Talanta 123 (2014) 207–217

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Analytical strategies based on multiple headspace extraction for the quantitative analysis of aroma components in mushrooms

I. San Román [a,*], M.L. Alonso [a], L. Bartolomé [b], R.M. Alonso [a], R. Fañanás [c]

[a] Analytical Chemistry Department, Faculty of Science and Technology, University of the Basque Country/EHU, P.O. Box 644, 48080 Bilbao, Spain
[b] Central Analysis Service (SGIker), Faculty of Science and Technology, University of the Basque Country/EHU, P.O. Box 644, 48080 Bilbao, Spain
[c] DTS-OABE Company, Orozko, Biscay, Spain

ARTICLE INFO

Article history:
Received 29 October 2013
Received in revised form
13 January 2014
Accepted 20 January 2014
Available online 6 February 2014

Keywords:
Multiple headspace extraction
Headspace analysis
HS-SPME
Mushroom
Volatile compounds
GC/MS

ABSTRACT

Headspace (HS) and headspace solid phase microextraction (HS-SPME) analysis by gas chromatography–mass spectrometry (GC/MS) have been found to be suitable methods for the analysis of volatile organic compounds. The objectives of this paper are to study the possibilities of multiple headspace extraction (MHE) for the quantitative determination of volatile compounds in mushroom samples and to compare the results obtained using three different sample treatment techniques. For this purpose, HS with two different injection techniques (pressure-loop system and gas-tight syringe autosampling system) and HS-SPME have been studied. Three processes were optimized for the analysis of 20 volatile compounds by experimental design technique based on Central Composite Design (CCD) and Full Factorial Design depending on the used methodology. Once the designs were finished, a trade off among optimum conditions for each compound analyzed was reached.

At optimum conditions, appropriate extraction time and sample amount for the three techniques used were established. Finally, the methods were validated in terms of linearity, detection and quantitation limits and repeatability. The most suitable method was then applied to the quantitative analysis of seven mushroom samples.

A detailed comparison of the analytical performance characteristics of HS and HS-SPME as sample treatment techniques for final GC/MS determination is given. In addition, MHE has been proved to be an adequate technique to avoid matrix effects in complex samples quantitation. Its applicability to the determination of volatile mushroom components, along with its limitations, is discussed in this work.
© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Volatile components contributing to mushroom aroma have been widely studied. Analytical methodology for determination of volatile compounds in vegetable matrices is continuously improving due to the important role of these compounds in organoleptic, chemical and nutritional characteristics [1,2]. Due to the fact that aromas present in mushrooms belong to different chemical families (esters, ketones, aldehydes, alcohols, terpens, phenols, and their derivatives), optimization of multicomponent sample preparation procedure is a difficult task. Moreover, there are significant differences in the behavior of the analytes between real samples and standard solutions since distribution constants depend on the composition of each one. In spite of analytical efforts, quantitation of aromas is rather problematic, and in most cases, not fully satisfactory results are obtained [3,4].

Although different sample treatment procedures have been used for the extraction of volatile compounds from mushroom samples [5–10], headspace extraction is now routinely used by scientists in a wide range of disciplines [11]. It is well known that HS is a non-quantitative extraction technique, thus being necessary to calibrate using extracted spiked blank samples. Unfortunately, this is not easy when determining the aroma profile in vegetables as no blank samples can be obtained [12]. A stepped procedure called multiple headspace extraction (MHE), whose theoretical bases were established in the earlier 80's, has been proposed as an alternative to overcome some typical difficulties as the matrix-effects [13–18]. MHE technique is based on the calculation of the area value corresponding to an exhaustive extraction of the analytes from a few steps of consecutive extractions (3 or 4) of the same sample. Thus, the matrix-effect is already eliminated even though obtained area value equivalent to a complete extraction depends only on the amount of analyte and not on the composition of the sample matrix or on the

* Corresponding author. Tel.: +34 94 601 33 66; fax: +34 94 601 35 00.
E-mail addresses: itxasosanroman@hotmail.com, itxaso.sanroman@ehu.es (I. San Román).

http://dx.doi.org/10.1016/j.talanta.2014.01.021
0039-9140 © 2014 Elsevier B.V. All rights reserved.

standards matrix. As extensively described by Kolb [19], the total area can be calculated according to Eq. (1):

$$A_T = \Sigma A_i = A_1/(1-e^{-q}) \quad (1)$$

where A_T is the total area, A_i is the peak area of the ith step, A_1 is the area of the first extraction and q is a constant which describes how fast the extraction process proceeds. The value of q can be experimentally obtained by plotting the neperian logarithms of the area values versus the number i of extraction steps, in fact, at equilibrium. A straight regression line is obtained and the slope of this straight line corresponds to q value [19].

$$\ln A_i = -q(i-1) + \ln A_1 \quad (2)$$

From the value of the slope we obtain the quotient Q:

$$Q = e^{-q} \quad (3)$$

Once obtained the A_T value, the real concentration of the target compounds in the original matrix can be gathered from a simultaneous external calibration graph, constructed apart with standard compounds by MHE.

On the other hand, SPME seems to be another attractive alternative for this kind of analysis. Introduced by Pawliszyn [20] SPME is a rapid solvent-free sampling technique that is well suited to the determination of volatile compounds by gas chromatography (GC). Since its introduction, many papers have dealt with the use of SPME for the determination of volatile compounds in the headspace of samples. It is also an excellent tool for comparative studies and semiquantitative determinations [21,22]. Since its development, this technique has become very popular for determining volatile and semi-volatile compounds due to its advantages over conventional extraction methods.

SPME can also be performed in a stepped fashion; this procedure is known as multiple headspace solid phase microextraction (MHS–SPME). The theoretical foundation of this combined technique under equilibrium was reported by Ezquerro et al [23]. MHS–SPME also employs the peak areas of a few consecutive extractions to calculate the amount of analyte of a complete extraction, but this time the analytes are partitioned in a three-phase system (sample matrix, headspace and fiber coating). In this case, the quotient Q is named as β, which has a value between zero and unity ($0<\beta<1$). β can be obtained from linear regression analysis of the logarithmic form of Eq. (2) as it is previously mentioned [23].

In the present study, the potentiality of multiple headspace extraction for the quantitative determination of volatile compounds in complex matrix samples (mushroom) using external solvent calibration has been investigated.

In this work, the multiple extraction method was applied to a particular mushroom species, which is growing up in our territory more and more, *Clathrus archeri*, which is commonly known as the octopus or cuttlefish stinkhorn [24]. *C. archeri* (Phallaceae), is a species native to Africa and Australasia although it is now also naturalized in Europe and North America. The knowledge of volatile compounds concentrations and proportions in this mushroom species will give us valuable information for later use in agroindustrial products. Although 22 volatile compounds had been already identified and qualitatively determined in *C. archeri* samples in previous work [25], to our knowledge, this is the first application of MHE and MHS–SPME to quantitative determination of aroma components of this mushroom. For the quantitative determination of *C. archeri* volatile compounds three different sample treatment techniques have been investigated: HS with two different injection techniques (pressure-loop system and gas-tight syringe autosampling system) and HS-SPME [12,15]. The two HS injection techniques discussed in this work showed differences, therefore a comparison between them has been done.

The extraction processes were optimized and validated in terms of linearity, precision, limits of detection and quantitation and by comparison of the quantitative results obtained by the three techniques. A detailed comparison of the analytical performance characteristics of MHE and MHS–SPME as aroma extraction techniques is given.

2. Experimental

2.1. Chemicals, materials and samples

Individual standard solutions in methanol (HPLC gradient grade, 99.8%, obtained from Prolabo (Leuven, Belgium)) were prepared from volatile compounds studied, 1-butanol (>99%), 1-pentanol (>99%), 6-methyl-5-hepten-2-one (98%), dimethyl trisulfide (98%), acetid acid (100%), 1-octen-3-ol (98%), 1-heptanol (99%), 2-methyl propanoic acid (99%), propanoic acid (99%), butanoic acid (>99%), pentanoic acid (99%), diphenyl ether (99%) and p-cresol (99%), all were supplied by Sigma Aldrich (St. Louis, MO, USA) while limonene/ocimene mixture (90%), isoamyl alcohol (98%), phenol (99%), 2-phenylethanol (100%), indole (99%) and 2-methyl butanoic acid (98%), were obtained by Alfa Aesar (Karlsruhe, Germany). Acetic acid was purchased from Merck (Madrid, Spain). The compounds selection for the study was based on literature [25] and previous work. In it, a qualitative analysis of *C. archeri* was performed and compounds with potential contribution to its aroma were selected. All standard solutions were stored at 4 °C in sealed glass vials completely filled (without headspace) to avoid analyte losses.

Samples of the wild species of *C. archeri* were collected during summer and autumn of 2010, 2011 and 2012 in the forests of Basque Country, Spain. Prior to analysis, two different sample pretreatments were carried out. One part of the mushroom samples were immediately transferred to the laboratory, mincing with a cryogenic grinder (SPEX SamplePrep 6770 Freezer/mill, Metuchen, New Jersey) and analyzed wet with qualitative and quantitative purposes. Other part of the samples was kept in glass bottles, frozen, triturated and freeze-dried at low temperatures (−46/−52 °C) and pressures (0.17/0.22 mbar) in a Cryodos-50 freeze-drier (Telstar, Spain).

For HS-SPME extraction, SPME fibers coated with 85 μm polyacrilate (PA), 100 μm polydimethylsiloxane (PDMS), 75 μm carboxen–polydimethylsiloxane (CAR/PDMS), 65 μm polydimethylsiloxane–divinylbenzene (PDMS/DVB) and 50/30 μm divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR/PDMS) obtained from Supelco (Bellefonte PA, USA) were used. All of them were thermally conditioned in accordance with the manufacturer's recommendations.

2.2. Optimization of the HS and HS-SPME extraction procedures

HS and HS-SPME extraction parameters can affect the extraction process, and in order to get the highest recovery of the analytes, the optimization of parameters such as extraction temperature, extraction time, fiber type, sample amount, desorption time and stirring speed was performed. Depending on the extraction method used, different kind and number of parameters have to be optimized. If only few factors are involved in the optimization, the most suitable design is a factorial design. Thus, a central composite design (CCD) methodology was used in order to optimize the extraction process in the case of headspace extraction pressure loop system with three variables. The variables and its low, central and high levels were: extraction temperature (Temperature; 60, 75, 90 °C), loop fill time (Loop fill *t*; 0.015, 0.1, 0.20 min) and vial pressure time (Vial press *t*; 0.20, 0.35, 0.50 min). In the case of headspace gas-tight syringe

autosampling system, only two variables were optimized by means of Full Factorial Design at two levels: extraction temperature (Temperature; 60, 90 °C) and stirring speed (Stirring; 250, 750 °C). Finally, CCD with three variables was also applied for headspace solid phase microextraction to obtain the optimun values for significant variables once the most suitable fiber type was chosen. The variables and its low, central and high levels were: extraction temperature (Temperature; 30, 45, 60 °C), stirring speed (Stirring; 250, 500, 750 rpm) and desorption time (Desorption time: 1, 5, 10 min). 10 μL of the standard solution prepared in methanol (5 mg L^{-1}) were placed in 20 mL sealed vial for all the experimental designs and incubated for 30 min [26].

The analysis of the obtained data was carried out with The Unscrambler® program and an ANOVA test was applied to test model significance. The rest of the conditions were set according to the literature and previous experience [11,27].

2.3. Optimization of sample amount and extraction time in MHE

Once extraction temperature, fiber type, desorption time and stirring speed had been optimized, the extraction time and sample amount were optimized by the univariant method, using real wet and freeze-dried samples. The sample amount was established taking into account the linearity of the plots of Eq. (2) as well as the slope (q or $\ln\beta$) for each compound and their detectability as it will be explained later.

Once the sample amount was established, extraction time have to be determined. Therefore, different sample amounts of wet mushroom (0.05, 0.1, 0.2 g) at different extraction times (10, 30, 50, 60, 70, 80, 100 min) were studied. For this purpose, each sample amount was extracted four times under optimized conditions at the different times to establish when the linearity of lnAi versus number of extractions $(i-1)$ plots was achieved.

In the same way, the extraction time of the standard solutions was established for each method once the optimum conditions were fixed by extracting, in this case, 10 μL of 5 μg L^{-1} standard solution at different times (5, 10, 15, 20, 25, 30 min).

Finally, with the aim of improving the results of the sample peak areas in the extraction time profile and trying to achieve better response from the volatile fatty acids avoiding a complex derivatisation process, the same study was repeated with freeze-dried mushroom samples. This time, different freeze dried sample amounts (0.01, 0.05, 0.1 g) at different extraction times (10, 30, 40, 50, 60, 70, 80, 100 min) were extracted under optimum conditions using the three proposed methods.

2.4. Features of the method

The MHS and MHS–SPME methods developed have been validated in terms of linearity, repeatability and limits of detection and quantitation. After optimization of the HS and HS-SPME variables, a linearity study was carried out. The linearity of the total peak area versus the concentration of the volatile compounds was studied for standard solutions in methanol. 10 μL of different concentration standard solutions (0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5 and 5 mg L^{-1}) were placed in a 20 mL sealed vial and extracted four times under optimized conditions. Linearity was evaluated by means of the number of compounds with an exponential decay of the peak areas and by the correlation coefficient of the linear plot lnAi versus $(i-1)$ obtained. Repeatability was evaluated in four freeze dried mushroom samples, in different ripeness stages and analyzed five different days with each extraction technique ($n=21$). Repeatability was expressed as RSD (%) of the calculated concentrations. Finally, the quantitative results obtained with the three techniques were compared.

2.5. Instrumentation

Static headspace analysis by loop pressure system was performed using an Agilent 7694E headspace sampler. For headspace sampling by syringe autosampling system and for headspace-solid phase microextraction sampling, PAL COMBI-xt sample injector was used.

An Agilent 6890 series gas chromatograph equipped with a split/splitless injector, autosampler and a 5973-N mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA) was used. The GC separation column was a 30 m × 0.25 mm × 0.25 μm DB-FFAP capillary column. The injector and detector temperatures were set at 250 °C and 300 °C respectively. Oven temperature was programmed with an initial temperature of 50 °C for 4 min, followed by an increase at a rate of 15 °C min^{-1} to 160 °C, finally, oven temperature was increased at a rate of 2 °C min^{-1} up to 200 °C. The carrier gas was helium (99.999%) at a flow rate of 1.4 mL min^{-1}. Data were acquired in selected ion monitoring (SIM) mode using a specific ion for each of the quantified compounds.

In the HS loop pressure system the injections were performed in split mode (10:1) and in splitless for HS syringe autosampling system and HS-SPME method.

3. Results and discussion

3.1. Optimization of the HS and HS-SPME extraction procedures

A selection of the most suitable conditions for the extraction processes were carried out in order to get the highest signal (maximum sensitivity) along with an acceptable chromatographic resolution. Therefore, three experimental designs were carried out. The analysis of the responses was carried out with The Unscrambler® program and an ANOVA test was applied to test model significance. In HS loop pressure system optimization, the design results did not show significant effects for any variable (extraction temperature, loop file time and vial pressure time) and compound ($p > 0.05$). Although non-significant effects have been observed, the response of the target compounds obtained is higher in the design center points. The extraction was favoured with the temperature increment, nevertheless, when the extraction was performed at temperatures ≥ 80 °C the chromatographic resolution got worse. Therefore medium conditions were selected as optimum: an extraction temperature of 75 °C, a loop fill time of 0.1 min and a vial pressure time of 0.35 min. At these conditions, the highest chromatographic signal as well as good chromatographic resolution was obtained. Once the extraction temperature was established, the sample loop and transfer line temperatures were set at 15 °C and 25 °C higher than the oven temperature, respectively [28]. Other variables as loop equilibration time and injection time have not been optimized because they did not affect the analytical signal in a significant way [29,30].

With regard to the HS syringe autosampling system optimization, a full factorial design was carried out with two variables: extraction temperature and stirring speed. The results show that temperature, stirring speed and their interaction were significant parameters only for five compounds (1-butanol, limonene, (Z)-β-ocimene, isoamyl alcohol and 1-pentanol). In the results obtained it can be observed that at low temperature the highest stirring speed improved the extraction, but not at high temperatures. A stirring speed of 500 rpm and an extraction temperature of 75 °C were selected due to better chromatographic resolution obtained. Moreover, in the headspace analysis, medium temperatures are recommended to avoid the over-pressurization of the sample vial.

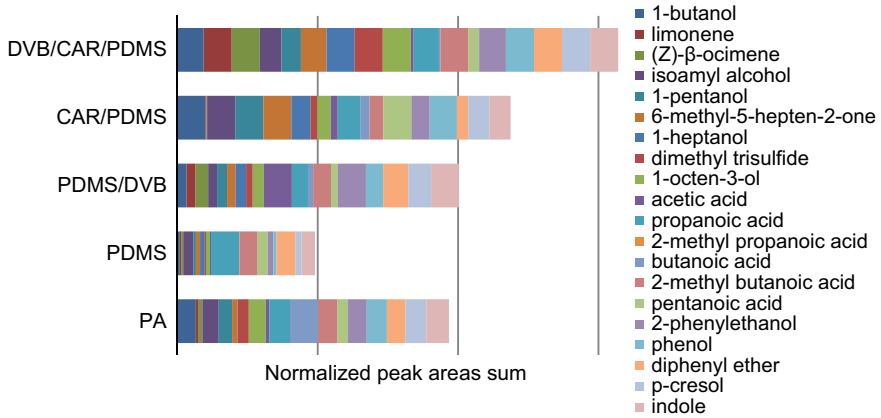

Fig. 1. Sum of total normalized peak areas of each mushroom aroma compound extracted with different SPME fibers: 85 μm PA, 100 μm PDMS, 65 μm PDMS/DVB, 75 μm CAR/PDMS and a 50/30 μm DVB/CAR/PDMS.

For both HS methodologies high extraction temperatures (75 °C) improved the extraction without introducing new compounds or modifying them.

Finally, three factors were selected to optimize HS-SPME extraction: extraction temperature, stirring speed and desorption time.

Before starting with the application of experimental design, five fibers were evaluated: PA, PDMS, CAR/PDMS, PDMS/DVB and DVB/CAR/PDMS. These fibers cover all the possibilities due to their apolar, polar or bipolar nature [12,21,26,31]. The results (see Fig. 1.) revealed that the less effective fiber was PDMS, followed by PA and PDMS/DVB. On the other hand, the chromatograms obtained with the CAR/PDMS fiber showed peak tails and lack of resolution for the first eluted compounds. As higher chromatographic signals with better resolution were obtained using the 50/30 μm DVB/CAR/PDMS fiber for most of the compounds, it was used in all remaining experiments.

Once the fiber was selected, the experimental design results revealed (see Supplementary material) that temperature had a statistical significant effect for all studied compounds ($p < 0.05$). Temperature may enhance or worsen the extraction process. For instance, extraction efficiency was larger at 50 °C than at 30 or 60 °C, so a temperature of 50 °C was selected as optimum. The interaction between stirring speed and desorption time had also a significant effect for most of the compounds. The extraction was also favoured by longer desorption time and higher stirring speed. Therefore both parameters were set at their studied highest levels; 10 min and 750 rpm, respectively. Response surfaces of some of them are shown in Supplementary material as an example. Similar strategy and plots were obtained for the rest of the compounds.

3.2. Optimization of sample amount and extraction time in MHE

Once these variables had been optimized, the extraction time and sample amount were studied. In the quantitative analysis by MHE under equilibrium conditions the equilibration time is essential to achieve a good linearity in the total peak area versus extraction number plots. The mass of mushroom placed in the vial must be appropriate to observe an exponential decay of the peak area with the number of extractions. If the mass is too low, sensitivity problems (due to small chromatographic signals) and repeatability problems (if the sample is not very homogeneous) could occur. If the mass is too large, bad correlation coefficients and no exponential decay could be found.

In the case of wet mushroom samples, results for all of the studied methods showed that the amount analyzed affected the number of compounds with a linear decay. The highest sample amounts analyzed (0.1 and 0.2 g) showed a lower number of compounds with a linear decay, probably due to a saturation of the headspace in the successive extractions. A linear decay for most of the compounds only was obtained at extraction times of 30 and 40 min when a sample mass of 0.05 g was used. Under these conditions, 11 compounds showed a linear decay with correlation coefficient higher than 0.78 in the case of HS extraction methods and 13 compounds with correlation higher than 0.86 in the case of HS-SPME. However, it was remarkable that none of the acid compounds showed a linear decay in wet samples and therefore, they cannot be analyzed by MHE. It is worth mentioning that the response of volatile fatty acids can also be improved with a derivatisation procedure [32].

In addition, taking into account the results obtained by MHS–SPME as a reference, the correlation coefficients for compounds that showed good linearity were similar in most of the studied extraction times. For wet mushroom samples, linearity obtained from the successive extraction at each extraction time was maintained from 30 min to the largest studied time.

With regard to the graphical representations of chromatographic peak area versus extraction time different trends were observed: For standard compounds (Fig. 2a), the peak area (proportional to the amount of analyte extracted) increased by increasing the extraction time until it reached a plateau. On the contrary, for wet mushroom samples (Fig. 2b) a random behavior could be observed, probably due to the complexity and the heterogeneity of the sample. This trend could be explained by the uncontrollable water content present in different parts of mushroom although the sample used was the same. With regard to the results obtained for the freeze dried mushroom samples, the extraction time curve (Fig. 2c) shows that for almost all compounds the peak areas increased by increasing the extraction time until it reached a maximum at about 60 min, which is an expected trend. However, in some cases (6-methyl-5-hepten-2-one, phenol, 1-octen-3-ol and dimethyl trisulfide), the longer extraction time the lower chromatographic signal was obtained. Therefore a compromise must be reached.

When a further study was carried out not only checking the extraction profile, but also taking into account the linearity of the compounds in each extraction time, good linearity for all the 20 compounds was achieved but only by means of MHS–SPME (Table 1). In this particular case the best conditions were at extraction times between 10 and 50 min for 0.01 g of freeze dried sample and also at an extraction time of 40 min for 0.05 g sample.

In MHS–SPME method a 40 min extraction time was selected because wider sample amount range with linearity for all the compounds could be obtained. In the case of HS syringe autosampling and HS loop pressure methods, the best results were

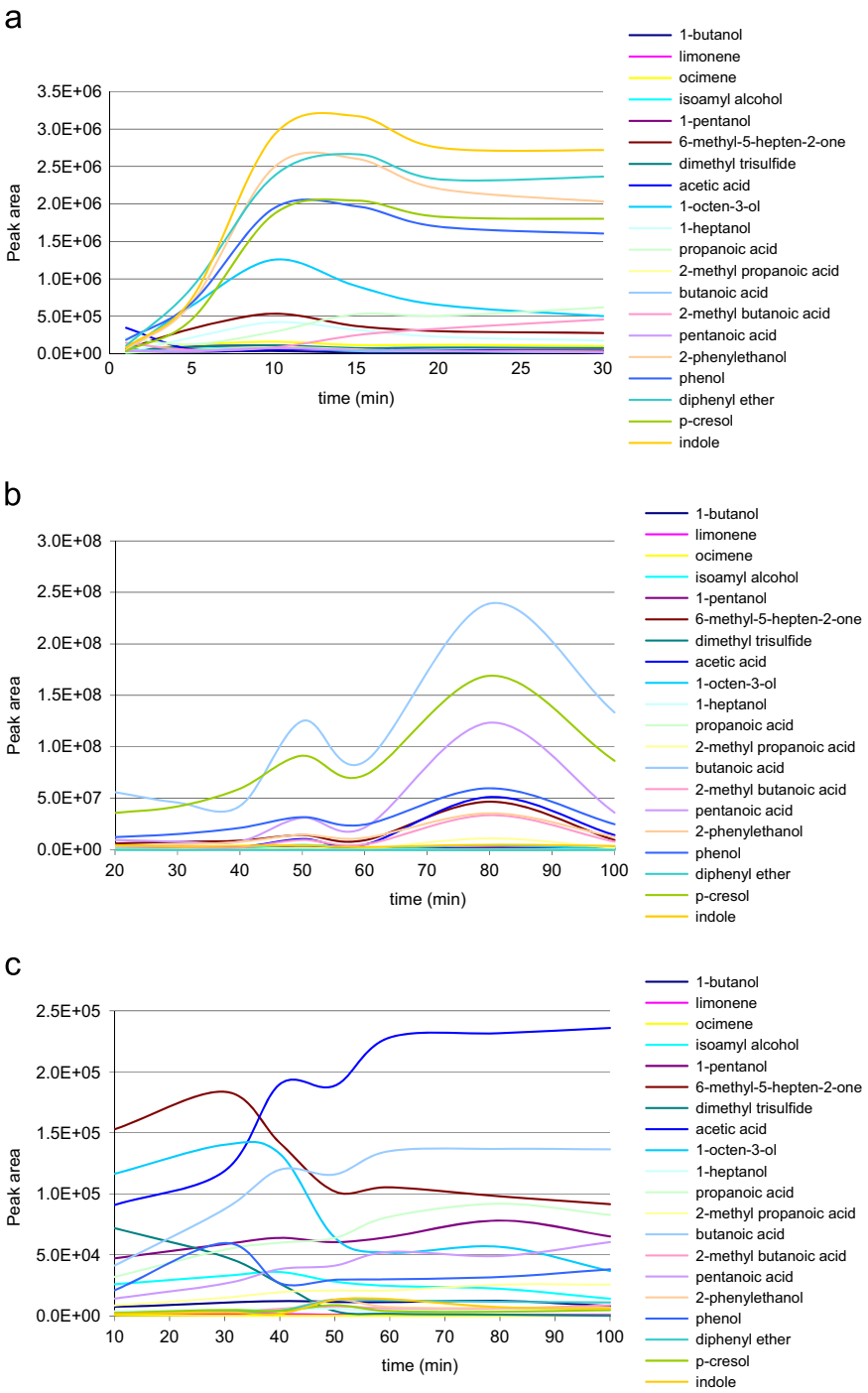

Fig. 2. HS-SPME extraction time profile of volatile compounds from (a) 5 mg L^{-1} standard solution (b) wet mushroom sample and (c) freeze-dried mushroom sample.

obtained with 0.05 g of mushroom sample at 50 and 30 min of extraction time, respectively. As can be seen in Table 1, in any case good linearity results were found for all the compounds (14 compounds for HS syringe autosampling system and 18 compounds for HS loop system). Moreover, in general no linear decay was observed when the highest sample amount was used (0.1 g). This effect could be due to the larger amount of sample used, the higher volatile compounds concentration was obtained, and therefore, an exhaustive extraction with good linearity results was more complicate to perform. With regard to extraction time, the best results for all techniques were observed between 30 and 50 min. As expected, these extraction times are higher than the equilibrium times (see Fig. 2a) selected for the standards due to the fact that volatile compounds are absorbed within the mushroom sample.

Taking into account that better results were obtained by using freeze dried mushroom samples, these were used for all remaining experiments. Moreover, by freeze dry process, it was proved that β parameters (or Q quotient in the case of HS extraction) were maintained much more constant along different analysis than with the wet samples.

In summary, an extraction time of 40 min and a sample mass of 0.01 g of freeze dried mushroom were selected as optimum for HS-SPME extraction where all of the compounds achieve a good linearity and good resolution. On the other hand, 30 and 50 min with a sample amount of 0.05 g were selected for HS loop pressure

Table 1
Extraction time and sample amount optimization for freeze-dried mushroom sample. Best conditions for MHE and MHS–SPME methods were highlighted in bold.

Method	Sample amount (g)	Extraction time (min)	no. of compounds that show linearity	R^2
HS-SPME	**0.01**	10	20	0.987–0.999
		30	20	0.778–0.999
		40	**20**	**0.848–0.999**
		50	20	0.778–0.999
		60	19	0.796–0.999
		80	19	0.826–0.999
		100	17	0.829–0.999
	0.05	10	18	0.856–0.999
		30	19	0.907–0.999
		40	20	0.784–0.998
		50	18	0.892–0.998
		60	18	0.888–0.999
		80	17	0.773–0.998
		100	16	0.772–0.997
	0.1	10	13	0.854–0.999
		30	16	0.876–0.999
		40	12	0.729–0.999
		50	16	0.778–0.993
		60	16	0.782–0.972
		80	14	0.833–0.982
		100	11	0.766–0.966
HS syringe autosampling system	0.01	10	10	0.809–0.998
		30	9	0.873–0.999
		40	5	0.907–0.999
		50	5	0.972–0.999
		60	6	0.814–0.995
		80	6	0.906–0.997
		100	7	0.763–0.967
	0.05	10	12	0.838–0.999
		30	5	0.915–0.999
		40	9	0.769–0.998
		50	**14**	**0.757–0.984**
		60	11	0.755–0.998
		80	9	0.839–0.997
		100	4	0.857–0.974
	0.1	10	5	0.797–0.999
		30	6	0.915–0.999
		40	6	0.950–0.998
		50	7	0.912–0.999
		60	4	0.769–0.999
		80	5	0.774–0.996
		100	9	0.777–0.997
HS loop pressure system	0.01	10	11	0.843–0.999
		30	11	0.809–0.998
		40	11	0.788–0.999
		50	10	0.818–0.998
		60	10	0.900–0.999
		100	15	0.853–0.999
	0.05	10	13	0.829–0.999
		30	**18**	**0.770–0.999**
		40	14	0.860–0.999
		50	9	0.760–0.997
		60	9	0.908–0.999
		100	14	0.810–0.992

and HS syringe autosampling systems, respectively. Table 2 shows summarized optimum conditions selected for the extraction methods.

3.3. Features of the methods

The MHS and MHS–SPME methods developed have been validated in terms of linearity, repeatability and limits of detection and quantitation (LODs and LOQs). Table 3 shows the linear ranges, the LODs and LOQs and the correlation coefficients (R^2) for the exponential decay of the peak area of the volatile compounds studied.

As it could be expected, depending on the compound studied and the methodology used, different linear behavior was observed in the ranges tested. When linear behavior was obtained, regression coefficients were between 0.704 and 0.999. The results show (Table 3) that the best linearity was obtained when HS loop pressure system technique was used (all the compounds show the maximum linearity range except 1-butanol, limonene, isoamyl alcohol and 1-heptanol), although better mean correlation coefficients were achieved when HS-SPME was applied. For HS syringe system, the results were similar to those obtained by HS-SPME in terms of linearity and regression coefficients values (R^2).

LODs and LOQs were calculated from the peak area value for the first extraction of a blank plus three and ten times the standard deviation of five blank replicates respectively [33]. These parameters were calculated in this way due to the impossibility to obtain an experimental decay from a blank sample. For the calculation of the LOD and LOQ concentrations, a linear calibration was obtained by representing the total area against the standard concentration used before for the linearity study, where, the slope obtained in the multiple extractions was used to calculate the total area present in the vial using Eq. (1). Finally, the chromatographic signal of the blanks was interpolated in the linear calibration to determine the concentrations of LOD and LOQ of each methodology.

Calibration curves showed adequate correlation coefficients with values higher than 0.98 with exception of acids, which showed limitations in different aspects of the employed multiple extraction methodologies. The three methods allowed reaching limits of detection between 0.16–2.08 ng, 0.01–0.36 ng and 0.01–0.21 ng for HS loop pressure system, HS syringe autosampling system and for HS-SPME respectively, except, once more, for acids. Acids presented LODs between 0.22–4.19 ng, 0.38–5.37 ng and 0.02–3.20 ng for the three methodologies. When the LODs and LOQs were compared it could be seen that the lowest values for a higher number of compounds were achieved with HS-SPME method. HS loop pressure extraction gives the highest and therefore, the worst LODs and LOQs except for 2-methyl propanoic and pentanoic acids. HS syringe autosampling method offers the best LOQs for 7 compounds but only 4 of them (dimethyl trisulfide, butanoic acid, 2-methyl butanoic acid and diphenyl ether) followed linear behavior. The next best values for the other three non-linear compounds (1-butanol, (Z)-β-ocimene and 6-methyl-5-hepten-2-one) were obtained with HS-SPME method so, therefore, 14 out of the 20 compounds analyzed had the best LOQs by means of HS-SPME methodology (see Table 1).

As already mentioned before, the aroma was composed by different families of compounds. If we study the sensitivity of the methods with respect to this classification, it can be concluded that the worst quantitation limits were found in volatile fatty acids such as propanoic, butanoic, pentanoic and 2-methyl butanoic acids (1–4 ng), while the best ones have been found for monoterpenoids and irregular terpenes such as limonene, (Z)-β-ocimene and 6-methyl-5 hepten-2-one (<0.2 ng). In summary, the results show (Table 3) that the best LODs and LOQs were obtained when HS-SPME was used. By this method, the detection limits obtained for some of the studied volatile compounds were lower than the olfactory threshold of these compounds [34]. On the contrary, headspace loop pressure system technique showed the worst sensitivity. It should be pointed out that the chromatographic determination by HS loop pressure system was carried out in split mode (1:10), therefore, it could be expected to achieve higher LOD and LOQ values by this technique.

After that, an evaluation of the robustness of Q and β values was carried out with different ripeness stage freeze-dried mushroom

Table 2
Selected conditions for Headspace loop pressure system (HS 7694E), Headspace syringe autosampling system (HS CTC-PAL) and Headspace-solid phase microextraction (HS-SPME CTC-PAL).

HS loop pressure system		HS syringe autosampling system		HS-SPME	
Injection volume	3 μL	Injection volume	1000 μL	Pre incubation time	5 min (300 s)
Oven temperature	75 °C	Incubation temperature	75 °C	Incubation temperature	50 °C
Transfer line temperature	90 °C	Incubation time	40 min (2400 s)	Stirring speed	750 rpm
Loop temperature	100 °C	Syringe temperature	95 °C	Stirring On time	5 s
Vial equilibration time	50 min	Stirring speed	500 rpm	Stirring Off time	2 s
GC cycle time	30 min	Fill speed	100 μL s^{-1}	Vial penetration	31 mm
Stirring level	Off	Fill strokes	0	Extraction time	40 min (2400 s)
Loop fill time	0.10 min	Pullup delay	3500 s	Injection penetration	57 mm
Loop equilibration time	0.05 min	Injection speed	500 μL s^{-1}	Desorption time	10 min (600 s)
Inject time:	3 min	Pre injection delay	200 ms	Post Fib Cond temperature	10 min (600 s)
Vial pressurization time	0.35 min	Post injection delay	400 ms	GC run time	40 min (2400 s)
Sample amount	0.05 g	GC run time	50 min (3000 s)	Sample amount	0.01 g
		Sample amount	0.05 g		

Table 3
Linearity study with mushroom aroma compound standard solutions in methanol. Studied linearity range was 0.005–5 mg L^{-1} for all methodologies.

Compound	HS loop pressure system				HS syringe autosampling system				HS-SPME			
	Linear rangemg L^{-1}	R^2	LOD (ng)	LOQ (ng)	Linear range (mg L^{-1})	R^2	LOD (ng)	LOQ (ng)	Linear range (mg L^{-1})	R^2	LOD (ng)	LOQ (ng)
1-Butanol	0.01–5	0.814–0.931	0.46	1.53	0.005–5	0.892–0.981	0.03	0.11	0.005–2.5	0.946–0.983	0.17	0.57
Limonene	0.01–5	0.823–0.999	0.96	3.20	0.1–5	0.987–0.999	0.10	0.32	0.005–5	0.858–0.998	0.06	0.19
(Z)-β-ocimene	0.005–5	0.835–0.999	0.38	1.27	0.1–5	0.964–0.994	0.04	0.14	0.005–5	0.885–0.999	0.04	0.14
Isoamyl alcohol	0.05–5	0.753–0.890	0.14	0.46	0.01–5	0.750–0.979	0.02	0.07	0.005–2.5	0.852–0.999	0.02	0.06
1-Pentanol	0.005–5	0.797–0.955	0.33	1.11	0.01–5	0.901–0.988	0.04	0.14	0.005–1	0.897–0.981	0.08	0.26
6-Methyl-5-hepten-2-one	0.005–5	0.738–0.982	0.92	3.07	0.005–5	0.745–0.996	0.01	0.03	0.005–5	0.947–0.993	0.02	0.08
Dimethyl trisulfide	0.005–5	0.788–0.973	0.11	0.37	0.01–5	0.911–0.980	0.01	0.02	0.5–5	0.984–0.996	0.23	0.76
Acetic acid	0.005–5	0.704–0.989	1.91	6.36	0.05–2.5	0.827–0.999	0.70	2.34	0.5–5	0.900–0.967	0.02	0.06
1-Octen-3-ol	0.005–5	0.719–0.995	0.53	1.76	0.1–5	0.760–0.992	0.11	0.38	0.005–5	0.768–0.999	0.01	0.03
1-Heptanol	0.01–5	0.890–0.982	1.00	3.35	0.01–5	0.816–0.962	0.24	0.80	0.005–5	0.805–0.999	0.04	0.13
Propanoic acid	0.005–5	0.870–0.984	1.28	4.27	0.005–5	0.709–0.992	0.85	2.84	0.01–5	0.792–0.983	0.29	0.96
2-Methyl propanoic acid	0.005–5	0.830–0.999	0.22	0.74	0.5–5	0.821–0.917	5.37	17.89	0.01–5	0.870–0.985	0.37	1.24
Butanoic acid	0.005–5	0.735–0.999	4.19	13.96	0.005–5	0.852–0.988	1.16	3.87	0.01–5	0.859–0.960	1.62	5.39
2-Methyl butanoic acid	0.005–5	0.729–0.999	0.44	1.46	0.05–5	0.954–0.992	0.38	1.27	0.1–5	0.889–0.934	3.20	10.65
Pentanoic acid	0.005–5	0.925–0.994	2.66	8.86	2.5–5	0.895–0.999	3.15	10.49	0.005–5	0.785–0.992	2.89	9.63
2-Phenylethanol	0.005–5	0.719–0.971	2.08	6.94	0.005–5	0.894–0.999	0.25	0.82	0.005–5	0.822–0.996	0.06	0.21
Phenol	0.005–5	0.719–961	1.06	3.52	0.005–5	0.835–0.991	0.23	0.77	0.005–5	0.863–0.975	0.08	0.28
Diphenyl ether	0.005–5	0.721–0.939	0.23	0.76	0.005–5	0.947–0.984	0.11	0.36	0.01–5	0.873–0.981	0.21	0.71
p-Cresol	0.005–5	0.803–0.996	0.64	2.14	0.005–5	0.914–0.993	0.36	1.20	0.005–5	0.899–0.994	0.02	0.07
Indole	0.005–5	0.846–0.985	0.16	0.53	0.005–5	0.851–0.996	0.11	0.38	0.005–5	0.918–0.985	0.06	0.18

samples. 3 replicates of 4 different freeze-dried mushroom samples were analyzed in five different days for each extraction method. Two samples out of four correspond to ripe mushrooms while the other two samples were unripe mushroom eggs. In Table 4 it can be observed the mean Q and β values and their standard deviation (s). The relative standard deviation of the Q values for HS loop pressure system was between 1% and 29%, which was higher than HS syringe autosampling system values (1–17%). In the case of HS-SPME, the β deviation was in the same order than HS syringe system (4–18%). This fact shows that the β or Q values seem to be constant within a similar sample matrix although different ripeness stages had been considered for the study. The same study was carried out with a wet mushroom sample (values not showed) in which the relative standard deviation for Q and β values in all the methods compared for compounds with linear behavior (less than in freeze-dried samples) were much higher.

At the same time, precision of the overall analytical procedure has been evaluated as repeatability and it was expressed as RSD (%) of the calculated concentrations (See repeatability column in Table 4 and the concentrations obtained by MHE and MHS–SPME techniques in Table 5). In general the highest values were found with HS gas-tight syringe autosampling system. The problem corresponding to the lack of linearity in the quantitated samples reveals slightly worse repeatability values. As it can be seen in Tables 4 and 3 compounds (isoamyl alcohol, dimethyl trisulphide and 2-methyl propanoic acid), out of 18 compounds which showed linearity behavior in the MHE process, had RSD repeatability values higher than 30%. The gas-tight syringe autosampler could have repeatability problems because of possible sample loss. As the sample is transferred from the vial to the injection port, some of it may be lost because of the pressure differences between the vial and atmospheric conditions. In the case of the pressure-loop system, the loop can be thermostated to high temperatures (100 °C), which helps to lessen adsorption of higher molecular weight and sensitive compounds and the fixed volume of the sample loop helps to improve run-to-run repeatability. In this case, 2 (limonene and indole) out of 14 compounds showed repeatability values higher than 30%. HS-SPME method precision was satisfactory with lower coefficients of variation than other techniques. In this case, the RSD values were less than 25% except for

Table 4
Q and β values obtained in the MHE and MHS–SPME determinations of volatile compounds in freeze dried mushroom samples and their relative standard deviation. The results correspond to the analyses of 4 different mushroom samples at different ripeness stages. Three replicates per day during five different days were carried out by the three extraction techniques ($n=21$).

Compound	HS loop pressure system		HS syringe autosampling system		HS-SPME	
	$Q \pm s$	Repeatability (RSD%)	$Q \pm s$	Repeatability (RSD%)	$\beta \pm s$	Repeatability (RSD%)
1-Butanol	0.88 ± 0.12	18	N.L.	N.L.	0.79 ± 0.10	18
Limonene	0.57 ± 0.01	> 30	0.75 ± 0.07	27	0.63 ± 0.05	20
(Z)-β-ocimene	0.74 ± 0.05	< LOQ	N.L.	N.L.	0.67 ± 0.08	> 30
Isoamyl alcohol	0.86 ± 0.04	11	0.92 ± 0.09	> 30	0.63 ± 0.10	7
1-Pentanol	0.78 ± 0.02	23	N.L.	N.L.	0.81 ± 0.12	30
6-Methyl-5-hepten-2-one	0.56 ± 0.12	19	N.L.	N.L.	0.67 ± 0.05	15
Dimethyl trisulfide	0.75 ± 0.10	27	0.80 ± 0.02	> 30	0.47 ± 0.02	< LOQ
Acetic acid	N.L.	N.L.	N.L.	N.L.	0.89 ± 0.04	16
1-Octen-3-ol	0.59 ± 0.08	13	0.87 ± 0.13	23	0.64 ± 0.03	18
1-Heptanol	0.76 ± 0.10	< LOQ	0.96 ± 0.08	22	0.79 ± 0.06	19
Propanoic acid	0.77 ± 0.06	N.L.	N.L.	N.L.	0.82 ± 0.06	14
2-Methyl propanoic acid	0.88 ± 0.14	15	0.97 ± 0.05	> 30	0.80 ± 0.07	17
Butanoic acid	0.60 ± 0.12	N.L.	N.L.	N.L.	0.73 ± 0.05	25
2-Methyl butanoic acid	0.77 ± 0.03	13	N.L.	N.L.	0.65 ± 0.05	24
Pentanoic acid	0.98 ± 0.28	24	N.L.	N.L.	0.83 ± 0.11	18
2-Phenylethanol	0.58 ± 0.05	< LOQ	0.82 ± 0.08	16	0.77 ± 0.08	19
Phenol	0.81 ± 0.04	28	0.95 ± 0.03	19	0.82 ± 0.13	19
Diphenyl ether	0.82 ± 0.21	27	0.83 ± 0.14	8	0.81 ± 0.03	24
p-Cresol	0.74 ± 0.07	16	0.90 ± 0.06	27	0.79 ± 0.11	16
Indole	0.64 ± 0.02	> 30	0.84 ± 0.08	< LOQ	0.83 ± 0.07	25

NL: Non-linear.
< LOQ: Under limits of quantitation.

Table 5
Concentration of the volatile compounds ($\mu g\, g^{-1}$) and their relative standard deviation ($n=5$) identified by MHE and MHS–SPME–GC/MS of 3 freeze-dried mushroom samples: Sample A corresponds to a ripe mushroom while samples B and C were unripe mushroom eggs.

Compound	Concentration $\pm s$ ($\mu g\, g^{-1}$)								
	Sample A			Sample B			Sample C		
	HS loop	HS syringe	HS-SPME	HS loop	HS syringe	HS-SPME	HS loop	HS syringe	HS-SPME
1-Butanol	1.7 ± 0.4	NL	1.4 ± 0.2	0.6 ± 0.2	NL	0.5 ± 0.1	0.34 ± 0.05	NL	0.28 ± 0.05
Limonene	0.03 ± 0.01	0.02 ± 0.01	0.030 ± 0.005	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.010 ± 0.005	0.02 ± 0.01	0.02 ± 0.01
(Z)-β-ocimene	0.010 ± 0.005	< LOQ	NL	0.03 ± 0.01	NL	0.03 ± 0.01	< LOQ	NL	0.04 ± 0.02
Isoamyl alcohol	NL	NL	0.4 ± 0.1	0.13 ± 0.08	0.2 ± 0.1	0.2 ± 0.1	0.41 ± 0.04	NL	0.30 ± 0.01
1-Pentanol	1.1 ± 0.2	NL	1.1 ± 0.3	2.8 ± 0.7	NL	2.7 ± 0.3	1.59 ± 0.31	NL	1.4 ± 0.3
6-Methyl-5-hepten-2-one	26 ± 5	NL	18 ± 4	4.5 ± 0.3	NL	4.3 ± 0.6	0.15 ± 0.04	NL	0.15 ± 0.02
Dimethyl trisulfide	NL	0.02 ± 0.01	0.010 ± 0.002	0.22 ± 0.06	0.3 ± 0.1	< LOQ	0.07 ± 0.01	NL	< LOQ
Acetic acid	NL	NL	NL	NL	NL	113 ± 18	NL	NL	720 ± 69
1-Octen-3-ol	NL	0.13 ± 0.03	0.09 ± 0.02	3.0 ± 0.2	2.2 ± 0.6	3.3 ± 0.7	3.3 ± 1.0	NL	2.3 ± 0.5
1-Heptanol	ND	0.16 ± 0.02	0.09 ± 0.01	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.05	0.34 ± 0.04	0.31 ± 0.1	0.23 ± 0.07
Propanoic acid	NL	NL	1379 ± 192	NL	NL	2.8 ± 0.7	NL	NL	5.4 ± 1.5
2-Methyl propanoic acid	NL	NL	732 ± 54	4.4 ± 0.6	NL	4.1 ± 0.7	2.4 ± 0.4	NL	1.9 ± 0.2
Butanoic acid	NL	NL	4548 ± 772	NL	NL	11 ± 3	NL	NL	1.5 ± 0.5
2-Methyl butanoic acid	NL	NL	939 ± 226	3.0 ± 0.4	NL	2.5 ± 0.4	22.3 ± 0.5	NL	NL
Pentanoic acid	252 ± 60	NL	328 ± 41	NL	NL	30 ± 5	18.8 ± 2.0	NL	NL
2-Phenylethanol	< LOQ	0.05 ± 0.01	0.06 ± 0.01	< LOQ	< LOQ	0.020 ± 0.003	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.01
Phenol	5.4 ± 1.5	7.6 ± 1.7	8.2 ± 0.3	< LOQ	0.2 ± 0.05	0.15 ± 0.03	0.18 ± 0.06	0.19 ± 0.04	0.10 ± 0.03
Diphenyl ether	< LOQ	< LOQ	0.14 ± 0.04	< LOQ	< LOQ	0.16 ± 0.03	0.003 ± 0.001	0.003 ± 0.001	NL
p-Cresol	6.2 ± 1.0	7.3 ± 0.7	8.0 ± 0.4	0.010 ± 0.004	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.010 ± 0.005	0.02 ± 0.01
Indole	NL	NL	9 ± 2	< LOQ	< LOQ	0.02 ± 0.01	0.02 ± 0.01	< LOQ	0.02 ± 0.01

NL: Non-linear.
< LOQ: Under limits of quantitation.

(Z)-β-ocimene and 1-pentanol. These results are comparable to those obtained in other works for this kind of volatile compounds analysis [12].

Since no certified reference material was available for these compounds in mushrooms, it was thought that one way to validate the optimized MHE and MHS–SPME methods was to compare them with the same sample. In this way, the quantitative data obtained for three different freeze-dried mushroom samples by MHE and MHS–SPME techniques (see Table 5) were taking into account as method validation step. As the results show (see Table 5), the performances of the developed methods were comparable. The three techniques have been successfully applied to mushroom headspace analysis. All the studied methods enable the quantitation of volatile compounds which contribute significantly to the aroma of *C. archeri*. Comparable concentration ranges together with an aceptable repeatability was obtained for all the methodologies. Although the RSD values (see Table 4) were between 1 and 29% and in some cases were higher than 30%, the

concentration levels of the volatile compounds found in this mushroom species were, in several cases, in ng L^{-1} levels.

On the other hand, as it can be seen, the linearity of the multiple extractions depends directly on the amount of analyte. For that reason, the same analyte could be not quantitated in all the samples analyzed in the repeatability study although in other samples the quantitation had been possible. In addition, depends on the technique used there was different number of compounds which have showed linearity as previously happened. Taking this last fact into account, it could be stated that studied techniques could be complementary. Depend on the analyte amount the quantitation could be possible with one or other extraction technique. The best results were obtained by MHS–SPME which was the technique that achieved higher number of quantitated compounds. In all the cases that the quantitation had been possible the results obtained were comparable (in terms of concentration values) with all the evaluated techniques, indicating that the validation of the method by the different extraction methods comparison, had been successfully obtained.

3.4. MHE quantitative analysis of C. archeri mushroom samples

The developed MHS–SPME/GC method was applied for the quantitative analysis of seven different mushroom samples at different ripeness stages.

For the estimation of the concentrations of *C. archeri* mushroom aroma compounds by MHE, external standard calibration method was used. External standard calibration was carried out through the optimized MHS–SPME process similar to that for the sample proper. However, since an exhaustive gas extraction is performed with both samples (mushroom sample and multicomponent standard solution), this standard does not have to contain the same matrix as the sample. In this case, a simple vapour standard prepared in the vial using the TVT (Total Vaporization Technique) was used. This variant is called total vaporization technique when the whole sample, including the components of the matrix will evaporate [19]. With this purpose, 10 μL of 5 mg L^{-1} standard solution prepared in methanol were placed into 20 mL sealed vial and extracted four times under optimized conditions as it was done with the sample. Finally, the concentrations were estimated by means of previously mentioned equations.

Among analyzed samples (see Table 6), samples 1 and 2 were unripe mushroom eggs while sample 3 was a ripe mushroom egg (characteristic red colour already appeared while it is still inside the egg). Sample 4, 5 and 6 were ripe mushrooms (mushroom has already left the egg), while sample 7 corresponds to a mushroom in an advanced stage of putrefaction. As can be seen in Table 6 no significant differences were found between the two first samples (Sample 1 and 2). On the contrary, when the analysis of more ripe mushroom egg was done (sample 3), higher concentrations of the majority of the compounds were found. All the compounds except isoamyl alcohol, 2-phenylethanol and phenol were present in a higher concentration than before and, in some cases, the quantitation was not possible (acetic and propanoic acids). In the same way, even still higher concentrations were found in samples 4, 5 and 6. This was expected because these samples correspond to a ripe mushroom which is characterized by its strong and disgusting aroma. Finally, in sample 7 it can be seen that some of these compounds appear in lower concentration than before (2-methyl-5-hepten-2-one, diphenyl ether and indole). In this degradation stage the analysis shows that the mushroom has lost a portion of some of the compounds while few other's presence increases (1-butanol, isoamyl alcohol, 2-phenylethanol and p-cresol). In samples 3, 4, 5, and 6, some compounds did not have a concentration value because β parameter could not be assigned. This fact

Table 6
Concentration of the volatile compounds (μg g^{-1}) identified by MHS–SPME–GC/MS of 7 freeze dried mushroom samples: 1 and 2 were unripe mushroom eggs while sample 3 corresponds to a ripe mushroom egg (characteristic red colour already appeared while it is still inside the egg). Samples 4, 5 and 6 correspond to a ripe mushroom (mushroom has already left the egg) and 7 to a over ripe mushroom. Sample 6 was also analyzed wet (sample 6').

Compound	Concentration (μg g^{-1})							
	Freeze dried mushroom samples							Wet mushroom
								
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 6'
1-Butanol	0.08	0.06	1.0	0.6	1.2	**0.4**	9.2	**8.5**
Limonene	0.03	0.03	0.07	0.6	0.1	**0.05**	0.05	**0.6**
(Z)-β-ocimene	ND	ND	0.03	ND	ND	**0.02**	0.02	**0.07**
Isoamyl alcohol	0.9	0.6	0.5	0.4	0.1	**0.2**	18	**80**
1-Pentanol	0.1	0.1	3.8	NL	0.8	**0.5**	0.4	**4.0**
6-Methyl-5-hepten-2-one	0.12	0.03	56	44	62	**23**	3.0	**17.9**
Dimethyl trisulfide	ND	0.01	0.04	0.4	0.03	**0.2**	0.02	**2.5**
Acetic acid	661	719	NL	NL	NL	**NL**	4977	**NL**
1-Octen-3-ol	5.8	2.8	7.8	0.5	1.5	**0.6**	0.2	**40**
1-Heptanol	0.05	0.05	0.3	0.09	0.2	**0.1**	0.08	**0.8**
Propanoic acid	ND	7.6	NL	NL	0.4	**NL**	3.3	**NL**
2-Methyl propanoic acid	0.4	0.3	5.4	442	214	**229**	12	**NL**
Butanoic acid	0.2	0.1	4.7	5489	5152	**595**	1000	**NL**
2-Methyl butanoic acid	0.2	0.1	1.9	367	347	**204**	160	**NL**
Pentanoic acid	0.2	0.1	7.2	12	108	**7.7**	15	**NL**
2-Phenylethanol	0.04	0.03	0.009	0.09	0.07	**0.02**	4.1	**35**
Phenol	0.4	0.5	0.5	13	5.3	**2.0**	2.3	**23**
Diphenyl ether	0.03	0.03	0.1	0.1	0.3	**0.1**	0.02	**0.3**
p-Cresol	0.01	0.01	0.1	7.8	6.3	**4.6**	11	**73**
Indole	0.02	0.01	0.1	13	9.4	**8.5**	1.0	**26**

NL: Non-linear.
ND: Non detected.

occurred because the concentration of the compound was too high and no linear depletion was observed. In the first two samples, the highest concentrations were obtained for acetic acid followed by 1-octe-3-ol. In sample 3, acetic acid seems to be present in higher concentration than before, so high that depletion on the peak areas was not observed and therefore no quantitative results can be obtained. In this case, the presence of 6-methyl-5-hepten-one had gained importance among the rest of the compounds. When mushroom leaved the egg to continue growing outside, the concentration of the majority of the compounds goes up being the most predominant compounds 2-methyl propanoic, butanoic and 2-methyl butanoic acids, followed by 6-methyl-5-hepten-2-one, phenol, p-cresol and indole. In the case of sample 7, the highest concentration was for acetic acid, as in the first two samples, followed by butanoic and 2-methyl butanoic acids. In general, it can be seen that the composition of the compounds depends a lot on the stage of the mushroom and although similar stages were analyzed the result differed between them, therefore, the results were matrix-specific. Almost all of the compounds that appear in a higher concentration are related to a putrefactive aroma. In addition, analyzed mushroom appears to have a scent which mimics both carrion odours (e.g. dimethyl trisulphide) and faecal odours (e.g. phenol and p-cresol) [23].

Finally, a comparison between a freeze dried and a wet mushroom sample was carried out (See Table 6 Samples 6 and 6'). The analysis was repeated three times for each sample type. The results showed that for almost all the compounds, the concentration obtained in a freeze dried mushroom sample decreases on an average of 88% with respect to wet mushroom sample. One part of the compounds was lost in percentages between 88% and 99%, among them, the compounds which are moderately or totally soluble in water (1-butanol, limonene, 1-pentanol, dimethyl trisulfide, 1-octen-3-ol, 2-phenylethanol, phenol and p-cresol). The rest of the compounds were lost in percentages between 59% and 84% except for 6-methyl-5-hepten-2-one, for which concentration was maintained constant. These compounds are slightly soluble ((Z)-β-ocimene and 1-heptanol) or totally insoluble in water (6-methyl-5-hepten-2-one, diphenyl ether and indole). In this sense, the losses of volatile compounds in the freeze dried process could be related to their solubility in water.

Although mushrooms experienced huge losses of aromas during the freeze drying process, sample freeze drying give the advantage of mushroom storage. This species is available in the nature only for a few months along the year and therefore, a process for its conservation is needed. In addition, water elimination enables a wider applicability of the MHE approach and improves the repeatability. However, the response obtained for wet mushroom sample can be also considered since only acids can not been quantitated.

4. Conclusions

Three different methods based on multiple headspace extraction and multiple headspace-solid phase microextraction followed by gas chromatography–mass spectrometry analysis have been developed, optimized and validated for the determination of volatile compounds responsible for mushroom aroma. The optimization of the extraction conditions of the three methods was performed, showing the usefulness of the experimental design. The MHS and MHS–SPME methods developed have been validated in terms of linearity, repeatability and limits of detection and quantitation. In all the cases that the quantitation had been possible, the results obtained were comparable with all the evaluated techniques, indicating that the validation of the method by the different extraction methods comparison had been successfully applied. It is worth mention that the compounds that could not been quantitated with one of the techniques could quantitated with other of the evaluated techniques. Therefore, the techniques used can be supplementary. Moreover, as the employed extraction techniques are automated, by changing the extraction mode from HS to HS-SPME or vice versa it can quantitate any of the 20 studied volatile compounds.

The HS-SPME method offers better results in terms of sensitivity and precision followed by HS loop pressure and HS syringe autosampling systems. While in the HS determination do not require any extraction fiber which could reduce costs, in HS-SPME, by a proper selection of the SPME fiber certain selectivity and different sensitivity can be achieved. Although with HS-SPME shorter linear range concentration was observed, the best regression coefficients were achieved by means of HS-SPME followed by HS syringe and HS loop pressure systems.

With respect to the results obtained combining multiple successive extractions with different HS extraction methods, the most reproducible β parameters were obtained in the case of MHS–SPME for the highest number of compounds (20 analytes). In addition, HS-SPME affords the shortest time required to reach equilibrium. These results have been obtained when the sample has been previously freeze-dried. In the case of the MHS–SPME analysis on wet mushroom samples, the results were significantly worse. It appears that this latter combined technique can safely be recommended for the determination carried out in this work to increase sample throughput. To conclude, the presented data indicate a good agreement between MHE and MHS–SPME determinations of the aroma compounds and all the studied techniques have demonstrated its reliability through an acceptable repeatability for the analyzed samples of *C. archeri*.

MHS–SPME is an interesting approach that can be used to overcome matrix-effect errors in the quantitative determination of volatile compounds, particularly in solid samples (like mushrooms), to which the standard addition method is unsuited. The advantages of this method have already been described, however, it has certain limitations and drawbacks (shorter linear range of the other techniques studied and sometimes lack of linearity caused by matrix conditions). Since the volume of the extracting phase is reduced, a small amount of analyte is extracted by the fiber, so the amount of analyte processed must be small enough to produce significant depletion and, thus, obtain linear plots with a slope significantly different from zero. The amount of sample should therefore be a few micrograms, affecting repeatability as can be seen in Table 4. Although matrix-effects errors can be avoided by using the multiple-extraction approach, the matrix-effect is not totally eliminated as can be observed in the differences between wet and freeze-dried mushroom sample. In our case, the humidity content limits applicability of the MHE approach because it may cause deviations from linearity for some of the compounds.

By means of determining β parameters corresponding to each compound in selected extraction conditions, MHS–SPME has been found to be the most adequate technique to avoid matrix effects and to obtain acceptable quantitative results in mushrooms samples. In some cases, quantitative data were satisfactory but in others (e.g. highly concentrated volatiles) the application of MHS–SPME was unfeasible because the β parameters could not be calculated. This matter should be treated in more detail in further works focused mainly in mushrooms with higher concentrations of volatile compounds.

Acknowledgments

Authors thank the Basque Country Government and the University of Basque Country for financial support (SAIOTEK S-PE11UN134,

IT47-10, IT789-13 and UFI 11/23) and the SGIker technical support (UPV/EHU, MICINN, GV/EJ, ERDF and ESF). I. San Roman also thanks the University of Basque Country for her predoctoral grant. Authors also thank Dr. I. Salcedo and Santutxu's mycological society for her help with *Clathrus archeri* mushroom samples collection.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.01.021.

References

[1] B. Baccouri, S.B. Temime, E. Campeol, P.L. Cioni, D. Daoud, M. Zarrouk, Food. Chem. 102 (2007) 850–856.
[2] J. Beltrán, E. Serrano, F.J. López, A. Peruga, M. Valcárcel, S. Roselló, Anal. Bioanal. Chem. 385 (2006) 1255–1264.
[3] R. Costa, L. Tedone, S. De Grazia, P. Dugo, L. Mondello, Anal. Chim. Acta 770 (2013) 1–6.
[4] K.J. Gandhi, D.W. Gilmore, R.A. Haack, S.A. Katovich, S.J. Krauth, W.J. Mattson, J. C. Zasada, S.J. Seybold, J. Chem. Ecol. 35 (2009) 1384–1410.
[5] S. Rapior, S. Breheret, T. Talou, Y Pelissier., J.M. Bessiere, Mycologia 94 (3) (2002) 373–376.
[6] S. Rapior, F. Fons, J.M. Bessierre, Mycologia 24 (2) (2003) 159–166.
[7] W. Kabbaj, S. Breheret, J. Guimberteau, T. Talou, J.M. Olivier, M. Bensoussan, M. Sobal, Appl. Biochem. Biotech. 102–103 (2002) 463–469.
[8] D.B. Cho, H.Y. Seo, K.S. Kim, J. Food Sci. Nutr. 8 (4) (2003) 306–314.
[9] S. Zeppa, A.M. Gioacchini, C. Guidi, M. Guescini, R. Pierleoni, A. Zambonelli, V. Stocchi, Rapid Commun. Mass Spectrom. 18 (2) (2004) 199–205.
[10] S. Eri, N. Costa, Proc. Wartburg Symp. Flavour Chem. Biol. 21–23 (2004) 284–291.
[11] N.H. Snow, G.P. Bullock, J. Chromatogr. A 1217 (2010) 2726–2735.
[12] E. Serrano, J. Beltrán, F. Hernández, J. Chromatogr. A 1216 (2009) 127–133.
[13] M. Suzuki, S. Tsuge, T. Takeuchi, Anal. Chem. 42 (1970) 1705.
[14] C. McAuliffe, Chem. Tech. 1 (1971) 46–51.
[15] B. Kolb, Chromatographia 15 (9) (1982) 587–594.
[16] O. Ezquerro, G. Ortiz, B. Pons, M.T. Tena, J. Chromatogr. A 1035 (2004) 17–22.
[17] B. Kolb, M. Auer, P. Pospisil, Quantitative determination of residual solvents in printed packing foils following the process of multiple gas extraction, Angewandte Chromatographie—Applied Chromatography No. 35E, Perkin Elmer, Überlingen, 1981.
[18] B. Kolb, P. Pospisil, M. Auer, J. Chromatogr. A 204 (1981) 371–376.
[19] B. Kolb, L.S. Ettre (Eds.), Static Headspace—Gas Chromatography: Theory and Practice, Wiley-VCH, New York, 1997.
[20] C.L. Arthur, J. Pawliszyn, Anal. Chem. 62 (1990) 2145–2148.
[21] P. Guedes de Pinho, B. Ribeiro, F. Gonçalves, P. Baptista, P. Valentão, R. M. Seabra, P.B. Andrade, J. Agric. Food Chem. 56 (5) (2008) 1704–1712.
[22] J.C. Beaulieu, J.M. Lea, J. Agric. Food Chem. 54 (2006) 7789–7793.
[23] O. Ezquerro, B. Pons, M.T. Tena, J. Chromatogr. A 999 (2003) 155–164.
[24] E. Sarrionaindia, N. Abrego, R. Picón, I. Olabarria, I. Salcedo, Zizak 7 (2011) 7–18.
[25] S.D. Johnson, A. Jürgens, Afr. J. Bot. 76 (2010) 796–807.
[26] F. Maggi, F. Papa, G. Crstalli, G. Sagratini, S. Vitori, Food Chem. 123 (2010) 983–992.
[27] I. Arambarri, M. Lasa, R. Garcia, E. Millan, J. Chromatogr. A 1033 (2) (2004) 193–203.
[28] Operating Manual, Agilent 7694 Headspace Sampler, 2000, Publisher, Wilmington, USA.
[29] R. Splivallo, S. Bossi, M. Maffei, P. Bonfante, Phytochemistry 68 (2007) 2584–2598.
[30] C. Sanz, D. Ansorena, J. Bello, C. Cid, J. Agric. Food Chem. 49 (2001) 1364–1369.
[31] P. Diaz, E. Ibañez, G. Reglero, Food Sci. Technol. 42 (2009) 1253–1259.
[32] E. Stashenko, R. Martínez, Trends Anal. Chem. 23 (2004) 553–561.
[33] M. Flores, D. Hernandez, J Agric. Food Chem. 55 (2007) 8688–8695.
[34] A. Marco, J.L. Navarro, M. Flores, J. Agric. Food Chem. 55 (2007) 3058–3065.