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Optimization of a miniaturized solid-phase microextraction method followed by gas chromatography mass spectrometry for the determination of twenty four volatile and semivolatile compounds in honey from Galicia (NW Spain) and foreign countries

Lua Vazquez [a], Maria Celeiro [a], Meruyert Sergazina [a,b], Thierry Dagnac [c], Maria Llompart [a,*]

[a] CRETUS Institute, Department of Analytical Chemistry, Nutrition and Food Science, Universidade de Santiago de Compostela, E-15782, Santiago de Compostela, Spain
[b] Department of Chemistry, Institute of Natural Science and Geography, Abai Kazakh National Pedagogical University, Almaty, Kazakhstan
[c] Galician Agency for Food Quality - Agronomic Research Centre (AGACAL-CIAM) – Unit of Organic Contaminants, Apartado 10, E-15080, A Coruña, Spain

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ABSTRACT

An analytical miniaturized methodology based on solid-phase-microextraction (mini_SPME) followed by gas chromatography coupled to mass spectrometry (GC-MS) has been developed for the identification of volatile and semivolatile compounds in honey samples. The main influential experimental parameters, such as the type of fibre coating, extraction temperature, solvent addition, extraction mode, ionic strength, and sample dilution were optimized. A design of experiments (DOE) was conducted including twenty-four target compounds. The final extraction conditions comprised the use of 200 mg of honey mixed with 200 μL of water (100%, w/v), employing a DVB/CAR/PDMS fibre in the headspace mode at 100 °C for 30 min. The mini_SPME-GC-MS method was successfully validated in terms of linearity, repeatability, reproducibility and accuracy. Finally, it was applied to a broad range of varieties of real honey samples from Galicia (NW Spain), as well as some foreign honeys, demonstrating suitability.

1. Introduction

Honey is a product consumed worldwide and its use is justified by physicochemical, medicinal and nutritional characteristics providing therapeutic effects. Properties of each honey are conditioned by their botanical and geographical origin. It is important to guarantee the authenticity and quality as well as to identify frauds and prevents overpayments. The biological value of honey is due to the presence of sugars, proteins, amino acids, enzymes, organic acids, vitamins, minerals, phenolic and volatile compounds (Kaškonienė and Venskutonis, 2010; Kortesniemi et al., 2018; Bianchi et al., 2005; Verzera et al., 2014; da Silva et al., 2015).

The aromatic profile of honey is important since it forms the organoleptic characteristics, identity and quality of honey. Aroma compounds are present in honey as complex mixtures of volatile components of different chemical families, and this composition depends on the floral origin of the nectar extracted by bees (Rahman et al., 2017; Cuevas-Glory et al., 2007).

In concordance to the geographical origin of production, the European Union (EU) under the labels of Protected Designation of Origin (PDO) and Protected Geographical Identification (PGI) classify honey. Honeys with these classifications commonly have particular characteristics linked to a specific place or a local environment. Actually, Spain is the second country with the highest number of honeys registered in the EU of which five are PDO and one PGI (Galician honey NW Spain) (DOOR, 2019).

Different sample preparation techniques are found in the literature, as well as different types of analysis for honey characterization. The classic sample preparation, such as solid-phase extraction (SPE), ultrasound-assisted extraction (UAE) and liquid–liquid extraction (LLE) are used for the extraction of volatile, semivolatile and low volatile compounds from honey (Alissandrakis et al., 2009; Dobrinas et al., 2008; Vazquez et al., 2006). Most of these techniques are subject to inconveniences, requiring large amounts of solvents or highly toxic solvents. In addition, most methods are labor-intensive and time-consuming requiring multiple steps such as sonication,

* Corresponding author.
E-mail address: maria.llompart@usc.es (M. Llompart).

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Table 1
Target compounds, CAS number, molecular weight (MW), boiling point, retention time and quantification and confirmation ions. Precision of the mini_SPME process in honey is also included (two last columns).

Nº	Compounds	CAS Number	MW, g mol^{-1}	Boiling Point, °C	Retention time, min	Quantification ion	Confirmation ion	Precision, %	
								Repeatability (n = 3)	Reproducibility (n = 5)
1	Cis-Linalool oxide	1365-19-1	170.25	188	10.55	59	59, 94, 111	9.6	10
2	Furfural	98-01-1	96.08	161	10.81	96	96, 95, 39	15	19
3	α-Ionene	475-03-6	174.29	238	11.04	159	159, 174, 131	7.3	16
4	Trans-Linalool oxide	34995-77-2	170.25	188	11.16	59	59, 94, 111	5.3	8.2
5	Benzaldehyde	100-52-7	106.12	178	12.07	106	106, 77, 105	13	16
6	β-Linalool	78-70-6	154.25	198	12.23	93	93, 71, 55	11	14
IS	1-Octanol	111-87-5	130.23	195	12.35	56	56, 55, 41	14	17
7	Hotrienol	20053-88-7	152.23	228	13.26	71	71, 82, 67	3.2	12
8	Isophorone	78-59-1	138.21	215	13.48	82	82, 138, 54	5.0	8.0
9	4-Oxoisophorone	1125-21-9	152.19	214	15.23	96	96, 152, 68	3.0	5.8
10	1,1,5-Trimethyl-1,2-dihydronaphthalene (TDN)	30364-38-6	172.27	270	16.33	157	157, 142, 172	2.7	10
11	β-Damascenone	23696-85-7	190.28	275	17.73	69	69, 121, 41	8.2	8.6
12	Phenylfurane	13679-41-9	144.17	222	18.25	144	144, 115, 145	2.9	16
13	Benzyl alcohol	100-51-6	108.14	232	18.63	108	108, 79, 107	12	12
14	Phenylethyl alcohol	60-12-8	122.16	218	19.46	91	91, 92, 65	14	13
15	Anisic aldehyde	123-11-5	136.15	248	21.67	135	135, 136, 77	9.9	17
16	1-(2,3-Dimethylphenyl) ethanone (DMPE)	2142-71-4	148.20	245	22.85	133	133, 105, 148	2.8	8.3
17	Megastigmatrienone A (MTMA)	38818-55-2	190.28	311	24.27	190	190, 148, 175	3.3	5.3
18	γ-Eudesmol	15051-81-7	220.37	301	24.34	161	161, 189, 204	4.4	4.9
IS	Celestolide	13171-00-1	244.37	319	24.45	229	229, 244, 43	2.4	13
19	Megastigmatrienone B (MTMB)	38818-55-2	190.28	311	25.06	190	190, 148, 175	4.3	11
20	α -Eudesmol	473-16-5	220.37	301	25.34	59	59, 161, 189, 149	2.5	5.5
21	β-Eudesmol	473-15-4	220.37	301	25.53	59	59, 149, 108	5.0	8.6
22	α-Gurjunene	489-40-7	204.35	298	25.91	204	204, 161, 105	1.5	10
23	Megastigmatrienone C (MTMC)	38818-55-2	190.28	311	26.86	190	190, 148,175	7.1	14
24	5-Hydroxymethylfurfural (HMF)	67-47-0	126.11	291	29.61	97	97, 126, 41	20	12

centrifugation, filtration and even further clean-up.

Solid-phase microextraction (SPME) is one of the preferred techniques for extracting organic compounds. The main advantages of SPME include simplicity, high sensitivity, solvent-free, one step sample preparation and low cost per analysis. It has been successfully applied, mainly for aqueous samples, and widely used in environmental and food analysis (Llompart et al., 2019; Souza-Silva et al., 2015). Regarding honey analysis, some SPME methodologies for the isolation and identification of compounds from volatile and semivolatile fractions are reported in the literature (Alissandrakis et al., 2007; Belinato et al., 2021; Karabagias et al., 2020; Bianchin et al., 2014; Kortesniemi et al., 2018; Moniruzzaman et al., 2014; Pérez et al., 2002; Plutowska et al., 2011; Rodríguez-Flores et al., 2021; Wang et al., 2019). Most of them employ a high amount of honey and the optimization is based on systematically study of one parameter at a time; therefore, factor interactions are not considered. Gas chromatography combined with mass spectrometry (GC-MS) provides high separation efficiency and the reproducibility of generated mass spectra locates it as the preferred technique for the study of the aroma profile of honey (Rivellino et al., 2013; Špánik et al., 2014; Seisonen et al., 2015; Robotti et al., 2017; Soria et al., 2009). Combined SPME-GC-MS offers many advantages since the SPME fibre is directly transferred into the injector of the gas chromatograph for thermal desorption and analysis, and no intermediate steps are necessary. Also, the obtained mass spectra provide an unequivocal identification of the substances extracted by SPME.

The aim of this work is the development of a miniaturized and sustainable analytical methodology based on miniaturized SPME (mini_SPME) followed by GC-MS for the determination of volatile and semivolatile compounds in honey. This is the first time that 1.8 mL vials are proposed to carry out the SPME procedure employing a very low amount of honey. The most critical parameters (i.e. extraction temperature, fibre coating, addition of NaCl, sample dilution) were optimized by an experimental design strategy to obtain the highest extraction efficiency. Finally, the validated method was applied to a broad range of varieties of real honey samples, most of them from Galicia (NW, Spain), the only honey in Spain with Protected Geographical Identification.

2. Experimental

2.1. Honey samples

Different types of honey samples including several varieties such as multi-floral (MF), honeydew (HD), heather (HE), chestnut (CN) and blackberry (BL) were collected in Galicia (NW Spain). The samples were kindly supplied by I.X.P. Mel de Galicia. Eight honey samples from foreign countries (Italy (IT), France (FR), Kazakhstan (KZ) and Greece (GR)) including different varieties (multi-floral, lavender (LV), orange (OR) and thyme (TH)) were also analyzed. The samples were received in

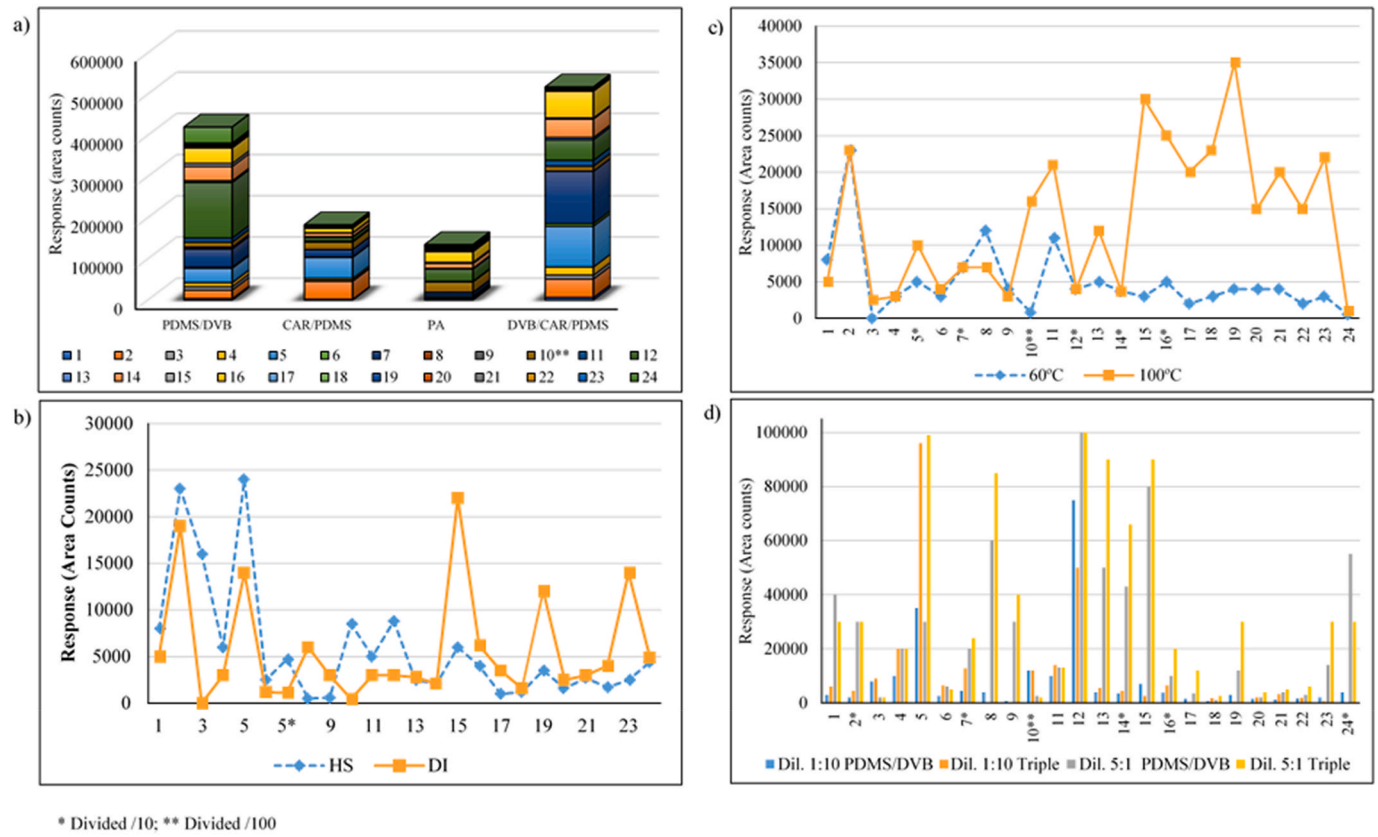


Fig. 1. Chromatographic responses for: a) Σ of individual areas obtained with the different fibre coatings (PDMS/DVB, PA, CAR/PDMS, DVB/CAR/PDMS); b) SPME mode (HS and DI) for PDMS/DVB fibre; c) extraction temperature (60 °C and 100 °C); d) dilution factor (1:10 and 5:1, w/v) for the target compounds (see compound codes in Table 1).

glass jars sealed with an aluminium cap, stored at controlled temperature (20 °C) and protected from light until analysis.

2.2. Reagents and materials

Methanol and ultrapure water MS grade were supplied by Scharlab (Barcelona, Spain). Acetone and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Linalool oxide, linalool, β-Damascenone, benzyl alcohol and eudesmol were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and phenylethyl alcohol by Chemservice (West Chester, PA, USA). 1-Octanol and celestolide, employed as internal standards (IS), were obtained by Merck and LGC standards (Teddington, UK), respectively. Individual stock solutions of each compound (concentration about 10,000 mg L^{-1}) were prepared in methanol. Further dilutions and mixtures were prepared in acetone. Stock solutions were stored in glass vials and protected from light at −20 °C. All solvents and reagents were of analytical grade.

Commercial 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB), 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 85 μm polyacrylate (PA) and 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fibres and a manual SPME holder were obtained from Supelco (Bellefonte, PA, USA). Prior the first use, the fibres were conditioned as recommended by the manufacturer, inserting them in the GC injector under helium flow at 250 °C (PDMS/DVB), 270 °C (DVB/CAR/PDMS), 280 °C (PA) and 300 °C (CAR/PDMS) for 30 min.

2.3. Solid-phase microextraction (SPME)

Under the optimized conditions (see Results and Discussion), 200 mg of honey were placed in a 1.8 mL glass vial. Then, 2 μL of a 20 mg L^{-1} acetonic solution containing the internal standards (1-Octanol and celestolide) were added giving a final concentration of 200 ng g^{-1}, and the sample was diluted by adding 200 μL of ultrapure water. The vial was sealed with an aluminium cap furnished with PTFE-faced septa and immersed into a water bath maintained at 100 °C. The samples were magnetically stirred using a tiny piece of clip (0.8 mm diameter, 3 mm length) made of stainless steel. After 2 min of sample equilibration, the DVB/CAR/PDMS (triple) fibre was exposed to the headspace over the sample (headspace mode, HS) for 30 min. Afterwards, the fibre was retracted into the needle of the holder syringe and immediately thermally desorbed at 270 °C in the GC injection port for 5 min, and GC-MS analysis was carried out.

2.4. GC-MS analysis

The GC–MS analysis was performed using an Agilent 7890A (GC)-Agilent 5975C inert MSD with triple axis detector from Agilent Technologies (Palo Alto, CA, USA). Separation was carried out on a DB-WAX capillary column (50 m × 0.20 mm i.d., 0.20 μm film thickness) obtained from Agilent Technologies. Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 0.6 mL min^{-1}. The GC oven temperature was programmed from 70 °C (held 1 min) to 120 °C at 10 °C min^{-1} and, finally, to 240 °C at 5 °C min^{-1} (held 2 min). The total run time was 32 min. Splitless mode was used for injection (1 min, 75 mL min^{-1}). The injector temperature was set at 270 °C. The mass spectrometer detector (MSD) was operated in the electron ionization (EI) positive mode (+70 eV), and the temperatures of the transfer line, the quadrupole and the ion source were set at 230 °C, 150 °C and 230 °C, respectively. Full Scan (FS) acquisition mode was employed monitoring mass/charge (*m/z*) fragments between 35 and 400. The system was operated by Agilent MSD ChemStation E.02.00.493 software. The

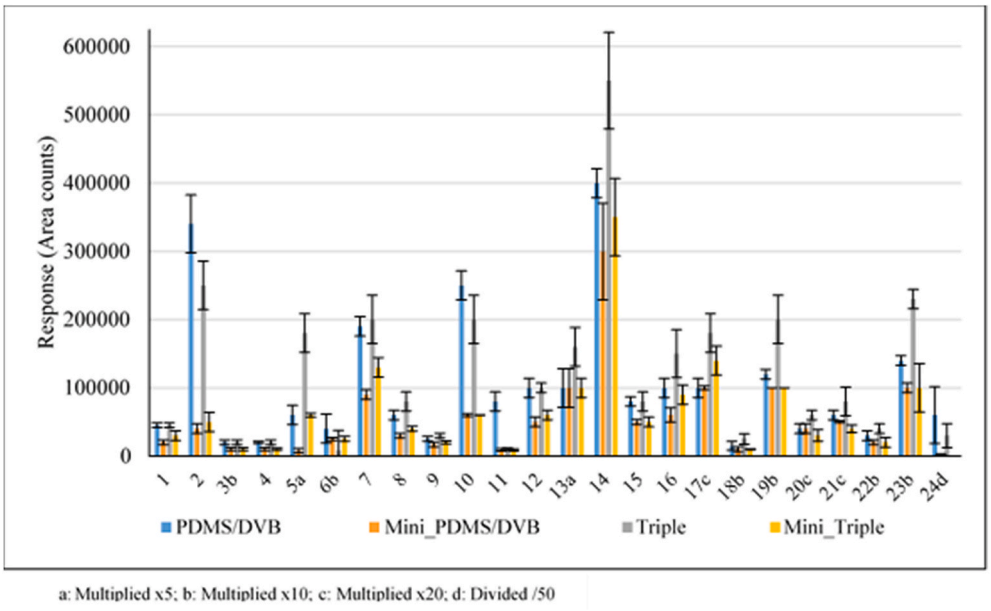


Fig. 2. Assessment of the miniaturization of the SPME procedure (see compound codes in Table 1).

quantification and identification ions for each compound are shown in Table 1. The analyte peak areas were normalized by that of the internal standards (area/area IS) x100 1-Octanol for the most volatile compounds (1–14 in Table 1), and celestolide, for the last eluted compounds (15–24 in Table 1).

2.5. Statistical analysis

Basic and descriptive analysis were performed using the software package Statgraphics Centurion XVIII (Manungistics, Rockville, MD, USA).

3. Results and discussion

3.1. Selection of the target compounds

A multi-floral honey sample was selected to carry out initial screening analysis. This honey variety is expected to contain a higher number of compounds than other types of honey since it is collected from a variety of crops, flowers, and herbs. Fig. S1 shows its chromatographic profile obtained by HS-SPME-GC-MS. The most abundant compounds as well as several compounds found in literature were selected as targets to conduct the optimization study, giving a total of 24 volatile and semivolatile species. Table 1 includes the target compounds and internal standards (1-Octanol and celestolide), CAS number, molecular weight, boiling point, retention time, as well as the quantification and identification ions for each identified or tentatively identified compound. Compound identification was based on comparison (match $>$ 80%) between the obtained experimental MS spectral and those provided by the commercial spectral library database (NIST version 2.0, National Institute of Standards and Technology). In addition, comparison with real standards available in the lab was also conducted.

3.2. Preliminary SPME experiments

The type of fibre coating is one of the most critical SPME parameters affecting the extraction efficiency. Since the target compounds present a broad range of polarity, the extraction efficiency of four types of SPME fibres was assessed: PDMS/DVB (medium polarity), DVB/CAR/PDMS (wide polarity range), PA (high polarity) and CAR/PDMS (high polarity). Experiments were carried out employing 1.2 g of multi-floral honey diluted 1:10 (w/v) in water and performing SPME in HS mode at 60 °C. The extraction time was 30 min. Results are summarized in Fig. 1a (see also Fig. S2). As can be seen, the fibres DVB/CAR/PDMS and PDMS/DVB showed the highest chromatographic responses for all compounds. In general, the most volatile compounds (e.g. compounds 1–7: cis and trans-Linalool oxide, furfural, α-Ionene, benzaldehyde, β-Linalool and hotrienol) showed clearly higher responses employing the DVB/CAR/PDMS fibre, whereas the use of the PDMS/DVB fibre showed similar or better results for the least volatile compounds, being even the only coating able to extract isophorone and 5-Hydroxymethylfurfural (see Fig. S2).

The extraction mode is a critical SPME parameter and HS and direct immersion (DI) modes were evaluated, employing the most suitable fibres, PDMS/DVB and DVB/CAR/PDMS. Experiments were performed under the same previously indicated conditions. Results for PDMS/DVB are shown in Fig. 1b. As it could be expected, higher responses were obtained in the HS mode for the most volatile compounds and, even one of the compounds, α-Ionene (number 3), was only detected by HS. On the other hand, for the least volatile compounds, the use of DI offered better responses. The background level was similar in the two modes. In view of the results, it can be concluded that both sampling modes could be used, being HS more suitable for volatiles and DI for semivolatiles. In the literature, most studies are based on HS-SPME and DI is not considered excluding few exceptions (Peña et al., 2004; Campillo et al., 2006), although it appears as an interesting approach, specially for semivolatile species.

The temperature is also an important factor to achieve a suitable extraction since it causes different effects on the SPME technique. As can be seen in Fig. 1c, performing the HS extraction at the highest temperature, 100 °C, gave much higher responses for 15 out of the 24 target compounds, especially the least volatile ones for which responses increased between 3 and 8 times. For the most volatile analytes (e.g. trans-Linalool oxide, furfural, β-Linalool, hotrienol), similar or slightly higher responses were obtained at 60 °C, excluding α-Ionone and benzylaldehyde which responses significantly improved at 100 °C. In other studies, higher temperatures than 70 °C were not tested appealing to the possibility of formation of artefacts or compound degradation (Moniruzzaman et al., 2014; Plutowska et al., 2011). Those undesirable effects were not observed in this study.

Honey is a viscous matter which makes not easy to work with. However, due to its high content of sugars and other soluble compounds,

Table 2
Experimental design: ANOVA table for main factors and interactions. Values in bold denote statistical significance (p-value < 0.05).

Compounds	Temperature (A)		Fibre (B)		NaCl (C)		Dilution (D)		AB		CD	
	F	P	F	P	F	P	F	P	F	P	F	P
Cis-Linalool oxide	2.54	0.172	0.14	0.726	0.01	0.916	0.02	0.893	2.65	0.165	4.73	0.082
Furfural	0.11	0.759	4.46	0.102	0.96	0.382	0.78	0.426	**13.98**	**0.020**	7.36	0.053
α-Ionene	**27.87**	**0.003**	0.68	0.447	0.66	0.453	3.47	0.122	6.35	0.053	**10.75**	**0.022**
Trans-Linalool oxide	4.25	0.094	0.33	0.591	0.09	0.778	0.07	0.799	3.53	0.119	4.97	0.076
Benzaldehyde	**13.00**	**0.015**	4.25	0.094	**7.61**	**0.040**	3.85	0.107	**20.26**	**0.006**	**28.26**	**0.003**
β-Linalool	4.98	0.076	1.28	0.309	2.72	0.160	2.94	0.147	3.65	0.114	**7.89**	**0.038**
Hotrienol	4.19	0.096	0.31	0.601	0.00	0.982	0.05	0.839	5.21	0.071	5.77	0.061
Isophorone	0.02	0.884	0.09	0.774	1.84	0.233	0.49	0.517	2.19	0.199	**9.41**	**0.028**
4-Oxoxisophorone	0.20	0.674	0.35	0.581	0.26	0.630	0.00	0.997	1.96	0.221	**6.76**	**0.048**
TDN	**8.54**	**0.032**	0.04	0.856	**16.55**	**0.010**	**13.21**	**0.015**	**8.61**	**0.033**	**20.21**	**0.006**
β-Damascenone	1.74	0.245	0.17	0.698	5.16	0.072	4.56	0.086	2.86	0.152	**9.62**	**0.027**
Phenylfurane	0.73	0.432	1.19	0.325	3.99	0.102	1.98	0.219	2.85	0.152	**8.22**	**0.035**
Benzyl alcohol	3.14	0.137	2.67	0.163	0.21	0.669	0.23	0.652	4.91	0.078	**7.55**	**0.040**
Phenylethyl alcohol	**11.26**	**0.020**	0.48	0.518	6.31	0.054	**9.72**	**0.026**	**12.52**	**0.017**	**47.90**	**0.001**
Anisic aldehyde	**25.05**	**0.004**	1.47	0.279	1.00	0.363	1.71	0.248	**26.92**	**0.004**	**46.98**	**0.001**
DMPE	**13.33**	**0.015**	1.04	0.355	2.13	0.204	1.30	0.305	**11.71**	**0.019**	**25.35**	**0.004**
MTMA	**20.57**	**0.006**	0.27	0.628	0.52	0.503	0.49	0.515	**16.74**	**0.009**	**18.73**	**0.008**
γ-Eudesmol	5.94	0.059	2.54	0.172	2.23	0.195	3.96	0.103	3.35	0.127	14.29	0.063
MTMB	**21.11**	**0.006**	0.21	0.669	0.53	0.498	0.41	0.551	**16.76**	**0.009**	**26.18**	**0.004**
α-Eudesmol	**17.54**	**0.013**	0.92	0.392	0.02	0.884	0.02	0.885	**13.41**	**0.022**	**9.23**	**0.039**
β-Eudesmol	**28.89**	**0.006**	0.04	0.860	**11.14**	**0.029**	**10.72**	**0.031**	**19.45**	**0.012**	**23.78**	**0.008**
α-Gurjunene	**17.67**	**0.014**	0.01	0.923	1.76	0.255	2.14	0.218	**13.88**	**0.020**	**18.77**	**0.012**
MTMC	**18.05**	**0.013**	0.42	0.552	0.03	0.864	0.12	0.750	**13.46**	**0.021**	**22.08**	**0.009**
HMF	**52.50**	**0.019**	**32.77**	**0.029**	**287.79**	**0.004**	15.64	0.058	**833.09**	**0.001**	8.89	0.097

it can be easily dissolved in water so that it is very common to mix the sample with water before accomplishing extraction (Bianchin et al., 2014; Soria et al., 2009). Considering previous studies, initially, two extreme dilution factors were evaluated 1:10 (w/v) and 5:1 (w/v). For the dilution factor 1:10 (w/v), the same amount of honey and water than in previous experiments were employed (1.2 g and 12 mL). For the dilution factor 5:1 (w/v), 2 g of honey and 400 μL of ultrapure water were used (Plutowska et al., 2011). Results are shown in Fig. 1d. As can be seen, higher chromatographic responses were clearly obtained for 18 of the 24 target compounds employing dilution 5:1 (w/v). It is worth noting that these experiments comparing the sample dilution were performed employing the same vial size.

Also, the addition of a small amount of an organic solvent could improve the extraction of organic compounds from complex matrices, so that this protocol was assessed for HS-SPME adding 2% of MeOH. As the chromatographic responses did not improve (data not shown), organic solvent addition was discarded, allowing a most environmentally friendly method.

3.3. Miniaturization

One of the trends in sample preparation consists on the miniaturization of the processes and techniques. Aiming at sustainable features, researchers seek the minimization of the cost and time of preparation as well as the production of the least amount of residues as possible and the development of procedures easy to implement in other laboratories. SPME has been proposed for the honey extraction but, until now, the miniaturization of the whole process has not been proposed. In fact, in most studies the amount of sample was set to several grams (Verzera et al., 2014; Rodríguez-Flores et al., 2021). With the purpose of miniaturizing the procedure, the extraction was carried out with 0.2 g of honey in conventional 1.8 mL glass vials usually employed for GC or LC analysis. A small piece of clip (0.8 mm diameter, 3 mm length) made of stainless steel was used to magnetically stir the sample, avoiding the use of magnetic bars with Teflon coating. The protocol cost is negligible since one clip is enough for performing up to 30 extractions. 40 μL of ultrapure water were added to the 0.2 g of honey since in the preliminary experiments the sample dilution 5:1 (w/v) showed higher responses. The extraction was performed in the HS mode. Fig. 2 compares the results for the miniaturized procedure and the previously obtained (2 g of honey), employing both PDMS/DVB and DVB/CAR/PDMS fibres. As can be seen, although the amount of honey is 10 times less in the miniaturized procedure, the chromatographic responses are quite comparable. The response decrease using the mini_SPME procedure was only 50% or lower (excluding furfural and 1,1,5-Trimethyl-1,2-dihydronaphtalene). For the PDMS/DVB fibre, the response was similar for both procedures and for many compounds such as benzaldehyde, benzyl alcohol, megastigmatrienone A, α-Eudesmol and β-Eudesmol (numbers 5, 13, 17, 20, 21, in Fig. 2).

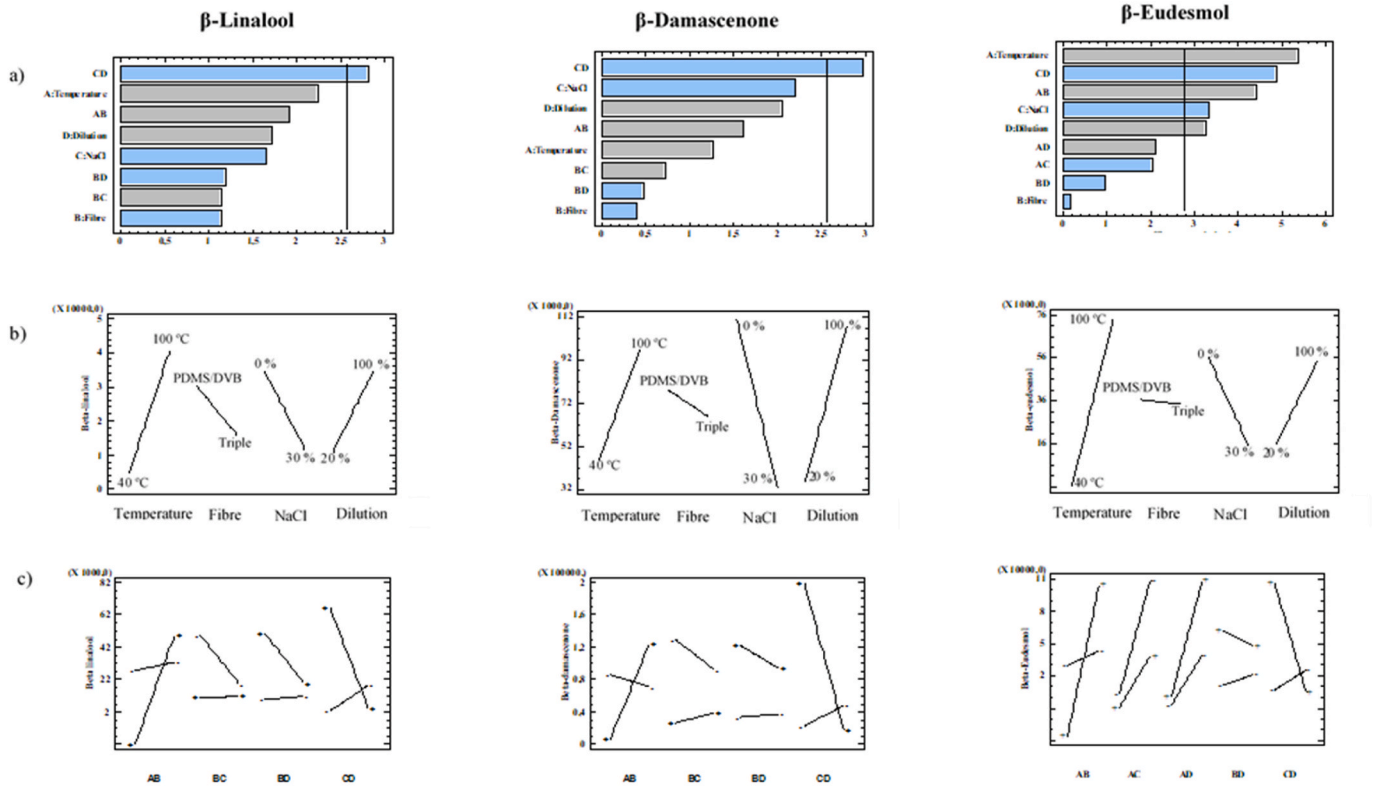
Therefore, the miniaturized method appears as an interesting alternative to perform honey analysis enabling the use of a very small amount of honey, up to 30 times less than in other studies (Rodríguez-Flores et al., 2021). Also, the sample handling is much easier because honey is a viscous stickly matrix and it is easily homogenized, thermostatized and stirred by means of the proposed procedure. Finally, the consumption of internal standard is much lower and residues generation are negligible.

3.3.1. Optimization by an experimental design approach

In an attempt to develop an efficient extraction using the mini_SPME technique, an experimental design approach was implemented. It is important to study not only the individual effects of the factors affecting the SPME process, but also the possible interaction effects between them.

Based on preliminary experiments and on the literature, four critical parameters that can affect the SPME efficiency were included in the experimental design: the temperature (Factor A), the SPME fibre coating (Factor B), the salting out effect (Factor C), and the addition of water (Factor D). The extraction temperature was studied at three levels between 40 °C and 100 °C. The other three factors were studied at two levels. The two fibres included were PDMS/DVB and DVB/CAR/PDMS. The sample dilution, which demonstrated to be an important factor in preliminary experiments, was also evaluated (20%–100%, w/v). Finally, the addition of salt (NaCl) (Factor C) was considered (0%–30%, w/v).

A mixed level fractional factorial design ($3{\cdot}2^{3-1}$) was chosen, including two central points, giving rise to a total of 14 experiments that were only performed in the HS mode since the low honey dilution in water for some experiments could damage the fibre. The sample amount



The code (-) and (+) of the factors correspond to the low and high level of the factors (A: Temperature, C: NaCl and D: Dilution). For factor D (Fibre), the code (-) correspond to PDMS/DVB and (+) to Triple.

Fig. 3. a) Pareto charts, b) main effects and c) interaction plots for β-Linalool, β-Damascenone and β-Eudesmol.

was fixed at 0.2 g and the extraction time at 30 min. The purpose was the development of a miniaturized and sensitive high throughput procedure so that 30 min appears as a reasonable compromise to achieve good signals taking into account the chromatographic run time (32 min). The experimental responses were the peak areas of the 24 target compounds (see Table 1).

The results of the analysis of variance (ANOVA) are shown in Table 2. For the sake of simplicity, only main factors as well as significant second order interactions were included in the table. The ANOVA describes the impact of the studied factors on the obtained responses. The F-ratios measure the contribution of each factor and the interaction on the variance of the response, and the *p*-value tests the statistical significance. Factors or interactions with *p*-values lower than 0.05, denote statistical significance at the 95% confidence level.

As can be seen in Table 2, the temperature (Factor A) was the most relevant factor being statistically significant for 13 out of the 24 target compounds, whereas the fibre (Factor B) was only significant for one compound (5-Hydroxymethylfurfural). In fact, in preliminary experiments, both fibres also showed similar responses in most cases. The NaCl addition (Factor C) and sample dilution (Factor D) were statistically significant for only 4 and 3 compounds, respectively. Nevertheless, the interaction of these factors CD was very important, being the most influential interaction for many analytes (see F and p values in Table 2). In addition, the AB interaction, temperature-fibre, was also statistically significant for 13 compounds. The other second order interactions were not relevant.

Some of the graphical tools provided by the statistical software easily visualize the influence of the factors, such as the Pareto charts, the main effects and the interaction plots. In Fig. 3 some representative examples are included. In the Pareto charts (Fig. 3a), the length of each bar is proportional to the effect of the corresponding factor. The vertical line represents the statistical significance bound (95% confidence level). The main effects plots (Fig. 3b) show the main effects with a line drawn between the low (−) and the high (+) level of the factor. The length of the line is proportional to the magnitude of the effect. As it was commented, the extraction temperature (A) was significant for 13 compounds (see Table 2, and, as example, the pareto for β-Eudesmol Fig. 3a). In all cases, higher responses were obtained performing the extraction at 100 °C, as can be seen in the main effect plots in Fig. 3b. On the other hand, the fibre (B) was not a significant factor, and both fibres might initially be suitable to perform extraction, although it would be necessary to evaluate the interaction temperature-fibre (AB) since it was significant for half of the compounds. The NaCl addition (C) and sample dilution (D) were also non-significant in almost all cases; however, CD interaction was significant for most compounds and, therefore, it must be considered. Some interaction plots, helping to easily visualize optimal conditions, are shown in Fig. 3c. For 19 compounds, the interaction salt addition-sample dilution (CD) was statistically significant (see ANOVA in Table 2); the highest responses were obtained without NaCl addition (0%) and with a dilution factor of 100% (see CD in Fig. 3c). Therefore, these conditions should be selected as the most favourable ones. The interaction extraction temperature-type of fibre (AB) was statistically significant for 13 compounds, the optimal conditions involving the use of the DVB/CAR/PDMS (triple) fibre at 100 °C in all cases (see AB in Fig. 3c). This study highlights the importance of evaluating not only the main effects, but also interaction effects, since quite often, second order factors are more important than first order factors, and influence the selection of the optimal parameter values.

In brief, and based on the obtained results, the most favourable SPME conditions for conducting the miniaturized methodology (mini_SPME) implies the use of DVB/CAR/PDMS (triple) fibre at 100 °C, the dilution 1:1 (w/v) of the honey in water (200 mg honey/200 μL ultrapure water) without NaCl addition. These conditions were selected for the next studies, including the method validation.

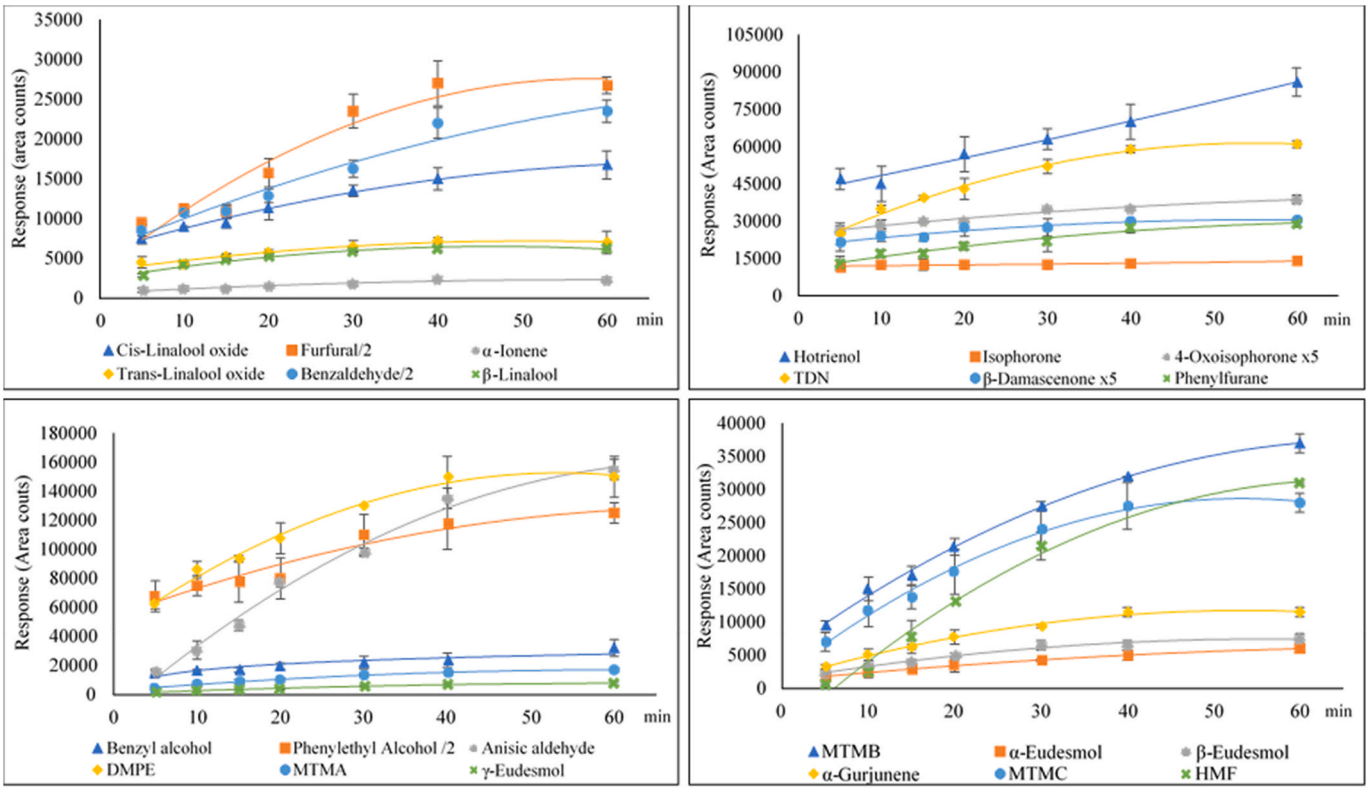


Fig. 4. Time profile curves studied at 5, 10, 15, 20, 30, 40 and 60 min.

3.4. Extraction time profile

In SPME, the time needed to reach equilibrium depends, among others, on the properties of the target analytes, the sample matrix and the fibre coating. Under the optimized experimental conditions, several extraction times between 5 and 60 min (5, 10, 15, 20, 30, 40, 60 min) were tested. Time profile curves for the 24 compounds are depicted in Fig. 4. As can be seen, the time needed to reach equilibrium depends on the compound. In general, the first eluted compounds reach equilibrium in about 30 min of exposure, and most of the remaining ones reach equilibrium in the interval studied (60 min), demonstrating that the extraction process is quite fast. An extraction time of 30 min appears to be a good compromise to define a sensitive and high throughput extraction.

3.5. Mini_SPME-GC-MS performance

Method performance was evaluated in terms of linearity, precision (repeatability, reproducibility), and accuracy.

For the precision study, the multi-floral honey used for conducting the optimization experiments was employed. This sample contains all target compounds and, in this way, precision could be evaluated in a more realistic way than that based on the use of spiked samples. Honey extraction was carried out over different days, and intra-day (n = 3) and inter-day (n = 5) precision were assessed (Table 1 (two last columns)). Relative standard deviation (RSD) values were lower than 10% and 13% for most compounds, respectively.

The linearity assessment included 9 of the target compounds that were available in the lab. Standard solutions containing these 9 compounds, cis and trans-Linalool oxide, β-Linalool, β-Damascenone, benzyl alcohol, phenylethyl alcohol, α, β and γ-Eudesmol, were prepared in acetone. The honey sample was spiked with different amounts of the target compounds covering a concentration range from 5 to 5000 ng g^{-1} (final concentrations in honey). Each calibration level was analyzed by triplicate (n = 3). The results are shown in Table 3. Coefficients of determination (R^2) higher than 0.9939 were obtained in all cases. The limits of detection (LODs) were calculated as the compound concentration giving a signal-to-noise ratio of three (S/N = 3) and are also summarized in Table 3. They were between 0.05 and 6.90 ng g^{-1}.

Recovery studies were carried out in five samples including different honey varieties: honeydew (HD), chestnut (CN), multi-floral (MF) and heather (HE) honey. Previous analysis of the samples evidenced the

Table 3
Mini_SPME-GC-MS validation: linearity, recoveries in 4 different types of honey, precision (RSD, %) and LODs.

Compounds	Linearity		Recoveries (RSD), %					Precision, %	LOD, ng g^{-1}
	R^2	Range, ng g^{-1}	HD3	MF1	CN2	HE	Mean between samples	RSD Within sample	
Cis-Linalool oxide	0.9968	5–5000	107 (5)	92.7 (5.0)	91.1 (7.9)	92.9 (2.1)	95.9 (7.4)	4.9	0.05
Trans-Linalool oxide	0.9962	5–5000	107 (4)	94.3 (6.3)	90.3 (8.0)	93.1 (2.3)	96.2 (7.4)	5.3	0.05
β-Linalool	0.9939	5–5000	102 (1)	90.3 (8.2)	88.5 (1.5)	101 (1)	95.4 (6.9)	3.0	0.3
β-Damascenone	0.9978	50–5000	105 (11)	107 (11)	109 (2)	113 (7)	109 (3)	7.8	6.9
Benzyl alcohol	0.9965	100–5000	89 (16)	93.4 (3.8)	81.2 (0.8)	70 (12)	83 (10)	8.1	4.3
Phenylethyl alcohol	0.9945	100–5000	88 (10)	91.7 (5.3)	75.9 (0.4)	62 (16)	79 (13)	7.9	1.1
γ-Eudesmol	0.9990	5–500	114 (5)	118 (10)	117 (6)	106 (13)	113 (5)	8.3	0.7
α-Eudesmol	0.9983	5–500	101 (1)	116 (11)	98.6 (8.4)	107 (9)	106 (8)	7.4	1.0
β-Eudesmol	0.9986	5–500	101 (5)	118 (12)	97 (10)	109 (9)	106 (9)	9.0	0.7

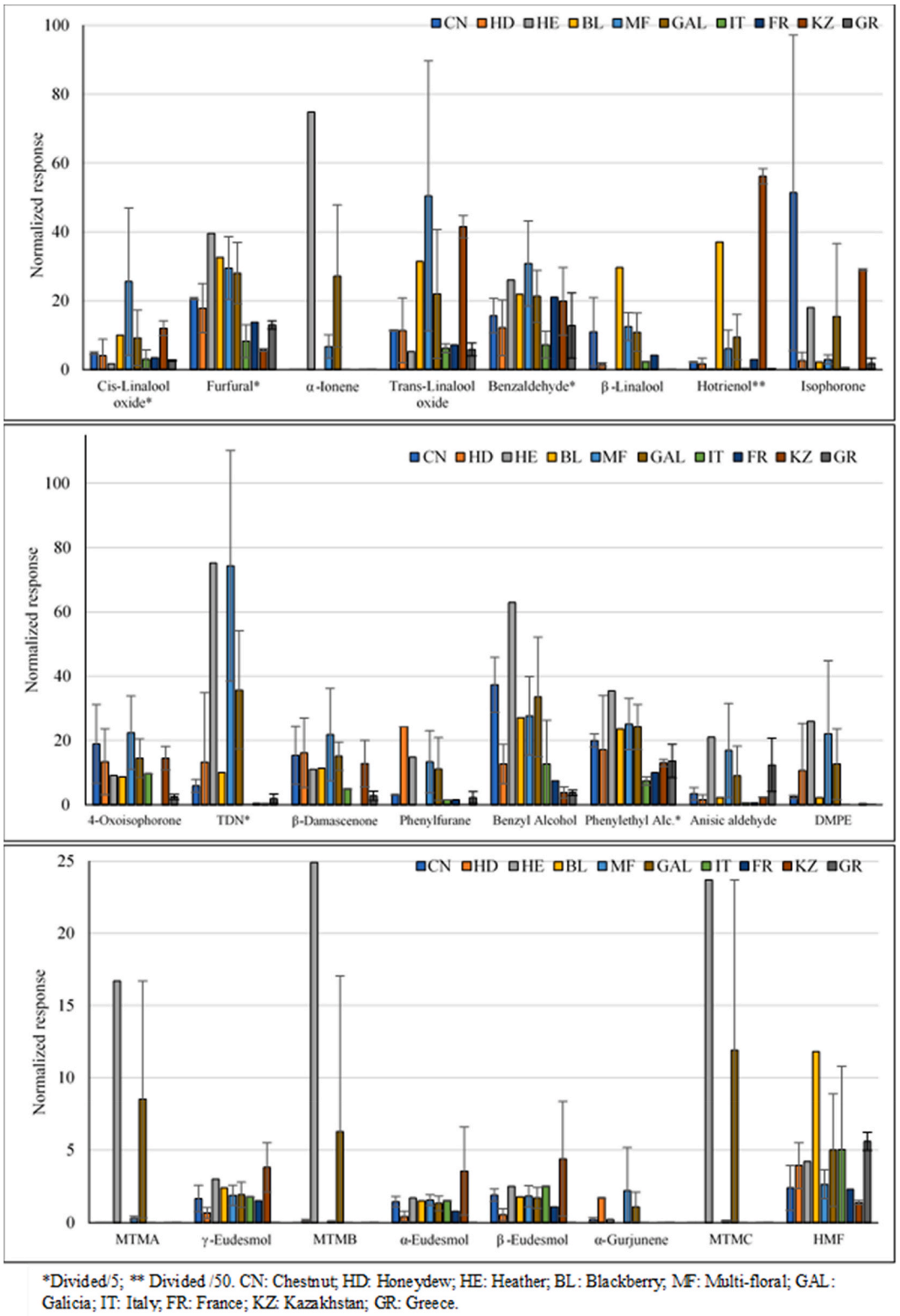


Fig. 5. Normalized response of target compounds for different types of honey.

presence of some of the target compounds, so that these initial concentrations were withdrawn to calculate recoveries. The spiked level was 5 μg g^{-1} (500 ng g^{-1} for α, β and γ-Eudesmol). Method accuracy was satisfactory with mean recoveries, considering all the types of honey, between 79% and 113%, as well as good precision with RSD values <12% in most cases (Table 3). Only few studies demonstrated linearity and accuracy (Campillo et al., 2006; Peña et al., 2004) like those we achieve in the present study. In addition, no matrix effects were found for the different types of honey, demonstrating the suitability of the matrix matched calibration using standards prepared in honey.

Although the growing interest in green analytical chemistry (GAC) is a positive phenomenon, an appropriate balance with analytical performance should be desired (Nowak and Kościelniak, 2019). This methodology was successfully validated complying with sustainable features

Table 4a
Concentration (ng g^{-1}) of the target compounds in Galician honeys. CN: Chestnut; HD: Honeydew; HE: Heather; BL: Blackberry; MF: Multi-floral.

	CN1	CN2	HD1	HD2	HD3	HE	BL	MF1	MF2	MF3	MF4	MF5
Cis-Linalool oxide	229	226	484	76.2	87.6	49.2	516	108	2028	2564	689	138
Trans-Linalool oxide	116	125	249	60.2	111	35.7	360	77.4	903	978	346	89.2
β-Linalool	6.1	55.0	5.2		4.3		86.5		31.3	36.4	16.3	28.5
β-Damascenone	537	1450	972	2192	568	456	778	1465	623	669	809	1853
Benzyl alcohol	1570	1245	729	653	328	1549	1114	1246	334	1118	730	885
Phenylethyl alcohol	1131	1079	2146	680	390	1276	1415	1077	709	1216	1918	958
γ-Eudesmol	67.7	78.3	30.6	77.4	29.6	95.9	118	80.1	37.6	66.3	97.4	42.2
α-Eudesmol	45.7	34.0	15.4	30.5		31.8	42.8	35.7	24.4	27.6	41.9	21.8
β-Eudesmol	44.5	30.9	11.7	30.1	8.4	32.7	35.8	35.3	11.8	18.8	34.5	25.6

Table 4b
Concentration (ng g^{-1}) of the target compounds in the foreign honeys. IT: Italy; FR: France; KZ: Kazakhstan; GR: Greece; CN: Chestnut; OR: Orange; LV: Lavender; MF: Multi-floral TH: Thyme.

	IT1 (CN)	IT2 (OR)	FR1 (LV)	KZ1 (MF)	KZ2 (MF)	GR1 (MF)	GR2 (MF)	GR3 (TH)
Cis-Linalool oxide	146	47.4	251	376	514	96.7	88.4	
Trans-Linalool oxide	66.0	49.5	80.2	312	369	56.0	36.5	
β-Linalool	9.9	5.6						
β-Damascenone		275		367	903	178	85.4	41.0
Benzyl alcohol	249	743	126	73.1	150	121	114	74.5
Phenylethyl alcohol	483	318	494	576	539	739	629	308
γ-Eudesmol	52.7	28.7		134	68.3			
α-Eudesmol	15.6	13.7		87.7	20.9			
β-Eudesmol	15.1	16.8		79.5	17.2			

as its low cost- and time-effectiveness as well as the negligible generation of residues.

3.6. Application to real samples

The developed mini_SPME-GC-MS method was applied to the analysis of 12 honey samples collected in Galicia (Spain) including honeydew (HD), multi-floral (MF), chestnut (CN), blackberry (BL) and heather honey (HE). Eight other samples from four different countries such as Kazakhstan, Italy, France and Greece were also analyzed. The 24 target compounds were identified in the 20 analyzed honey samples and the results expressed as normalized areas are summarized in Table S1. Mean values for each kind of honey are represented in Fig. 5. The concentrations of the nine compounds included in the quantification study are shown in Table 4. The highest contents were observed in the Galician honeys (Table 4a) in which compounds like phenylethyl alcohol, benzyl alcohol and β-Damascenone, reached concentrations higher than 1500 ng g^{-1}. In addition, cis-Linalool oxide was detected at concentrations up to 2000 ng g^{-1} in samples MF2 and MF3. Regarding foreign honeys (Table 4b), the highest concentrations were reached in Kazakhstan honey samples, although benzyl alcohol and phenylethyl alcohol were also found at high concentration in the orange Italian honey and the multi-floral Greek honeys, respectively. β-Linalool was only found in practically all Galician honeys and Italian honeys.

Mean values for each kind of honey are depicted in Fig. 5 in which the first 5 bars correspond to the different varieties of Galician honeys (CN, HD, HE, BL, MF), the sixth bar is the mean value considering all Galician samples (GAL) and the last 4 bars represent the average value for each foreign country (IT, FR, KZ, GR). Regarding Galician samples 16 of the 24 target compounds were identified in all samples and two of the samples, MF3 and MF4, contained all compounds. As can be seen, seventeen out of the twenty-four compounds were detected in the two Kazakhstan (KZ) honeys, fifteen in the French and chestnut Italian honeys, fourteen in the two multi-floral Greek honeys and eleven in the thyme variety, whereas only ten compounds were detected in the Italian orange honey. As can be seen in Fig. 5, the relative concentrations of the studied compounds was generally higher in Galician honeys than in the foreign ones, highlighting 1,1,5-Trimethyl-1,2-dihydronaphtalene (TDN) and 1-(2,3-Dimethylphenyl)ethanone (DMPE), as well as compounds that were even not found in foreign honeys such as α-Ionene, megastigmatrienone A, B and C (MTMA, MTMB, MTMC) and α-Gurjunene. As it can be seen, heather (HE) variety reach the highest abundance of α-Ionene and megastigmatrienone A, B and C. A statistical approach would be further necessary to check whether the above mentioned compounds could be considered as markers for Galician honeys.

The method could also identify 5-Hydroxymethylfurfural (HMF) in all analyzed samples, compound that can be formed in bad storage conditions and considered as a marker of quality deterioration (Shapla et al., 2018).

Non-target species identification was also conducted. δ-Decalactone and 4-Hydroxy-4-methyl-2-pentanone were only found in Galician honey samples. On the other hand, thymol and carvacrol were only identified in Greek honeys, as well as benzothiazole. This compound was also identified in the Kazakhstan honeys as well as 1,2-Dihydrolinalool, epoxylinalool and 1,4-Dimethylindanyl acetate, compounds that were not identified in other samples. Benzeneacetaldehyde, different acids (octanoic acid, nonanoic acid, decanoic acid, hexadecanoic acid, dodecanoic acid …), benzyl nitrile, 3-Methy-l-2-butanol, quinoline derivatives (4-Methylquinoline, isoquinoline, 4-Quinolinecarboxaldehyde), 2-Aminoacetophenone or cinnamaldehyde, were present in the honey samples from all zones, as well.

4. Conclusions

A miniaturized solid-phase microextraction methodology (mini_SPME) followed by gas chromatography-mass spectrometry (GC-MS) was optimized for the simultaneous analysis of different volatile and semivolatile compounds in honeys. In order to achieve an efficient extraction, some parameters such as temperature, type of fibre, dilution and salt addition were studied by means of an experimental design, including 24 volatile and semivolatile compounds. The optimal conditions were based on the use of DVB/CAR/PDMS fibre in the headspace mode, mixing 200 mg of honey with 200 μL of water. Extraction kinetic curves were obtained demonstrating that most of the target analytes reached equilibrium within the interval studied (60 min). A fibre exposure of 30 min was selected as a good compromise for ensuring good sensitivity and high analytical throughput. The method was

validated showing good performance in terms of repeatability, reproducibility and linearity. Recoveries were evaluated in four different types of honey, including multi-floral, chestnut, honeydew and heather honeys obtaining satisfactory values, in general above 80%. Twenty-four target compounds were investigated in twelve Galician honeys and eight foreign honeys and nine compounds were quantified, demonstrating the suitability of the proposed method. Non-target analysis was also performed. Some identified compounds might be specific markers regarding the geographical and botanic origins of the honey. Nevertheless, a larger panel of samples and a rigorous statistical approach would be necessary for marker identification and classification purposes. In conclusion, the developed methodology demonstrates suitability for honey analysis and it is in line with the principles of GAC minimizing the overall impact of the extraction process.

Author statement

Lua Vazquez: Formal analysis, Validation, Investigation, Data curation, Software, Writing - original draft, Writing - review & editing. **Maria Celeiro**: Investigation, Writing - review & editing. **Meruyert Sergazina**: Formal analysis, Validation, Investigation, Data curation, Software. **Thierry Dagnac:** Methodology, Resources, Visualization, Writing - review & editing, Project administration, Funding acquisition. **Maria Llompart**: Conceptualization, Methodology, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2021.100451.

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