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Optimization by experimental design of headspace sorptive extraction and solid-phase microextraction for the determination of terpenes in spices

Zélie Triaux[1,2] · Hugues Petitjean[2] · Eric Marchioni[1] · Damien Steyer[3] · Christophe Marcic[1]

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Abstract
Headspace sorptive extraction (HSSE) was compared with solid-phase microextraction (SPME), a reference method for the extraction of volatiles. HSSE and SPME were optimized by experimental design for the extraction of terpenes and terpenoids from spices. The different extraction parameters studied were the extraction time, the extraction temperature, the sample mass, and the equilibrium time (only for SPME). Extracts were obtained by HSSE and SPME from six spices: cinnamon, cumin, thyme, fennel seeds, nutmeg, and clove. Those extracts were analyzed by gas chromatography coupled to a mass spectrometer (GC-MS) qualitatively and quantitatively. The quantitative analysis was conducted using 28 standards. For each standard, calibration curves, limits of detection (LOD) and quantification (LOQ) were determined. The study shows that HSSE is selective, sensitive, and highly repeatable method for the extraction of volatile terpenes and terpenoids from spices and allows to produce extracts concentrated in the μg/g range of terpenes.

Keywords HSSE · SPME · Terpenes · Quantitative analysis · GC-MS · Experimental design

Introduction

Spices are widely used in cooking as flavors. Besides, their essential oils are also known to have numerous biological activities such as antioxidant (Yashin et al. 2017), anti-inflammatory (Opara and Chohan 2014), antidiabetic (Srinivasan 2005), and anti-tumorigenic (Kaefer and Milner 2008; Rakhi et al. 2018). Those plants are rich in terpenes and terpenoids, secondary metabolites responsible for their aroma but also for their bioactivities. With 25,000 known structures, terpenes belong to the widest family of natural compounds (Zwenger and Basu 2008). They are classified depending on the number of isoprene units in their chemical structure. Monoterpenes, terpenes composed of two isoprene units, are volatile compounds and the main constituents of essential oils. Those compounds have been widely reported as the source of the various bioactivities of essential oils (De Sousa 2011). When the chemical composition of spices varies, due to different parameters (plant variety, harvest, drying, storage…), the organoleptic properties and bioactivities of their essential oils varies (Forney and Song 2017). Thus, developing a robust analytical method for the analysis of flavors and more specifically the analysis of monoterpenes in plants is important.

Different methods are conventionally used for flavor analysis such as distillation, supercritical fluid extraction (SFE), soxhlet extraction, solvent-assisted flavor evaporation (SAFE). However, those methods are often time-consuming, usually require a large volume of organic solvent thus having a negative impact on the environment and most of them have low extraction efficiency. In the past decade, microextraction methods such as static-headspace extraction, solid-phase microextraction (SPME), single drop microextraction (SDME), hollow fiber liquid phase microextraction (HF-LPME), and stir bar sorptive extraction (SBSE) have been increasingly used for food flavor analysis (Jeleń et al. 2012). Those techniques are eco-friendly as they are often solventless (or use a minimal amount of solvent), rapid as in most cases no sample preparation is needed and have better selectivity especially when used in headspace mode.

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✉ Christophe Marcic
christophe.marcic@unistra.fr

[1] Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France

[2] Benephyt, 16 rue d'Austerlitz, 67000 Strasbourg, France

[3] Twistaroma, 74 Route du Rhin, 67400 Illkirch-Graffenstaden, France

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SBSE was first introduced by Baltussen et al. in 1999 (Baltussen et al. 1999) as a novel extraction technique. In SBSE, a magnetic stir bar enclosed in a glass tube, usually coated with polydimethylsiloxane (PDMS), is immersed in a liquid sample. The targeted compounds in the sample are extracted by sorption onto the stir bar. After the extraction step, the extracted molecules are desorbed from the stir bar in a minimum volume of solvent or thermally. The analytes are then often analyzed by gas chromatography coupled to a mass spectrometer (GC-MS). This method allows to concentrate compounds found in a large volume of sample. Instead of placing the stir bar into the sample, it can also be suspended in the headspace of the sample, when dealing with solid samples for example. This use of SBSE is known as headspace sorptive extraction (HSSE). In comparison to SPME, a more common microextraction technique, the stir bar used in HSSE has a larger amount of extracting phase than the fiber used in SPME, providing higher recoveries and sensitivities (David and Sandra 2007). Even though HSSE is more selective toward volatiles and semi-volatiles compounds than SBSE, only few studied describe its use (Prieto et al. 2010) and has never been described for the analysis of spices to our knowledge.

Quantitative analysis of bioactive compounds is essential for the evaluation of their biological activities. Monoterpenes, and in general all bioactive compounds, are active at a certain concentration, can be toxic beyond this concentration and inert under it (Fonsêca et al. 2016). For a lot of authors, quantitative analysis consists of measuring the relative percentage abundance of each compound in the extract. For others, it consists of using a standard from the same chemical family of the analyte and calculating the relative concentration of the analytes to the one of the standard, this approach is also known as semi-quantitative analysis. Few authors conduct complete quantitative analysis by using analytical standards for each analyte (Bicchi et al. 2008). However, even when complete quantitative analyses are led, it is usually on no more than ten compounds of interest (Abilleira et al. 2010). Quantitative analysis of monoterpenes is rarely done for HSSE (Weldegergis et al. 2007; Hevia et al. 2016; Barba et al. 2017). The sensibility of the HSSE procedure is different for each terpene depending on their air/stir bar distribution coefficients. The sensibility of each terpene is also different regarding to the analysis procedure; signals generated by GC-MS are dependent not only on the concentration of the analytes but also on their chemical structures (Bicchi et al. 2008).

To be able to conduct a complete quantitative analysis with the most standards, the extraction procedure must be well optimized so that the solid/air equilibrium is reached for all analytes. In fact, the optimization of the extraction parameters (time, temperature, shaking rate) is a critical stage in analytical chemistry even more so when dealing with adsorption techniques. Those techniques rely on an equilibrium between the gas phase and the sorbent phase which needs to be reached in order to improve the repeatability of the extraction method (Robotti et al. 2017). The most common way to optimize an extraction method is to experiment with one-variable-at-a-time (OVAT) while holding all others fixed (Weldegergis et al. 2007; Rodrigues et al. 2012; Cacho et al. 2015). Even though this procedure can lead to the best possible extraction conditions, it does not consider the potential interactions between the factors and it often requires a high number of experiments. A more effective method is to study factors simultaneously using the design of experiments approach. Chemometric optimization process allows to tease out which factors have a significant impact on the response, to distinguish interactions between the factors and to determine which combination of levels produces the optimum while minimizing the number of experiments needed (Stalikas et al. 2009).

In this study, HSSE was used to extract, identify, and quantify monoterpenes from six different spices namely cinnamon, cumin, thyme, fennel seeds, nutmeg, and clove. As they are known to contain a wide range of terpenes (Adiani et al. 2015; Mancini et al. 2015; Santana De Oliveira et al. 2016; Abdelwahab et al. 2017), the spices were used as a plant model rich in monoterpenes to develop an efficient extraction method of those volatiles compounds. HSSE was compared with SPME, a reference extraction method for the analysis of food flavor. HSSE and SPME were optimized by experimental design prior to the comparative study. Quantitative analyses of the extracts were conducted by GC-MS.

Experimental

Reagents and Materials

Milli-Q water (18.2 MΩ) was generated by Millipore synergy system (Molsheim, France). All solvents were of analytical grade (VWR Chemicals, Fontenay-sous-Bois, France). α-pinene (99%), β-pinene (99%), camphene (95%), p-cymene (99%), 3-carene (≥ 90%), linalool (97%), limonene (97%), pulegone (97%), 4-terpineol (≥ 95%), caryophyllene (≥ 98.5%), menthone (97%), camphor (96%), menthol (99%), borneol (≥ 99%), estragole (98%), α-humulene (96%), farnesene (mixture of isomers), eucalyptol (99%), cuminaldehyde (98%), eugenol (99%), carvacrol (98%) and thymol (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). β-citronellol (≥ 95%), anethole (≥ 98%), and α-terpineol (≥ 97%) were purchased from Fluka (Buchs, Switzerland). Geraniol (98%) was purchased from Carl Roth GmbH (Karlsruhe, Germany). Nitrogen was of 4.5 grade and helium of 6.0 grade (Sol France, Saint-Ouen l'Aumone, France).

Cinnamon (*Cinnamomum verum*, Chamsyl), cumin (*Cuminum cyminum*, Conquête des saveurs), thyme (*Thymus vulgaris*, Chamsyl), fennel seeds (*Foeniculum vulgare*,

Ducros), nutmeg (*Myristica fragrans*, Ducros), and clove (*Syzygium aromaticum*, Ducros) were all bought from a local shop. For the extractions, the food matrices were used as bought.

SPME Conditions

All SPME extractions were conducted automatically using a Combi-pal (CTC Analytics AG, Switzerland) autosampler. A known amount of sample was placed in a 20-mL headspace vial (23 × 75 mm) which was closed with PTFE-lined silicon septa and metallic screw caps. The vial was then automatically put in an agitated (250 rpm) incubator to maintain a constant temperature during the determined equilibrium time. The SPME needle then pierced automatically the septum of the vial and the fiber was extended through the needle in the headspace of the vial. A 1-cm fused silica fiber coated with 100 μm of polydimethylsiloxane (PDMS) was used for the extractions (Supelco, Bellefonte, USA). Before use, the fiber was conditioned during 30 min at 250 °C according to the manufacturer's instructions. The fiber was exposed to the headspace of the sample during different time periods and was then withdrawn into the needle, removed from the vial, and thermally desorbed at 210 °C during 2 min directly in the split/splitless injection port of the GC. The obtained extracts were analyzed by GC-MS in split mode (1:50) and each extraction was performed in triplicate.

The extraction temperature (50–80 °C), the equilibrium time (5–11 min), the sample mass (40–80 mg), and the extraction time (10–90 min) were optimized by experimental design.

HSSE Conditions

Ten-millimeter long stir bars coated with a 1-mm film thick layer of polydimethylsiloxane (PDMS) (Gerstel, Mülheim an der Ruhr, Germany) were used. A weighed amount of sample was placed in a 20-mL headspace vial (23 × 75 mm) which was closed with PTFE-lined silicon septa and metallic screw caps. The magnetic stir bar was attached with a clean metal paper clip by simple magnetic force in the headspace of the vial. The HSSE extraction was then carried out in an agitated (250 rpm) incubator at constant temperature. Once the sorption step was over, the stir bar was removed from the headspace and inserted in 100 μL of ethanol in a 250-μL insert (29 × 5.7 mm) placed in a 2-mL vial closed with a screw cap for the desorption step. The stir bar was then desorbed under ultrasonic treatment during 30 min. After removal of the stir bar, 1 μL of the obtained extract spiked with an internal standard to follow the variations of the analytical method was injected in the GC-MS in split mode (1:20). Each extraction was performed in triplicate.

The extraction temperature (50–90 °C), the extraction time (30–120 min), and the sample mass (40–80 mg) were optimized by experimental design.

Prior to each use, each stir bar was preconditioned following the same cleaning procedure. First the stir bar was washed under ultrasound for 30 min in a 1-mL dichloromethane-methanol mixture (50:50 v/v) and then in 1 mL acetonitrile for another 30 min. Finally, the stir bar was placed in 1 mL acetonitrile overnight. To check the reliability of the cleaning procedure, after having gone through the washing steps, the stir bar was desorbed once more in 100 μL of ethanol for 30 min under sonication, last solvent being then analyzed by GC-MS in splitless mode. No compounds of the previous extract were detected in the "washing solvent" which validates the cleaning procedure. This cleaning step could be improved by using a thermal desorption unit, no solvent would be needed and it would be time-saving.

Experimental Design and Definition of the Response

The optimal extraction parameters for SPME and HSSE were determined by establishing an experimental design approach. The corresponding experiments defined by the designs were done on nutmeg as it contains the widest range of terpenes and terpenoids among the spices studied. For data manipulation, JMP® Statistical Discovery™ 8 (SAS Institute) was used. Each experimental design had 28 responses corresponding to the 28 compounds identified in nutmeg (Fig. 1). The measured property of the response is the chromatographic peak area of the corresponding compound. Those compounds were chosen as they have different chemical properties (mainly polarities and boiling temperatures), the aim being to optimize the extraction methods in order to extract the higher concentration of terpenes and terpenoids. First, a two-level full factorial design 2^k (with k the number of parameters) was built to evaluate which parameters had a significant impact on the responses and to calculate the interactions between the parameters. The number of experiments for this design is equal to 2^k + the number of central points. The data obtained by those experiments was fitted according to a second-order interaction model which corresponds to the following equation (Bezerra et al. 2008) (Eq. (1)):

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where y is the response (the area of a selected peak), x_i the studied factors, β_0 the constant, β_i the coefficients of the linear parameters, β_{ij} the coefficients of the interaction parameters, and ε the experimental error.

The influence of the interactions of third order (or higher) were neglected as they are considered very small in most cases (Lundstedt et al. 1998).

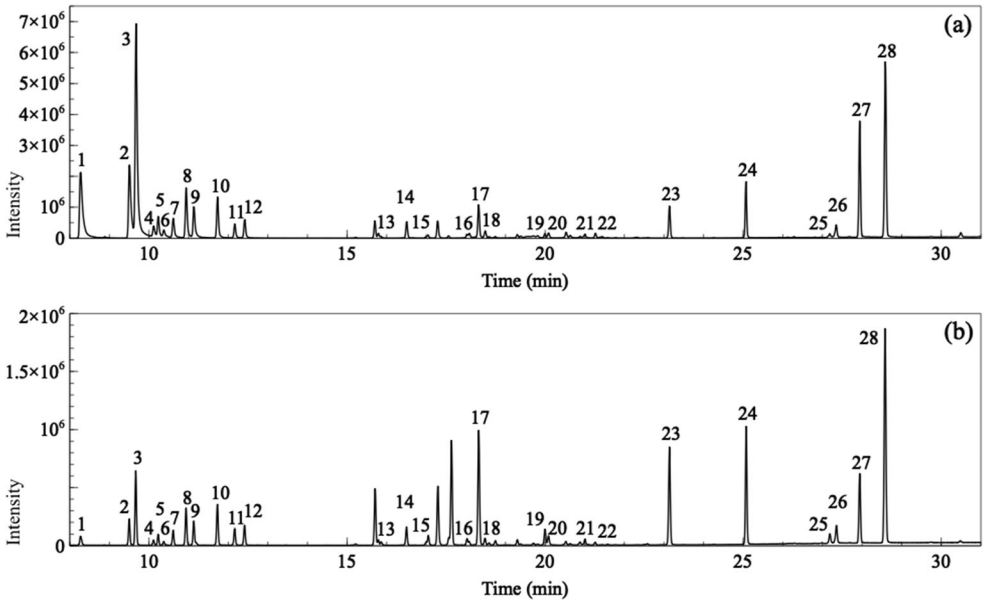

Fig. 1 Total ion chromatogram obtained for nutmeg extracts by **a** SPME and **b** HSSE. Selected compounds for the optimization by experimental design. 1, α-Pinene; 2, β-pinene; 3, sabinene; 4, 3-carene; 5, β-myrcene; 6, α-phellandrene; 7, 4-carene; 8, limonene; 9, β-phellandrene; 10, γ-terpinene; 11, p-cymene; 12, terpinolene; 13, α-cubebene; 14, copaene; 15, linalool; 16, bornyl acetate; 17, 4-terpineol; 18, caryophyllene; 19, α-terpineol; 20, α-terpineol acetate; 21, geranyl acetate; 22, δ-cadinene; 23, safrole; 24, methyl eugenol; 25, eugenol; 26, isoeugenol methyl ether; 27, elemicin; 28, myristicine

Then, choosing only the significant parameters identified by the first design, the optimal extraction conditions were determined using a face centered design. For the model corresponding to this design, quadratic terms are added in order to determine a maximum, i.e., the optimal conditions. The data obtained from the experiments of this design was fitted to the following quadratic equation (Bezerra et al. 2008) (Eq. (2)):

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \varepsilon \qquad (2)$$

where β_{ii} represents the coefficients of the quadratic parameters.

A face-centered design is a factorial design augmented with a group of points placed on the center of each face of the factorial space (corresponding to a square for a 2^2 design). The identification of the optimal extraction parameters which optimize all 28 responses simultaneously was found using the desirability function approach (Bezerra et al. 2008). The first step of this methodology is to build a desirability function for each individual response. This is done by transforming the chromatographic peak areas of each compound into a dimensionless individual desirability scale. This scale ranges between $d = 0$, lowest peak area obtained, and $d = 1$, highest peak area obtained in the experiments conducted in the experimental design. An overall desirability function was then drawn from the 28 partial desirability functions to find a global optimum corresponding to the best compromise for maximizing simultaneously all the different responses studied.

The experimental data was fitted by least squares. To evaluate the adequacy of the model corresponding to the experimental design, three values were calculated: the goodness of the fit R^2, the goodness of prediction Q^2, and the lack of fit (LoF). Values of $R^2 > 0.8$ and $Q^2 \geq 0.5$ were considered acceptable in our study (Lundstedt et al. 1998). The lack of fit compares the model error to the experimental error by an F-test. If P-value < 0.05, the F-test is significant and there is a significant lack of fit of the corresponding response by the model. The statistical significance of each parameter and the interactions between them were studied by using an analysis of variance (ANOVA) for a 95% confidence level of the parameter's coefficients for each response (i.e., if P-value < 0.05, the parameter has a statistical significance on the response).

GC-MS Analysis

Analyses were performed with a 450-GC/240-MS (Varian, Les Ulis, France) GC-MS system equipped with a DB-WAX capillary column (60 m × 0.25 mm × 0.15 μm) (Agilent Technologies, Les Ulis, France). Helium was used as the carrier gas at a flow rate of 1 mL/min. The split/splitless injector temperature was set to 210 °C. The oven temperature was held at 40 °C for 1 min, increased to 100 °C at 10 °C/min, heated to 130 °C at 5 °C/min, heated to 15 °C at 10 °C/min, heated to 180 °C at 5 °C/min, heated to 230 °C at 10 °C/min and then held isothermal at 230 °C for 5 min. For MS, the electron multiplier was set to 70 eV. The temperature of the ion source

was 150 °C and the transfer line was set at 200 °C. Detection was performed in the scan mode in the range of 50–200 *m*/*z*.

Sample Characterization and Quantification

Terpenes were identified in the different extracts on the basis of their mass spectra and retention indexes (RI). Recorded mass spectra were compared with those from the National Institute of Standards and Technology (NIST 2.0, USA) database considering a match factor higher than 800 a good match. Retention indexes (RI) were calculated (Van den Dool and Kratz 1963) and compared with the ones in the literature for DB-WAX type columns.

Quantification analysis of the extracts was conducted using 28 terpene standards (Supporting Information Fig. 1). Not all the terpenes identified in the different samples were quantified, only the ones corresponding to the 28 standards. For each standard, solutions with a concentration range from 0.1 to 50 μg/g were prepared to draw the calibration curves. Two milliliters of the standard solution at different concentrations were placed in a headspace vial and extracted by both HSSE and SPME. Ten points were used to draw the calibration curves (two calibration curves for each standard corresponding to two ranges of linearity). Each concentration was analyzed in triplicate. Standards were quantified according to the peak area of the corresponding compound's selected target ion (the main ion of the mass spectra of the compound or a characteristic one). The limits of detection (LOD) and limits of quantification (LOQ), defined as the lowest concentrations detected at a signal-to-noise ratio of three or ten respectively, were measured for each standard. The lowest concentration used to draw the calibration curves was chosen above the LOQ.

Results and Discussion

Optimization of SPME Conditions

Screening by a 2^4 Full Factorial Design Nineteen experiments were conducted according to conditions fixed by a 2^4 full factorial design (including 3 central points) to study the significative influence of equilibrium time (5–11 min), extraction temperature (50–70 °C), extraction time (10–26 min), and sample mass (40–80 mg) on SPME efficiency. The intervals studied for each variable were selected according to previous work (Jeleń and Gracka 2015; Patel et al. 2016).

The results of this first screening design are shown in Table 1. There was no significant lack of fit ($P > 0.05$) observed for any of the responses. Sixty-four percent of the responses were well described by the chosen model ($R^2 > 0.8$ and $Q^2 > 0.5$) which was considered enough to validate this model. Extraction temperature (T) and extraction time (t_{ext}) were the most significant factors in the extraction process. Increasing the extraction time from 10 to 26 min increases the peak area of 79% of the responses. For the extraction temperature, increasing this factor from 50 to 70 °C had a positive effect on 57% of the responses but had a negative one on 39% of the responses which was expected. More precisely, increasing the extraction temperature, decreases the extraction efficiency of highly volatile compounds and on the contrary increasing the extraction temperature, increases the extraction efficiency of less volatile compounds. This can be explained by the fact that at a higher temperature the concentration of less volatile compounds in the headspace will increase while higher volatile compounds will desorb from the fiber due to a decrease of their distribution constant (Wardencki et al. 2007). Moreover, as the concentration of low volatile compounds increases in the headspace and eventually on the fiber, a potential competition between volatiles on the liquid stationary phase might appear (Barba et al. 2017). This phenomenon is well observed here as each response of the experimental design corresponds to a compound and not to the sum of the peak areas as it is usually done.

Equilibrium time (t_{eq}) and sample mass (M) did not seem to affect or only slightly affect some of the compounds studied (4% for t_{eq} and 25% for M). Those parameters were fixed at 10 min and 70 mg respectively. No significant interactions between the selected factors were observed.

Optimization by a Face Centered Design For an optimization design, the number of parameters must be kept as small as possible to avoid complex models (Guerrero et al. 2006). As only the extraction temperature (T) and the extraction time (t_{ext}) were found significant in the screening design (3.1.1.), those two parameters were selected to find the optimum extraction conditions by a 2^2 face centered design. The studied intervals were 60–80 °C for the extraction temperature and 30–60 min for the extraction time, corresponding to a design of eleven experiments (including three central points). The values of those intervals were increased according to the results obtained previously, as the optimal conditions were not found in those previous intervals.

The model of this face centered design was validated for 68% of the responses (no LoF, $R^2 > 0.8$ and $Q^2 > 0.5$) (Supplementary Information Table 1). To find the optimal conditions, the desirability function approach was used. The partial desirability was defined as maximizing the peak area of the corresponding compound, i.e., the maximum area obtained for a certain compound in certain conditions of the design is defined as $d = 1$. All partial desirability functions were similarly weighed to 1. The overall desirability was calculated to reach the most favorable extraction parameters by simultaneously maximizing the peak area for the 28 responses. It is used to find the best compromise to extract all the different compounds as well as possible. Only the partial desirability of the responses well fitted by the model were used to calculate the overall desirability of the design. The contour plot (Fig. 2) shows that the highest

Table 1 Significant regression coefficients and fitting parameters for all 28 responses of the 2^4 full factorial design for SPME extraction conditions

Compound	T	t_{eq}	t_{ext}	M	Tt_{eq}	Tt_{ext}	$t_{eq}t_{ext}$	TM	$t_{eq}M$	$t_{ext}M$	R^2	Q^2	LoF
α-Pinene	−[a]	ns[b]	ns	+[c]	ns	ns	ns	ns	ns	ns	0.941	0.748	ns
β-Pinene	−	ns	ns	+	ns	ns	ns	ns	ns	ns	0.890	0.573	ns
Sabinene	−	ns	+	+	ns	ns	ns	+	ns	ns	0.933	0.691	ns
3-Carene	−	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.852	< 0.500	ns
β-Myrcene	−	ns	ns	+	ns	ns	ns	ns	ns	ns	0.857	< 0.500	ns
α-Phellandrene	−	ns	ns	ns	ns	ns	ns	ns	ns	ns	< 0.800	< 0.500	ns
4-Carene	−	ns	+	+	+	ns	ns	ns	ns	ns	0.861	< 0.500	ns
Limonene	−	ns	+	+	ns	ns	ns	ns	ns	ns	0.818	< 0.500	ns
β-Phellandrene	−	ns	ns	ns	ns	ns	ns	+	ns	ns	< 0.800	< 0.500	ns
γ-Terpinene	−	ns	+	+	+	ns	ns	ns	ns	ns	0.834	< 0.500	ns
p-Cymene	−	ns	+	ns	ns	ns	ns	ns	ns	ns	0.812	< 0.500	ns
Terpinolene	ns	ns	+	ns	ns	ns	ns	ns	ns	ns	< 0.800	< 0.500	ns
α-Cubebene	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.971	0.883	ns
Copaene	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.947	0.732	ns
Linalool	+	ns	+	ns	ns	ns	+	ns	ns	ns	0.898	< 0.500	ns
Bornyl acetate	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.870	0.532	ns
4-Terpineol	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.911	0.598	ns
Caryophyllene	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.964	0.837	ns
α-Terpineol	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.936	0.723	ns
α-Terpineol acetate	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.951	0.805	ns
Geranyl acetate	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.963	0.891	ns
δ-Cadinene	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.931	0.846	ns
Safrole	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.918	0.695	ns
Methyl eugenol	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.921	0.807	ns
Eugenol	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.900	0.684	ns
Isoeugenol methyl ether	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.939	0.873	ns
Elemicin	+	+	+	ns	ns	+	ns	ns	ns	ns	0.984	0.938	ns
Myristicine	+	ns	+	ns	ns	ns	ns	ns	ns	ns	0.933	0.860	ns

T extraction temperature, t_{eq} equilibrium time, t_{ext} extraction time, *M* sample mass, *LoF* lack of fit

[a] Negative effect

[b] Not significant

[c] Positive effect

overall desirability ($D > 0.741$) is found at the maximum values of the selected parameters: 80 °C and 60 min. In our case, it is difficult to obtain an overall desirability closer to 1 as a wide range of compounds from different chemical families with different volatilities is studied. A higher temperature was not tested to prevent from compound degradation and loses of highly volatile analytes. Increasing the extraction time beyond 60 min did not significantly increase the responses (data not shown). In fact, as SPME is based on absorption, when the equilibrium between the solid phase and the fiber is achieved, the exposure time does not increase the extraction efficiency (Wardencki et al. 2007). Finally, the optimal extraction conditions selected were 70 mg sample mass, 10 min equilibrium time, 80 °C extraction temperature, and 60 min extraction time.

After the extraction and desorption of nutmeg in the optimal conditions above, a blank analysis (empty vial) was conducted to assess the residual amount of analytes on the fiber. After 2 min of desorption at 210 °C in the GC injector, the residual compounds were near or below the LOD. It was assumed that the desorption time was well selected and that in this period of time 100% of the analytes were desorbed.

Optimization of HSSE Conditions

Screening by a 2^3 Full Factorial Design The influences of extraction temperature (50–70 °C), extraction time (30–90 min), and sample mass (40–80 mg) were studied using a 2^3 full factorial design. The data of 11 extractions defined by the design conditions (including 3 central points) was collected.

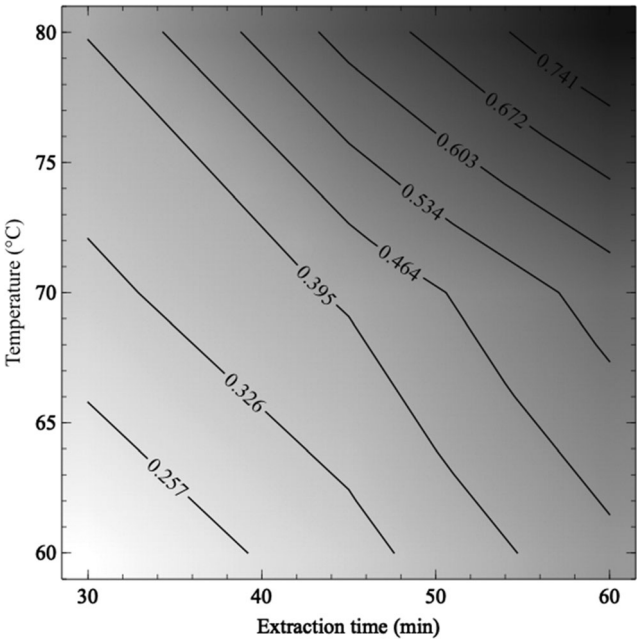

Fig. 2 Contour plots from the face centered design of SPME extracts of nutmeg showing the effect of extraction temperature and extraction time on the overall desirability

The intervals of the parameters were selected according to prior studies (Bicchi et al. 2005; Cacho et al. 2015) but also upon the results of the SPME optimization previously exposed in this work.

The results of the model's fitting are shown in Table 2. No significant LoF was observed for any of the responses. The explained variation R^2 of all 28 responses was higher than the acceptable value ($R^2 > 0.8$), which means that the model explained most of the experimental variability for 100% of the responses. The prediction variation Q^2 is acceptable for 54% of the responses; the variations of the highly volatile compounds are not well predicted by this model. In fact, a factor or an interaction might be missing in the model to better explain the variations of those compounds. Nevertheless, the aim of this first design is not to predict the optimal conditions but to study the parameters influence, it was considered that the model was well adapted to do so (no LoF and $R^2 > 0.8$ for 100% of the responses). Like the results obtained for SPME, two parameters showed a significant influence on the responses: the extraction temperature and the extraction time. However, for HSSE, the increase of temperature from 50 to 70 °C had a positive impact on 57% of the responses but a negative impact on only 14% of the responses; it was non-significant for most of the highly volatile compounds (29%). In fact, a PDMS stir bar has 10 times more PDMS amount than a SPME fiber due to its dimension (Bicchi et al. 2002); more compounds can be absorbed on the PDMS phase. Even though the concentration of less volatile compounds increases in the headspace, the higher volatile ones can still have sites to occupy, until exhaustion of the sites where a competition will begin. As expected, increasing the extraction time from 30 to 90 min increases the extraction efficiency (for 57% of the responses).

The sample mass had a significant influence on the HSSE for less than half the responses. This parameter was fixed at 80 mg (highest value of the interval), as its influence was positive for 39% of the responses.

Optimization by a Face Centered Design A 2^2 face centered design was built to find the optimal extraction conditions of HSSE. The factors used for this design was the ones that showed a significant influence on the extraction efficiency in the previous design: the extraction temperature (70–90 °C) and the extraction time (60–120 min). The values of those intervals were increased according to the results previously obtained. Eleven experiments (including three central points) were conducted according to the conditions set by the face centered design.

The results obtained to validate the design's model are presented in Table 2 of the supplementary information. Eighty-six percent of the responses were well fitted by the model (no LoF, $R^2 > 0.8$, $Q^2 > 0.5$), which was considered enough to use this model to optimize the extraction conditions. The partial desirability was defined as maximizing the peak area of the corresponding compound, same as for the SPME optimization. The overall desirability was then calculated from the partial desirabilities of the well-predicted compounds by the model. α-Cubebene, linalool, 4-terpineol, and α-terpineol were poorly predicted by the model ($Q^2 < 0.5$, Table 2) and were thus excluded from the calculation of the overall desirability. The contour plot of the overall desirability (Fig. 3) shows that the best compromise for the extraction parameters is 90 min extraction time and 80 °C extraction temperature. The value of the overall desirability at these conditions ($D > 0.534$) is not as high as expected. Indeed, the same phenomenon as described before can be observed: half of the studied compounds are well extracted at low temperatures while the other half is better extracted at higher temperatures. Finding a compromise to extract well those different compounds will inevitably lead to the decrease of the overall desirability. The decrease of the extraction efficiency after 80 °C shows compound degradation and loses of highly volatiles compounds at higher temperatures, which is a why an extraction temperature of 90 °C was not tested for SPME. The final optimal extraction parameters for HSSE were selected as follows: 80 mg sample mass, 80 °C extraction temperature, and 90 min extraction time.

After the extraction conditions were optimized, different desorption parameters were tested, namely the desorption solvent and the desorption time. Different desorption solvents were used for the desorption of the analytes: water, ethanol, methanol, and acetonitrile (data not shown). Water was rapidly discarded as its desorption efficiency was quite poor compared with the other solvents tested. In fact, PDMS is a non-polar phase, mainly non-polar compounds

Table 2 Significant regression coefficients and fitting parameters for all 28 responses of the 2^3 full factorial design for HSSE extraction conditions

Compound	T	t_{ext}	M	Tt_{ext}	TM	$t_{ext}M$	R^2	Q^2	LoF
α-Pinene	–[a]	ns[b]	+[c]	ns	–	–	0.979	< 0.500	ns
β-Pinene	–	ns	+	ns	ns	ns	0.946	< 0.500	ns
Sabinene	–	ns	+	ns	ns	ns	0.943	< 0.500	ns
3-Carene	ns	ns	+	ns	ns	ns	0.902	< 0.500	ns
β-Myrcene	–	ns	+	ns	ns	ns	0.906	< 0.500	ns
α-Phellandrene	ns	ns	+	ns	ns	ns	0.881	< 0.500	ns
4-Carene	ns	ns	+	ns	ns	ns	0.871	< 0.500	ns
Limonene	ns	ns	+	ns	ns	ns	0.860	< 0.500	ns
β-Phellandrene	ns	ns	+	ns	ns	ns	0.891	< 0.500	ns
γ-Terpinene	ns	ns	+	ns	ns	ns	0.798	< 0.500	ns
p-Cymene	ns	ns	+	ns	ns	ns	0.838	< 0.500	ns
Terpinolene	ns	ns	ns	ns	ns	ns	0.792	< 0.500	ns
α-Cubebene	+	+	ns	ns	ns	ns	0.978	0.864	ns
Copaene	+	+	ns	ns	ns	ns	0.984	0.966	ns
Linalool	+	+	ns	ns	ns	ns	0.932	< 0.500	ns
Bornyl acetate	+	+	ns	ns	ns	ns	0.986	0.978	ns
4-Terpineol	+	+	ns	ns	ns	ns	0.974	0.885	ns
Caryophyllene	+	+	ns	ns	ns	ns	0.984	0.943	ns
α-Terpineol	+	+	ns	ns	ns	ns	0.984	0.957	ns
α-Terpineol acetate	+	+	ns	ns	ns	ns	0.981	0.906	ns
Gernyl acetate	+	+	ns	ns	ns	ns	0.978	0.782	ns
δ-Cadinene	+	+	ns	ns	ns	ns	0.983	0.809	ns
Safrole	+	+	ns	ns	ns	ns	0.985	0.977	ns
Methyl eugenol	+	+	ns	ns	ns	ns	0.980	0.804	ns
Eugenol	+	+	ns	ns	ns	ns	0.969	0.659	ns
Isoeugenol methylether	+	+	ns	+	ns	ns	0.989	0.796	ns
Elemicin	+	+	ns	ns	ns	ns	0.964	0.606	ns
Myristicine	+	+	ns	ns	ns	ns	0.975	0.735	ns

T extraction temperature, t_{ext} extraction time, *M* sample mass, *LoF* lack of fit

[a] Negative effect

[b] Not significant

[c] Positive effect

will adsorb on this phase and according to the "like dissolves like" rule, water is too polar to desorb those compounds. Between the three remaining solvents, the chromatographic profiles obtained for the extracts were similar, but the peak areas of the ethanol extract were higher than for the other two solvents, ethanol was thus selected for the desorption. Different desorption periods were tested: 15 min, 30 min, 45 min, and 60 min (data not shown). After 30 min ultrasonic treatment, no significant improvement of extraction efficiency was obtained at higher extraction time. Therefore, it was considered that after 30 min desorption in ethanol, most of the analytes were desorbed from the PDMS stir bar.

Calibration and Limits of Detection

After the determination of the optimal extraction parameters for SPME and HSSE, the method performances were evaluated by determining the linearity of response, the repeatability and the limits of detection (LOD) and quantification (LOQ) for the targeted terpenoids. Table 3 shows the calibration parameters of the 28 terpenoids standards studied for both extraction methods (SPME and HSSE). Most

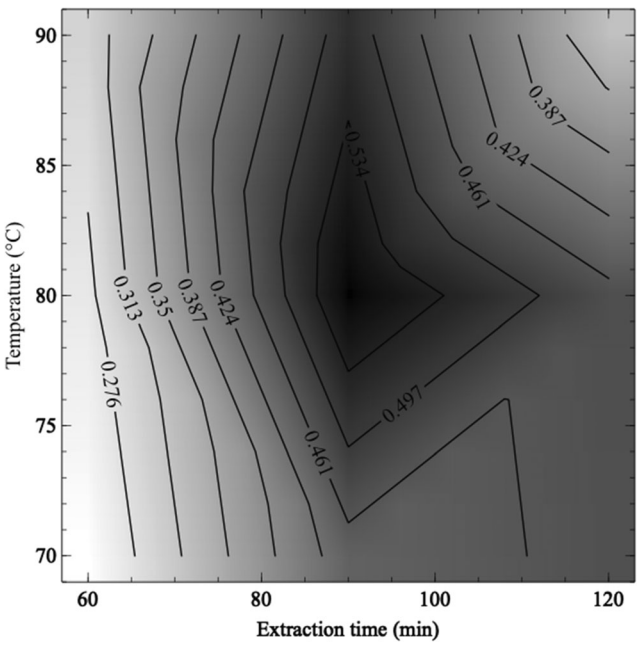

Fig. 3 Contour plots from the face centered design of HSSE extracts of nutmeg showing the effect of extraction temperature and extraction time on the overall desirability

Table 3 Calibration parameters of standards for SPME and HSSE: retention index (RI), ion extracted from the TIC analysis, relative standard deviation (RSD) calculated on a 3 μg/g standard mixture, concentration ranges, coefficient of determination (R^2), and limit of quantification (LOQ)

Compound	RI	Ion (*m*/*z*)	SPME				HSSE			
			RSD	Concentration range (μg/g)	R^2	LOQ (μg/g)	RSD	Concentration range (μg/g)	R^2	LOQ (μg/g)
α-Pinene	1049	93	13.5	[0.5;3]	0.991	0.2	0.8	[0.4;3]	0.995	0.1
				[3;50]	0.997			[3;50]	0.997	
Camphene	1085	93	11.2	[0.5;3]	0.996	0.1	0.2	[0.4;3]	0.996	0.1
				[3;50]	0.996			[3;50]	0.991	
β-Pinene	1119	93	5.0	[0.5;3]	0.996	0.1	6.9	[0.1;3]	0.995	0.1
				[3;50]	1.000			[3;50]	0.999	
3-Carene	1148	93	5.2	[0.1;3]	0.994	0.1	5.0	[0.1;3]	0.996	0.1
				[3;50]	1.000			[3;50]	1.000	
Limonene	1186	67	8.5	[0.1;3]	0.998	0.1	2.7	[0.4;3]	0.997	0.2
				[3;50]	0.996			[3;50]	0.994	
Eucalyptol	1202	139	6.6	[0.5;3]	0.999	0.1	8.1	[0.4;3]	0.998	0.2
				[3;50]	0.995			[3;50]	0.997	
p-Cymene	1258	119	3.1	[0.1;3]	0.999	0.02	2.1	[0.1;3]	0.993	0.03
				[3;50]	1.000			[3;50]	1.000	
Menthone	1481	139	6.2	[0.1;3]	0.999	0.1	5.3	[0.4;3]	0.995	0.1
				[3;50]	1.000			[3;50]	0.999	
Camphor	1529	108	4.2	[0.5;3]	0.999	0.2	11.3	[0.4;3]	0.998	0.3
				[3;50]	0.998			[3;50]	0.999	
Linalool	1540	93	13.3	[0.7;3]	0.993	0.7	5.4	[0.4;3]	0.996	0.2
				[3;50]	0.999			[3;50]	0.999	
4-Terpineol	1595	71	4.5	[0.7;3]	0.996	0.5	6.5	[0.7;3]	0.999	0.5
				[3;50]	0.995			[3;50]	0.999	
Caryophyllene	1603	133	4.7	[0.1;3]	0.999	0.02	3.4	[0.4;3]	0.997	0.2
				[3;50]	0.999			[3;50]	0.999	
(Z)-β-Farnesene	1620	161	12.4	[0.5;3]	0.999	0.2	5.7	–	–	–
				[3;50]	0.999			[3;50]	0.999	3.0
Menthol	1630	81	7.2	[0.5;3]	0.992	0.2	8.6	[0.1;3]	0.996	0.05
				[3;50]	0.997			[3;50]	0.999	
Pulegone	1638	152	6.4	[0.5;3]	0.998	0.1	5.6	[0.4;3]	0.993	0.2
				[3;50]	1.000			[3;50]	0.999	
(E)-β-Farnesene	1646	161	10.2	[0.5;3]	0.996	0.1	14.0	–	–	–
				[3;50]	0.997			[3.50]	0.999	1.8
Estragole	1657	148	3.9	[0.1;3]	0.999	0.03	9.7	[0.1;3]	0.991	0.04
				[3;50]	0.999			[3;50]	0.999	
α-Humulene	1667	147	11.5	[0.1;3]	0.998	0.02	9.6	[0.4;3]	0.997	0.2
				[3;50]	0.999			[3;50]	0.999	
α-Terpineol	1674	93	5.2	–	–	–	6.0	[0.4;3]	0.996	0.2
				[3;50]	0.999	1.2		[3;50]	0.998	
Borneol	1685	95	10.1	[0.5;3]	0.990	0.4	9.7	[0.4;3]	0.997	0.1
				[3;50]	0.999			[3;50]	0.999	
α-Farnesene	1729	161	12.0	[0.7;3]	0.997	0.5	3.2	–	–	–
				[3;50]	0.999			[10;50]	0.997	8.7
β-Citronellol	1739	67	3.1	[0.5;3]	0.996	0.4	8.3	[0.4;3]	0.997	0.2
				[3;50]	1.000			[3;50]	0.997	
Cuminaldehyde	1793	133	5.8	[0.5;3]	0.995	0.4	10.5	[0.4;3]	0.994	0.2
				[3;50]	1.000			[3;50]	0.997	
Anethole	1827	117	6.8	[0.1;3]	0.997	0.1	6.8	[0.4;3]	0.998	0.3
				[3;50]	0.991			[3;50]	0.999	
Geraniol	1842	69	8.4	[3;50]	0.999	1.3	10.6	[3;50]	0.995	0.9
Thymol	2138	135	8.1	[0.5;3]	0.996	0.1	9.0	[0.4;3]	0.999	0.3
				[3;50]	0.994			[3;50]	0.998	
Eugenol	2159	164	4.7	[0.5;3]	0.999	0.2	8.4	[0.7;3]	0.999	0.4
				[3;50]	0.997			[3;50]	0.992	
Carvacrol	2167	135	10.0	[0.5;3]	0.997	0.1	7.4	[0.4;3]	0.999	0.3
				[3;50]	0.995			[3;50]	0.998	

of the standards used for the calibration are monoterpenes or monoterpenoids, five sesquiterpenes were included ((Z)-β-farnesene, (E)-β-farnesene, α-farnesene, caryophyllene and α-humulene). All standards showed a satisfactory linearity within the concentration ranges tested, the regression coefficients (R^2) were all higher than

0.99 for both SPME and HSSE. The relative standard deviation (RSD) calculated at 3 μg/g standard mixture was lower than 15% for all standards which is acceptable for headspace extraction. As a matter of fact, two equilibriums are involved in headspace extraction: solid/gas equilibrium and gas/stationary phase equilibrium (Sghaier et al. 2016). This contributes to the challenge of the repeatability of this extraction method if it is not well standardized. The high repeatability of the two techniques is well shown here as the RSD was lower than 10% for 79% of the terpenoids studied by SPME and for 86% by HSSE, only few compounds exhibited an RSD between 10 and 15%. LODs and LOQs were calculated for both extraction methods for all 28 standards. Values of LODs are not shown in Table 3 as they are directly related to the LOQs by a factor of 3.3. LOQs are essential when doing quantitative analysis. LOQs were in the range of 0.02–1.3 and 0.03–8.7 μg/g for SPME and HSSE respectively. The values of LOQs calculated for both SPME and HSSE were similar for each compound except for the sesquiterpenes. Those compounds ((Z)-β-Farnesene, (E)-β-Farnesene, α-Farnesene, Caryophyllene, α-Humulene) showed a ten times higher sensibility by SPME (0.2, 0.1, 0.5, 0.02, and 0.02 μg/g resp.) than by HSSE (3, 1.8, 8.7, 0.2, and 0.2 μg/g resp.). This can be due to the lack of affinity between the desorption solvent (ethanol) and the apolar sesquiterpenes. On the contrary, linalool, menthol, and α-terpineol, which are more polar terpenoids, have slightly lower LOQs by HSSE (0.2, 0.05, and 0.2 μg/g resp.) than by SPME (0.7, 0.2 and 1 μg/g resp.). The LODs and the LOQs obtained for all terpenoids studied were low enough to analyze these compounds in real samples later on (3.4.).

SPME and HSSE Comparative Study

Optimized SPME and HSSE were used to extract terpenoids from six different spices: cinnamon, cumin, fennel seeds, clove, nutmeg, and thyme. Each sample was extracted by both methods in triplicate.

The chromatograms obtained for nutmeg using SPME and HSSE are shown in Fig. 1. As it can be seen, the same headspace profile is obtained for both extraction methods, the main difference remaining in the relative contribution of each compound. Furthermore, even if a higher split ratio was applied for SPME (1/50), the enrichment of volatiles was higher for SPME than for HSSE (split ratio 1/20). Due to this high sensibility, more terpenoids were identified in SPME extracts than in HSSE ones (Fig. 4). For all matrices studied, SPME extracted more terpenoids or as much as HSSE. The full identification of the compounds for each matrix is presented in the supporting information material. The quantitative analysis shows the same result: for most compounds, SPME extracts quantitatively more terpenoids than HSSE (Table 4). Twenty-five compounds of the 28 standards studied were found in the different matrices. This study shows that it was possible to conduct a robust quantitative analysis for both headspace extraction techniques.

There are two main differences between the two similar extraction techniques. The first one being the amount of PDMS used: the stir bar is covered with ten times more stationary phase than the fiber (55 μL against 0.6 μL (Bicchi et al. 2002)). The optimized extraction time for HSSE is thus longer (90 min against 60 min for SPME) as there is more PDMS phase to interact with the compounds. For the same reason, HSSE was expected to have a higher extraction efficiency than SPME because of the higher polymer amount that covers the bar. However, the second difference between the two extraction methods is the desorption step: thermal desorption for SPME and liquid desorption for HSSE. As observed on the different food matrices, HSSE has a lower extraction efficiency than SPME, it can be concluded that liquid desorption leads to more compound loses than thermal desorption. Nonetheless, HSSE coupled to liquid desorption still provides highly concentrated extracts of terpenoids while SPME coupled to thermal desorption remains an efficient and sensible analytical method of terpenoids. Besides providing a direct comparison of different extraction methods, our results highlight the high potential of the HSSE and SPME methods to extract a large number of terpenoids in different food matrices.

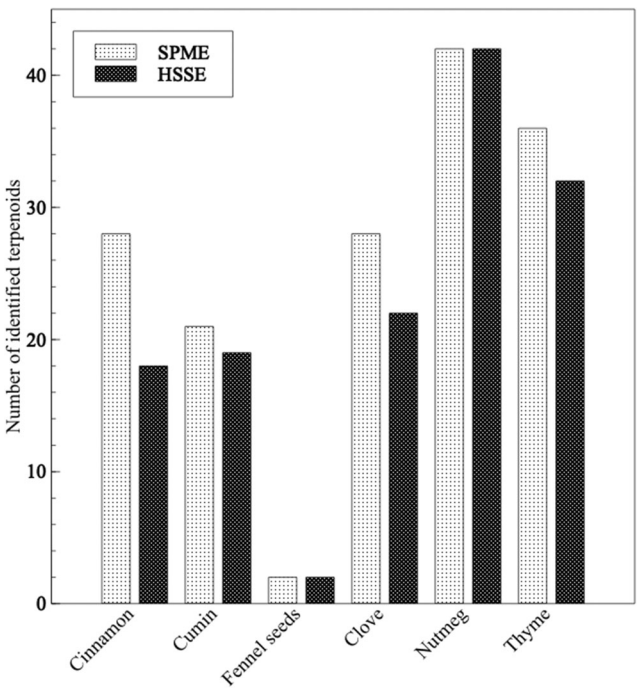

Fig. 4 Qualitative comparative study between SPME and HSSE for the extraction of terpenoids from seven different food matrixes

Table 4 Quantitative comparative study between SPME and HSSE for the extraction of terpenoids from seven different spices. Concentrations (μg/g) of terpenoids

Compound	Cinnamon		Cumin		Fennel seeds		Clove		Nutmeg		Thyme	
	SPME	HSSE	SPME	HSSE	SPME	HSSE	SPME	HSSE	SPME	HSSE	SPME	HSSE
α-Pinene	–[a]	–	–	–	–	–	–	–	986 ± 57	225 ± 25	60 ± 3	46 ± 9
Camphene	–	–	–	–	–	–	–	–	12.1 ± 0.1	3.8 ± 0.3	14.6 ± 0.9	16 ± 5
β-Pinene	–	–	–	2.4 ± 0.5	–	–	–	–	829 ± 57	400 ± 25	7.57 ± 0.09	6.0 ± 0.8
3-Carene	–	–	–	–	–	–	–	–	63 ± 4	39 ± 4	–	2.3 ± 0.1
Limonene	–	–	–	–	1.6 ± 0.1	< LOQ	1.37 ± 0.04	–	286 ± 14	363 ± 13	11.3 ± 0.4	13.3 ± 0.6
Eucalyptol	4.5 ± 0.1	4.3 ± 0.1	–	–	–	–	2.3 ± 0.3	–	–	24 ± 3	110 ± 4	54 ± 4
p-Cymene	–	–	5.1 ± 0.3	7.4 ± 0.4	–	–	< LOQ	5 ± 1	84 ± 4	86 ± 6	761 ± 13	738 ± 75
Camphor	–	–	–	–	–	–	–	–	–	–	157 ± 13	63 ± 13
Linalool	–	–	–	3.9 ± 0.3	–	–	16 ± 1	21 ± 5	214 ± 14	173 ± 8	2414 ± 329	1338 ± 88
4-Terpineol	18 ± 1	7.9 ± 0.4	13 ± 1	8 ± 1	–	–	–	–	1200 ± 71	1588 ± 75	139 ± 3	64 ± 4
Caryophyllene	3.5 ± 0.1	6.5 ± 0.3	1.8 ± 0.1	7.9 ± 0.5	–	–	686 ± 71	2738 ± 75	4.3 ± 0.3	24 ± 1	43 ± 1	88 ± 5
(Z)-β-Farnesene	–	–	–	–	–	–	–	86 ± 5	–	–	19 ± 1	–
(E)-β-Farnesene	–	–	22 ± 1	114 ± 9	–	–	–	–	11.0 ± 0.9	71 ± 5	14.7 ± 0.6	44 ± 4
Estragole	–	–	–	–	127 ± 16	39 ± 2	–	–	–	–	–	–
α-Humulene	1.3 ± 0.1	3.4 ± 0.1	–	–	–	–	73 ± 9	375 ± 25	–	5.4 ± 0.3	1.73 ± 0.09	5.5 ± 0.1
α-Terpineol	50 ± 1	22.5 ± 0.5	43.7 ± 0.9	11.3 ± 0.6	–	–	–	–	243 ± 14	338 ± 13	90.1 ± 0.9	58 ± 3
Borneol	84 ± 1	29 ± 1	–	–	–	–	–	–	–	7.6 ± 0.3	329 ± 14	188 ± 25
α-Farnesene	–	–	–	–	–	–	60 ± 4	263 ± 38	49 ± 3	151 ± 9	–	–
β-Citronellol	–	–	–	–	–	–	–	–	29.6 ± 0.7	17 ± 1	21 ± 7	12 ± 1
Cuminaldehyde	–	–	4043 ± 114	2588 ± 113	–	–	67 ± 10	31 ± 1	–	–	–	6.0 ± 0.3
Anethole	–	–	–	< LOQ	–	–	5.6 ± 0.9	3.6 ± 0.3	15.9 ± 1.3	< LOQ	–	–
Geraniol	43.9 ± 0.4	–	–	–	–	–	–	–	64 ± 3	28 ± 4	67 ± 1	29 ± 1
Thymol	–	–	19 ± 1	7.1 ± 0.6	–	–	–	–	–	–	9143 ± 357	7475 ± 238
Eugenol	–	–	–	–	–	–	125829 ± 10057	68338 ± 2900	291 ± 11	189 ± 8	–	–
Carvacrol	–	–	35.6 ± 0.6	16.4 ± 0.6	–	–	–	738 ± 50	–	51 ± 0.8	7300 ± 400	6425 ± 225

[a] Not extracted or not detected

Conclusions

Chemometric procedure is a very interesting approach for the optimization of extraction conditions. In this study, HSSE and SPME were successfully optimized by experimental design. This approach has made it possible to identify rapidly the influential parameters of the extraction: extraction time and temperature. The detection limits obtained for the compounds studied were adequate for their quantification in spices for both HSSE and SPME. Even though it was found out the SPME extracts more volatiles compounds then HSSE while HSSE showed high sensitivity and repeatability for the extraction of terpenes and terpenoids from spices.

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Compliance with Ethical Standards

Conflict of Interest Zélie Triaux declares that she has no conflict of interest. Hugues Petitjean declares that he has no conflict of interest. Eric Marchioni declares that he has no conflict of interest. Damien Steyer declares that he has no conflict of interest. Christophe Marcic declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals.

Informed Consent Not applicable.

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