Gerstel CIS6) injector operating in solvent vent mode at 4 °C. The TDU temperature was held at 40 °C for 0.10 min, raised to 300 °C at 720 °C/min and then held at this temperature for 10 min. The transfer line between the TDU and CIS units was kept constant at 300 °C. The desorption fow was set to 30 mL/min. After the complete desorption, the CIS6 temperature was increased to 250 °C at 12 °C/s and kept at this temperature for 5 min.

DHS conditions

DHS analysis was performed following the previously opti-mized methods [8, 14] with minor modifications. Briefy, 5.0 g of honey were dissolved in water (5 mL) in a 20 mL 75.5 × 22.5 mm DHS vial, which was tightly closed with a septum and allowed to equilibrate under agitation (500 rpm) for 5 min at 40 °C. The vial was then maintained at 40 °C for 15 min in a Gerstel MPS DHS station using a 20 mL/min fow of helium. Volatiles swept by nitrogen were trapped in a PDMS cartridge (Supelco, Milano, Italy) at 30 °C. Then, the PDMS cartridge was dried by fuxing through the trap an additional 120 mL of nitrogen (15 mL/min) at 30 °C. Then, the PDMS cartridge was desorbed for 2 min into a Gerstel CIS6 PTV injector operating at 250 °C in a splitless mode directly con-nected with a Cooling Injection System (Gerstel CIS6) injector operating in solvent vent mode at 4 °C. The TDU temperature was held at 40 °C for 0.10 min, raised to 300 °C at 720 °C/min and then held at this temperature for 10 min. The transfer line between the TDU and CIS units was kept constant at 300 °C. The desorption fow was set to 30 mL/min. After the complete desorption, the CIS6 temperature was increased to 250 °C at 12 °C/s and kept at this temperature for 5 min.

Retention indexes

A hydrocarbon mixture of *n*-alkanes (C_8–C_{23}) was analyzed separately under the same chromatographic conditions used on the HP-5MS and the VF-Wax capillary columns to calculate the retention indexes with the generalized equation by Van del Dool and Kartz, $I_x = 100[(t_x - t_n)/ (t_{n+1} - t_n) + n]$. Where t is the retention time, x is the ana-lyte, n is the number of carbons of alkane that elutes before analyte and $n + 1$ is the number of carbons of alkane that elutes after analyte.

Statistical analysis

The statistical analyses were performed comparing data with unpaired Student's t test, using SigmaStat v 3.5 soft-ware. The data were considered to follow a normal distribu-tion. A $p \leq 0.05$ was considered statistically significant.

Result and discussion

The general operating mode of an SBSE method includes two main general steps, namely a first static sorption/absorption of the analytes, followed by a back-extraction of the volatiles to the chromatographic system. This technique could be applied to the headspace volatiles (HS-SBSE or HSSE) or to the volatiles/semi-volatiles by immersion of the stirring bar in a liquid media (SBSE).

Some of the variables which infuence SBSE were considered for the optimization of extraction method applied to the honey solutions by one variable at time (OVAT) with the aim to find the best experimental conditions which allow the biggest TIC (total ion chromatogram) total area. The variables considered in the optimization were the con-centration of aqueous honey solutions and the addition of sodium chloride to the honey solution, used as matrix modifier. By contrast, the extraction temperature was fixed at 25 °C. The arithmetic mean of the chromatogram peak areas was used to generate the response as reported by Bianchin et al. [15].

Despite the fact that, in SPME, DHS or HSSE the temperature plays a key role in the extraction equilibrium of the solutes, in SBSE the effect of temperature is not usually considered during the optimization. Temperature variation could infuence the extraction in two opposite ways. Higher temperatures allow the equilibrium to be reached in a shorter time but, on the other hand, it also increases the sol-ubility of the analytes in water (according to Henry's law) and thus decreases the amount of extracted compounds. Therefore, the bulk of SBSE studies are usually carried out at room temperature [11].

Table 1 Experimental design for optimization of stir bar sorptive extraction at constant honey dilution ratio

	Dil ratio w/v	NaCl mol/L	Area $*10^9$	A %
Exp 1	1:2	0	6.36844	81.1
Exp 4	1:2	2	7.85319	100
Exp 7	1:2	4	2.90502	37.0
Exp 2	1:4	0	1.45543	39.4
Exp 5	1:4	2	3.69576	100
Exp 8	1:4	4	1.70416	46.1
Exp 3	1:6	0	1.30936	90.6
Exp 6	1:6	2	1.44418	100
Exp 9	1:6	4	1.25779	87.1

Table 2 Experimental design for optimization of stir bar sorptive extraction at constant NaCl concentration

	Dil ratio w/v	NaCl mol/L	Area $*10^9$	A %
Exp 1	1:2	0	6.36844	100
Exp 2	1:4	0	1.45543	22.8
Exp 3	1:6	0	1.30936	20.5
Exp 4	1:2	2	7.85319	100
Exp 5	1:4	2	3.69576	47.1
Exp 6	1:6	2	1.44418	18.4
Exp 7	1:2	4	2.92335	100
Exp 8	1:4	4	1.70416	58.0
Exp 9	1:6	4	1.25779	43.0

The matrix effect is one of the main parameters which affects the SBSE. Despite the fact that dilution of the sample could increase both the limit of detection and quantification (LOD and LOQ, respectively), it is also a useful method which infuences the matrix effect. Three concentrations of aqueous honey solution were tested (0.50, 0.25 and 0.17 g of honey per mL of water). Nine experiments were carried out: NaCl concentration was maintained constant at 0, 2 or 4 mol/L. For each NaCl concentration, three experiments were carried out at three different honey dilution (0.50, 0.25 and 0.17 g of honey per mL of water) as reported in Table 1. The biggest TIC total peak area was found for the most concentrated solution, 0.50 mg of honey per mL of water (Fig. 1a). This means that the high concentration of honey's sugars in the solution does not much affect the extraction of volatiles and then the higher honey concentration is the best choice in order to increase the sensitivity.

NaCl and methanol are commonly used in SBSE as matrix modifiers, with the salting-out effect of NaCl being employed to increase the extraction of polar compounds whereas the addition of methanol is used to increase the solubility of nonpolar solutes in water [16]. In addition, it is well known that the addition of salts in water solution decreases the solubility of several gasses and can be used to increase the extraction of the more polar compounds. The VOCs in honey represent a complex and heterogeneous class of compounds with different characteristics [6], and since the target of the analysis was the complete composition of the honey volatiles (which includes several slightly polar compounds) and considering the good capability of PDMS in extracting nonpolar compounds, NaCl was used as matrix modifier. Three concentrations of NaCl (0, 2.0 and 4.0 mol/L) were studied and for each concentration the arithmetic mean of total peak area of the TIC was monitored as reported in Table 2. The biggest TIC total peak area was registered when 2.0 mol/L of NaCl were added to the honey solution (Fig. 1b). As expected, the dual effect of NaCl on volatiles extraction is refected in our results, which indicated the middle concentration of NaCl to be the

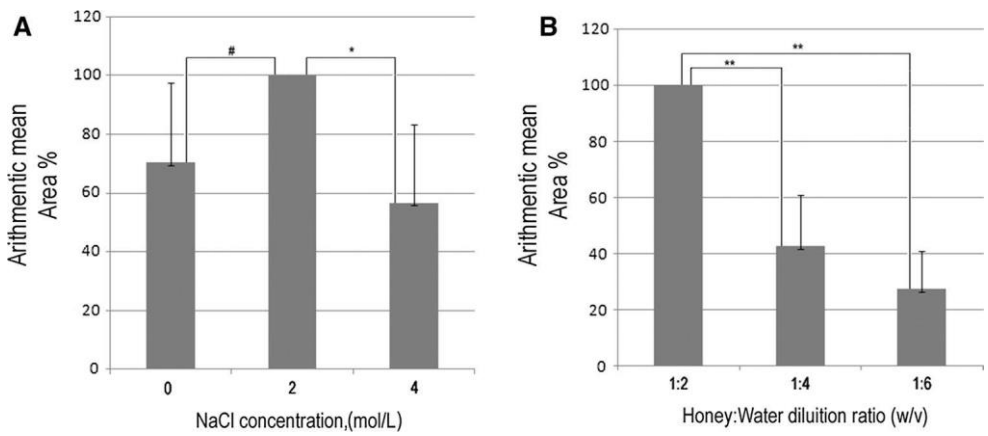

Fig. 1 Effect of salt concentra-tion (**a**) and dilution rate (**b**) on stir bar sorptive extraction; results are expressed as the mean relative area % response. Vertical segments represent standard deviation. # not sta-tistically significant, $*p < 0.01$, $**p < 0.05$

Table 3 Relative percent of compounds extracted by DHS, SBSE, SPME and USE followed by GC–MS analysis

Compounds	DHS	SBSE	SPME	RI_{HP5}	$RI_{VF\text{-}WAX}$	ID
Isoamyl alcohol	5.38	nd	nd	750#	1210	MS, RI, STD
Toluene	4.11	nd	nd	777#	1054	MS, RI, STD
Octen-1-ene*	2.79	nd	0.96	790#	840	MS, RI, STD
Octane	5.74	3.13	12.59	800	801	MS, RI, STD
Honane	nd	tr	0.65	900	901	MS, RI, STD
Heptanal	nd	0.93	0.44	902	1308	MS, RI, STD
α-thujene*	nd	nd	0.46	932	1038	MS, RI
2-Hydroxy-5-methyl-3-hexanone*	5.0	0.8	tr	947	1501	MS, RI
3-Hydroxy-5-methyl-2-hexanone*	4.79	0.5	tr	951	1497	MS, RI
Benzaldehyde	5.76	tr	0.12	960	1568	MS, RI, STD
5-hepten-2-one, 6-methyl-*	nd	1.26	nd	985	1357	MS, RI
p-cymene	0.37	0.60	3.00	1026	1293	MS, RI, STD
Limonene	nd	tr	2.06	1028	1218	MS, RI, STD
Benzyl alcohol	13.83	0.30	0.95	1037	1914	MS, RI, STD
Phenylacetaldehyde*	1.04	nd	nd	1044	1680	MS, RI
γ-terpinene	nd	nd	2.70	1062	1260	MS, RI, STD
Acetophenone	0.90	0.70	nd	1068	1696	MS, RI, STD
Octan-1-ol	nd	1.06	nd	1071	1471	MS, RI, STD
Linalool oxide furanoid cis*	0.93	nd	0.93	1074	1457	MS, RI
p-cymenene*	1.27	nd	0.69	1089	1460	MS, RI
Nonan-2-one*	nd	nd	0.45	1094	1406	MS, RI
Linalool	nd	nd	0.32	1101	1408	MS, RI, STD
Nonanal	18.51	6.77	39.66	1105	1414	MS, RI, STD
Phenylethyl alcohol*	0.84	0.35	0.22	1113	1955	MS, RI
Isophorone <4-keto>*	1.79	1.13	0.54	1144	1738	MS, RI
Nonan-1-ol*	13.93	14.30	22.74	1172	1672	MS, RI
para-cymen 8-ol*	0.84	0.26	0.47	1185	1878	MS, RI
Methyl salicilate	0.80	0.85	0.41	1193	1832	MS, RI, STD
Decanal*	0.73	9.54	1.62	1205	1522	MS, RI
trans-carveol*	nd	0.36	nd	1217	1561	MS, RI
Ethyl salicilate*	nd	0.42	0.19	1269	1865	MS, RI
Decan-1-ol*	nd	0.50	nd	1270	1774	MS, RI
Nonanoic acid*	nd	8.33	4.14	1273	2184	MS, RI
Decan-1-al*	nd	0.51	nd	1307	1629	MS, RI
Neryl acetone/geranyl acetone*	nd	2.65	nd	1443	1882	MS, RI
Dodecan-1ol*	nd	0.96	nd	1471	1981	MS, RI
Tridecanal*	nd	1.07	nd	1510	1843	MS, RI
cis-methyl dihydro jasmonate*	nd	1.50	nd	1666	>2300	MS, RI
Tricosane	nd	0.64	nd	2300	2302	MS, RI, STD
Total identified	89.36	59.41	96.30			

Compounds are listed according crescent retention times in HP5 column. ID: identification method, RI: retention index, nd: not detected, STD: pure standard co-injecton, * tentatively identified, #: calculated on the basis of C8–C9 alkane couple

best choice for the extraction of the whole composition of honey's volatiles.

The chemical composition of the Eucalyptus honey volatiles is reported in Table 3. Results are reported as TIC relative percent area. The use of internal normalization of the chromatogram, without considering any correction factor, has several limitations for obtaining quantitative data [17]. Conversely, when applied to the same sample, the TIC internal normalization serves as a useful tool for the comparison of several techniques applied to the same sample [18]. SBSE volatiles differ qualitatively and quantitatively from those obtained by the SPME and DHS methods.

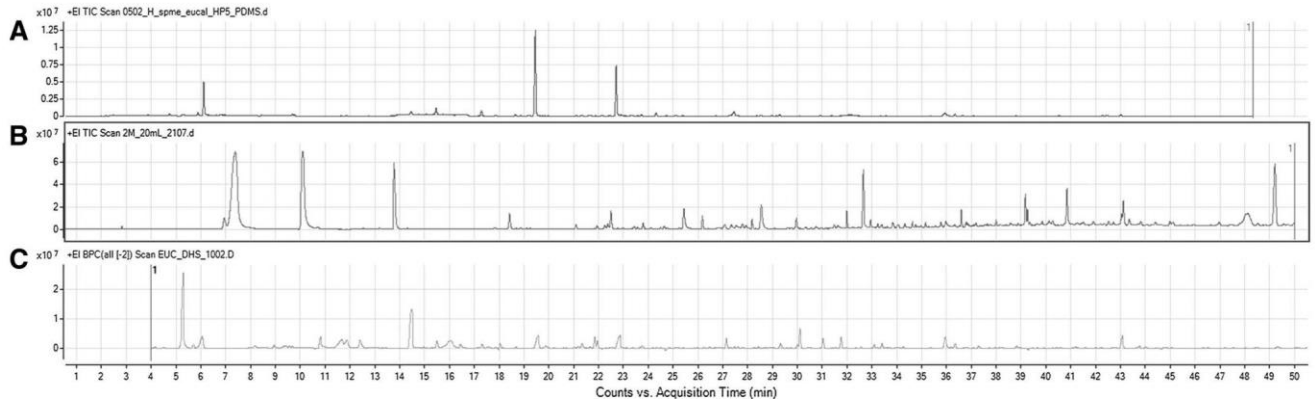

Fig. 2 Raw chromatograms of honey volatiles extracted by solid-phase microextraction technique (**a**), stir bar sorptive extraction technique (**b**) and by dynamic headspace technique (**c**). Deconvolution algorithm and blank subtraction were applied before elaboration data

Although several not identified compounds were detected by SBSE, the chemical composition of Eucalyptus honey volatiles extracted by all three techniques shows the presence of some typical foral markers: Castro-Varquez et al. [19] reported *p*-cymene and its derivate alcohol as markers for Eucalyptus honey, whereas Piasenzotto et al. [20] identified nonanoic acid and acetoin as foral markers. SBSE, DHS and SPME all showed the presence, in our sample of Eucalyptus honey, of *p*-cymene and *para*-cymen-8-ol; in addition SBSE and SPME revealed the presence of nonanoic acid. More recently, some authors [21, 22] indicated as foral markers for Eucalyptus honey also 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone which were detected by all three extraction techniques confirming the botanical source declared by beekeepers.

The Twister® stir bar is coated with PDMS, so direct comparisons were made with SPME using a fiber with a PDMS stationary extraction phase and with DHS trapped in a PDMS cartridge. In general, SBSE showed better sensitivity, in comparison with SPME and DHS for the extraction of less volatile components (Fig. 2). As reported in Table 3, over n-dodecane (retention index = 1200 on HP5 column) SBSE shows the presence of ten compounds, whereas SPME shows two compounds and DHS shows a fat chromatogram. Our results confirm a general trend reported in the literature, which show the higher sensitivity of SBSE in the extraction of less volatile compounds in comparison with HS methods [23, 24]. On the contrary by HS techniques, more sensitivity was found for the highly volatile compounds (Fig. 2), supporting previous literature data [8, 24]: isoamyl alcohol and toluene were detected only by DHS, octen-1-ene was detected by SPME and DHS while nonane and α-thujene were detected only by SPME technique.

Since in SBSE the stir bar is immersed in the solution there is a direct contact of solutes with the coating material, therefore SBSE extraction is similar to a liquid–liquid extraction with a nonpolar solvent. Jerkovic et al. [25] reported that, apart from the absence of thermally generated artifacts, the main advantages of honey ultra-sound assisted extraction (USE) is that it enables the extraction of the less volatile compounds. SBSE, like USE, does not require thermal treatment and has good sensitivity for the extraction of less volatile compounds. On the other hand, despite some new materials having recently been used to coat the Twister® stir bars [26], unlike SPME, only a limited number of coating phases are commercially avail-able, thus limiting the performance and applicability [27]. Several highly volatile compounds like isoamyl alcohol, 1-octene or toluene, were not detected by SBSE. In addi-tion, several polar compounds such as short-chain alde-hydes and alcohols, typically present in honeys [20] were not detected by either of extraction techniques, highlighting the disadvantages of PDMS stationary phase.

SBSE resulted all useful tools for honey VOCs analysis; anyway representative extract of honey volatiles is very difficult to obtain, and the isolation of volatile components from honey needs the application of several techniques. SPME and DHS in comparison to SBSE need shorter extraction times and have higher level of automation. Furthermore, DHS and SBSE need a TDU while SPME require just a GC injector.

Conclusion

The SBSE technique was successfully applied for the fractionation of VOCs from Eucalyptus honey. To the best of our knowledge, this is the first report on extraction of honey volatiles by SBSE. The variables of NaCl and honey concentration of the studied solutions were optimized in order to obtain the best chromatographic response. The more

concentrated honey solution resulted in the best response, whereas the middle (2 mol/L) concentration of NaCl, used as matrix modifier, shows the best result. SBSE confirmed its ability in the extraction of less volatile compounds, though further studies are required to explore the capability of new coating materials. Although SBSE revealed the presence of the main foral markers of Eucalyptus honey, to obtain a representative fingerprint of volatiles from a complex mixture such as honey, it would be better to utilize different techniques to extract different chemical families of compounds.

Compliance with ethical standards

Confict of interest The authors declare that there is no confict of interest regarding the publication of this paper.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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