Talanta 101 (2012) 177–186

Contents lists available at SciVerse ScienceDirect

Talanta

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

Development and validation of automatic HS-SPME with a gas chromatography-ion trap/mass spectrometry method for analysis of volatiles in wines

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ARTICLE INFO

Article history:
Received 12 April 2012
Received in revised form
21 August 2012
Accepted 22 August 2012
Available online 8 September 2012

Keywords:
Headspace solid-phase microextraction (HS-SPME)
Gas chromatography-Ion Trap/Mass Spectrometry (GC-IT/MS)
Wine
Volatile compounds
Central composite design

ABSTRACT

An automated headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-ion trap/mass spectrometry (GC-IT/MS) was developed in order to quantify a large number of volatile compounds in wines such as alcohols, ester, norisoprenoids and terpenes. The procedures were optimized for SPME fiber selection, pre-incubation temperature and time, extraction temperature and time, and salt addition. A central composite experimental design was used in the optimization of the extraction conditions. The volatile compounds showed optimal extraction using a DVB/CAR/PDMS fiber, incubation of 5 ml of wine with 2 g NaCl at 45 °C during 5 min, and subsequent extraction of 30 min at the same temperature. The method allowed the identification of 64 volatile compounds. Afterwards, the method was validated successfully for the most significant compounds and was applied to study the volatile composition of different white wines.

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1. Introduction

The aroma is one of the most important characteristics influencing wine quality and the consumer acceptance. The flavor of a wine is extremely complex, and is due to the presence of several classes of compounds, such as alcohols, terpenes, hydrocarbons, ketones, esters, acids, aldehydes, ethers, sulfur, nitrogen compounds and lactones. More than 1.000 aroma compounds have been identified, covering a wide range of polarities and volatilities [1]. Several factors influence the wine aroma: grape variety, grape ripeness, climate, soil, fermentation conditions, yeast and bacteria strains, production process, and aging.

Due to the complexity of wine matrix and relatively low concentrations of the aroma compounds, their analyses require some isolation/pre-concentration steps [2]. Among several extraction methods widely used for the extraction and determination of wine flavor compounds, the most frequent applied are those based on headspace (HS) analysis. Solid phase-microextraction (SPME) is a sample preparation technique based on sorption that constitutes a reliable tool for the analysis of organic volatile and semi-volatile compounds [3,4].

In the past, the SPME in wine analysis was focused on analysis of pesticide residues and other contaminants. Later, SPME was applied to the varietal characterization of wines and analysis of the wine bouquet using different fibers [5]. The wide range of fiber types allows a large diversity of compounds to be analyzed. Among the SPME fibers available, those containing liquid (PDMS) and solid (CAR/DVB) components are high sensitive [6].

There are several applications in wines headspace analysis for the SPME method: to evaluate a single compound, for pollutant analysis and for aromatic characterization [7]. The SPME procedure

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http://dx.doi.org/10.1016/j.talanta.2012.08.028

is simple to use, lasting less than 1 h to be completed and not requiring solvent extraction. The method is also less expensive and allows characterization of the HS in contact with the sample [3,4], representing the best the orthonasal perceptible aroma from wine. In spite of these advantages, the experimental conditions can vary according to a different number of variables. These variables are continuous and can be set in a range of variation, called the experimental domain. Statistical methods must be used to maximize a response modifying all variables at the same time. One of the useful designs for fitting quadratic response surface models is the central composite design (CCD). Using CCD, separate models are fitted for each response [8,9]. Correlated responses are picked up by the same principal component, while the independent ones are modeled by different principal components [9].

The objective of this study was to optimize an automated headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-ion trap/mass spectrometry (GC-IT/MS) in order to quantify a large number of volatile compounds in wines. Although HS-SPME has been widely used for the analyses of a range of compounds in wine [1,2,10–14], to our knowledge, this is the first time that a method for analysis of volatiles in wines comprises, simultaneously, the screen of fiber, optimization of HS-SPME extraction conditions, method validation and applicability. Moreover, the HS-SPME extraction conditions were optimized using the CCD. Twenty of the most relevant volatile compounds in white wines were tested for validation of the method comprising esters, terpenes, norisoprenoids, nerolidol and phenylethyl alcohol. Comparative studies of the volatile profile of white wines produced from different grape cultivars were performed.

2. Experimental

2.1. Chemicals and reagents

The volatile compounds studied were (CAS number in brackets): limonene (5989-54-8, Fluka), *cis*-linalool oxide (5989-33-3, Fluka), terpinolene (586-62-9, Aldrich), β-linalool (78-70-6, Sigma), β-terpineol (138-87-4, Sigma), α-terpineol (98-55-5, Sigma), nerol (106-25-2, Aldrich), geraniol (106-24-1, Sigma), α-ionone (6901-97-9, Aldrich), neryl acetate (141-12-8, Aldrich), β-ionone (6901-97-9, Aldrich), nerolidol (7212-44-4, Aldrich), ethyl butanoate (105-54-4, Merck), isoamyl acetate (123-92-21, Sigma), ethyl hexanoate (123-66,Sigma), hexyl acetate (142-92-7, Merck), diethyl succinate (123-25-1, Merck), ethyl octanoate (106-32-2, Merck), phenylethyl acetate (103-45-7, Merck) and phenylethyl alcohol (60-12-8, Sigma). A hydrocarbon mixture C_6–C_{20} was obtained from Fluka. NaCl and NaOH were purchased from Merck.

2.2. Samples description

2.2.1. Wines and vinification conditions

Eight Portuguese commercial white wines of 2009 vintage were used in the analysis: Loureiro, Alvarinho, Antão Vaz, Arinto, Fernão Pires, Sauvignon Blanc, Verdelho and Viosinho.

The same technological procedure was applied in the production of all wines. Grapes were picked at random and crushed, pressed and treated with sulfite solution (30 mg l^{-1}). After settling overnight at 5 °C, grape musts were racked. All experiments were carried out in duplicate. Fermentations were initiated by starter cultures of rehydrated *Saccharomyces cerevisiae*. Fermentations were carried out at 18 °C and considered complete when no variation of sugar content was observed (below 2 g l^{-1}) and before occurrence of malolactic fermentation. Wines were cold-stabilized and then the sulfite content was adjusted to 30 mg l^{-1} free.

2.2.2. Model synthetic solution

A model synthetic solution (11% ethanol (v v^{-1}) at pH 3.8) was used for the method validation. The solution was stored at 4 °C.

2.3. Method development

2.3.1. SPME fiber selection and procedure

The SPME fibers tested in this work were polydimethylsiloxane 100 μm (PDMS), polydimethylsiloxane/divinylbenzene 65 μm (PDMS/DVB), polyacrylate 85 μm (PA), divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm (DVB/CAR/PDMS), and carboxen/polydimethylsiloxane 75 μm (CAR/PDMS). All fibers were purchased from Supelco (Bellefonte, PA, USA). Prior to analyses, the fibers were conditioned at the manufacturer's recommended conditioning temperature. Fiber screening was carried out for the analysis of volatile compounds in Loureiro white wine.

For each fiber, in a vial of 20 ml, 0.5 g of NaCl were added to 5 ml of wine. After sampling, the HS-SPME procedures were performed using a Combi-PAL autosampler (Varian Pal Autosampler, Switzerland) and the Cycle Composer software (CTC Analytics System Software, Switzerland). The wine sample was continuous stirring at 250 rpm for 5 min at 45 °C. Therefore, the fiber was exposed to the headspace (HS) at 45 °C for 20 min, under continuous stirring (250 rpm). A desorption time into GC injector was 2 min at the appropriate temperature for each fiber in splitless mode.

2.3.2. Experimental design

The HS-SPME conditions were optimized using a central composite design (CCD, with $\alpha=2.000$), based on a 2^4 factorial design plus eight axial points plus five replicates in the center of the design. The variables chosen for HS-SPME optimization were the salt addition (NaCl, g), the extraction time (t_{ex}, min), the incubation time (t_{inc}, min) and the extraction temperature (T_{ex}, °C). Twenty nine experiments using Loureiro wine sample were generated by CCD and executed in randomized order. The factor levels and experimental domain are shown in Table 1.

2.4. Chromatographic conditions

GC-IT/MS analysis were performed on a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 4000 ion trap mass detector (USA) and a Saturn GC-IT/MS workstation software version 6.8. Chromatographic separation was achieved using a capillary column VF-5 ms (30 m × 0.25 mm × 0.25 μm) from Varian and a high purity helium C-60 (Gasin, Portugal) as carrier gas at a constant flow of 1.0 ml min^{-1}, in splitless injection mode. An initial oven temperature of 40 °C was held for 1 min, then increasing 5 °C min^{-1} to 250 °C (5 min) followed to

Table 1
Factor levels and experimental domain applied to optimize the HS-SPME experimental conditions.

Factor	Experimental domain				
	$-\alpha$[a]	−1	0	+1	$+\alpha$[a]
NaCl (g)	0	0.5	1.0	1.5	2
Extraction time (t_{ex} − min)	0.5	10.5	20.5	30.5	40.5
Incubation time (t_{inc} − min)	0	5	10	15	20
Extraction temperature(T_{ex} − °C)	35	40	45	50	55

[a] $\alpha=2.000$.

increase 5 °C min^{-1} to 300 °C (0 min). The ion trap detector was set as follow: the transfer line, manifold, and trap temperatures were 280 °C, 50 °C and 180 °C, respectively. All mass spectra were acquired in the electron impact (EI). The mass range was 35–600 m/z, with a scan rate of 6 scan s^{-1}. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto-tune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 m/z. The analysis was performed in full scan mode [4,14–16]. The selected ions used for qualitative analysis are presented in Table 2.

2.5. Method validation

2.5.1. Calibration and detection limits

Calibration curves were created for quantification of volatile compounds using the optimized HS-SPME sampling conditions with the injection into the GC-IT/MS. The linear ranges of the method were analyzed by performing calibration curves using different concentration levels of a model synthetic solution. All analyzes were performed in triplicate. The linearity of each compound was determined by evaluation of the regression curves (ratio of standard peak area against the standard concentration) and expressed by the squared determination coefficient (r^2).

The limits of detection (LOD) and quantification (LOQ) were determined from calibration curves data, following European Medicines Agency (EMEA) criteria [17]. The LOQ was defined as the lowest concentration of the calibration curve based on a signal-to-noise ratio of 10. To define the LOD, it was used a model synthetic solution containing small known concentrations of the standards aroma until reach the signal-to-noise ratio of 3 [18]. All the analyses were performed in triplicate.

2.5.2. Precision and accuracy

The intraday precision was evaluated after an analysis by GC-IT/MS, on the same day, of 3 different concentrations of the standard compounds in a model synthetic solution. The interday precision was determined by repeating the intraday precision study during 3 different days. All the analyses were performed in triplicate. Precision was calculated using the mean, standard deviation relative standard deviation (RSD, %) of those values.

Table 2
Compounds identified in wines: retention time (RT), retention indices (RI), identification methods (ID) and selected ions used as m/z identifiers.

No.	RT (min)	RI_{calc}[a]	RI_{lit}[b]	Compound	ID (fit/retrofit, %)[c]	Identifier Ions (m/z)
				Esters		
1	4.16	765	756	Ethyl isobutyrate	MS (79.0/83.0)	43/71/161
2	4.44	779	776	Isobutyl acetate	MS (78.2/87.9)	43/56/73/101
3	4.99	800	806	Ethyl butanoate	STD, MS	43/71/88/116
4	6.16	851	820	Methyl butyrate	MS (74.1/82.2)	57/102
5	6.23	855	854	Ethyl isovalerate	MS (82.1/91.8)	57/88
6	6.88	879	878	Isoamyl acetate	STD, MS	43/70
7	10.6	1008	1001	Ethyl hexanoate	STD, MS	43/88
8	10.8	1015	1015	Hexyl acetate	STD, MS	43/55/56
9	11.78	1047	1046	Ethyl-2-hexenoate	MS (74.1/82.3)	55/97/99
10	12.05	1056	1038	Ethyl 2-furoate	MS (79.2/85.3)	95/112/140
11	13.3	1097	1097	Propyl hexanoate	MS (86.2/89.5)	43/99/117
12	13.38	1100	1100	Ethyl heptanoate	MS (87.4/90.2)	88/113
13	15.4	1168	1437	Linalyl butyrate	MS (80.5/88.6)	93/121
14	15.8	1182	1182	Diethyl succinate	STD, MS	101/129
15	16.4	1201	1198	Ethyl octanoate	STD, MS	88/140
16	17.8	1227	1250	Isopentyl hexanoate	MS (72.2/85.3)	43/70/99
17	18.02	1231	1257	Phenylethyl acetate	STD, MS	43/104
18	18.25	1235	-	Diethyl malate	MS (79.1/82.8)	71/117
19	18.9	1247	-	Propyl octanoate	MS (79.9/81.2)	43/127/145
20	19.08	1250	1320	Ethyl nonanoate	MS (81.3/82.5)	88/101/141
21	19.8	1263	1310	Methyl decanoate	MS (78.8/81.9)	74/87
22	21.5	1295	-	Ethyl decanoate	MS (74.4/85.1)	88/157
23	21.54	1296	-	Ethyl *trans*-4-decenoate	MS (80.3/87.0)	69/88/110
24	23	1354	-	Isoamyl octanoate	MS (74.8/77.6)	70/127
25	24.1	1403	-	Propyl decanoate	MS (79.2/85.3)	43/61/173
26	26.57	1496	1494	Ethyl dodecanoate	MS (83.5/91.3)	88/101
27	30.97	1696	1694	Ethyl tetradecanoate	MS (82.3/90.1)	88/101
28	32.99	1795	1880	Ethyl pentadecanoate	MS (80.2/89.9)	88/101
29	34.5	1873	-	Ethyl 9-hexadecenoate	MS (82.0/85.1)	55/69/88
30	34.96	1897	1902	Ethyl hexadecanoate	MS (74.2/81.0)	88/157/284
				Terpenes		
31	8.79	947	939	α-Pinene	MS (86.6/89.3)	77/93/136
32	11.37	1033	1028	Limonene	STD, MS	67/93
33	11.53	1039	1092	-Pinene	MS (85.1/86.0)	93/131
34	11.88	1050	1045	β-*cis*-Ocimene	MS (84.1/88.2)	93/121
35	12.6	1074	1070	*cis*-Linalool oxide	STD, MS	43/59
36	13.07	1089	1088	Terpinolene	STD, MS	93/121/136
37	13.5	1104	1099	β-Linalool	STD, MS	73/91/121
38	13.6	1107	1106	Hotrienol	MS (82.3/88.1)	71/82/119
39	13.8	1114	1109	*cis*-Rose oxide	MS (81.9/86.6)	69/139/154
40	15	1151	1142	Nerol oxide	MS (81.5/82.2)	68/83
41	15.2	1155	1144	β-Terpineol	STD, MS	71/93/136
42	16.6	1205	1195	α-Terpineol	STD, MS	59/93/121
43	17.92	1229	1233	Nerol	STD, MS	69/93
44	18.11	1232	1276	Geraniol	STD, MS	69/123

Table 2 (*continued*)

No.	RT (min)	RI_{calc}[a]	RI_{lit}[b]	Compound	ID (fit/retrofit, %)[c]	Identifier Ions (m/z)
45	18.72	1243	-	Unidentified compound	-	69/121
46	18.74	1244	-	Unidentified compound	-	69/93
47	20.8	1282	-	Neryl acetate	STD, MS	69/93/121
				Sesquiterpen alcohols		
48	17.88	1228	-	Farnesol	MS (80.9/82.6)	69/81
49	25.8	1467	-	Nerolidol	STD, MS	69/93
50	28.8	1595	-	α-Bisabolol	MS (83.8/88.4)	43/69/119
				Norisoprenoids		
51	18.79	1245	-	Ionone	MS (83.7/87.8)	43/93/121/177
52	20.2	1271	-	α-Ionone	STD, MS	93/121/136/177
53	20.76	1281	1333	TDN[d]	MS (86.0/90.9)	142/157/172
54	21.4	1293	-	β-Damascenone	MS (81.7/83.1)	69/121
55	24	1399	-	β-Ionone	STD, MS	43/177
				Alcohols		
56	6.37	859	849	*cis*-3-Hexenol	MS (82.3/88.1)	55/67/82
57	6.71	873	870	1-Hexanol	MS (82.5/87.6)	56/69
58	11.7	1044	1050	Benzyl alcohol	MS (81.7/95.1)	79/108
59	14.1	1124	1113	Phenylethyl alcohol	STD, MS	91/122
				Acid		
60	10.2	995	984	Hexanoic acid	STD, MS	60/73
				Carbonyl compounds		
61	9.421	968	-	Benzaldehyde	MS (77.7/82.4)	77/105
62	9.74	979	-	Acetoin	MS (79.9/85.0)	43/45/48
63	13.2	1094	1093	2-Nonanone	MS (73.8/82.2)	43/58
64	19.87	1265	1321	*trans*-Methyloctalactone[e]	MS (85.0/90.3)	99
				Sulfur compound		
65	7.45	901	912	2-Furfurylthiol	MS (86.8/90.4)	53/81
				Phenol		
66	19.6	1260	1312	4-Vinylguaiacol	MS (81.5/82.2)	77/107/135/150

[a] RI_{calc}: retention indices calculated from C_8 to C_{20} *n*-linear alkanes with VF-5ms capillary column.
[b] RI_{lit}: retention indices reported in the literature for VF-5ms capillary column or equivalent.
[c] ID: identification methods. Compounds were identified by comparing their retention times with those of authentic compounds (STD) analyzed under the same conditions, and by comparison of the retention indices (as Retention Linear Indice) with those from literature data. The comparison of MS fragmentation pattern with those of pure compounds and mass spectra database performed using NIST 05 spectral database, considering fit and retrofit values >70%.
[d] TDN: 1,1,6-Trimethyl-1,2-dihydronaphthalene.
[e] *trans*-Methyloctalactone also named *trans*-whiskey lactone.

The accuracy of the method was determined through the calculation of the percent deviation between the calculated value and the nominal value [15,17,18].

2.6. Qualitative and quantitative analyses

The compounds identification was achieved by comparing the retention time and mass spectra obtained from sample by comparison with the standard compounds present in the model synthetic solution injected at the same conditions; by comparing retention times generated for each reference compound analyzed using a commercial hydrocarbon mixture (C_6–C_{20}) for determination of the retention indices (RI) in comparison with the retention indices described in the literature and by comparing the MS fragmentation present with the mass spectra present in the National Institute of Standards and Technology (NIST) MS 05 spectral database [4,14–16].

2.7. Statistical analyses

An analysis of variance (ANOVA) was applied to the experimental data; results were considered significant if the associated *p*-value was below 0.05. The significant differences were determined by Tukey tests. Those statistical analyses and the CCD were performed using Statistica, Version 7.0 (Statsoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. HS-SPME-GC–ITMS methodology

Volatiles present in 5 ml of white wine extracted by DVB/CAR/PDMS fiber at 45 °C for 20 min are presented in Table 2. Sixty-four (64) compounds were identified (Fig. 1), forty (40) were used in the selection of the fiber, and twenty (20) compounds were quantified.

3.1.1. Selection of fiber coating

The SPME fiber coating is one of the most important factors to evaluate the extraction efficiency [1,23], due to its strong chemical nature dependence of the extracted analyte, established by its polarity and volatility characteristics [1,20,21]. Five fibers (PDMS, PDMS/DVB, PA, CAR/PDMS, DVB/CAR/PDMS) were tested for their extraction efficiency of volatile compounds. These tests were performed using a Loureiro wine characterized by floral and fruity flavors associated to the presence of volatile compounds, namely terpenes, norisoprenoids and esters [22,23]. The performance of each fiber was determined based on the number of identified compounds and the response areas taking into account a set of 40 volatile compounds (23 esters, 11 terpenes, 2 alcohols, 2 norisoprenoids, 1 sesquiterpen alcohol and 1 acid). Results for the extraction efficacy of the different fibers are presented in Fig. 2A and B.

The DVB/CAR/PDMS and PDMS/DVB coatings had higher extraction of the volatile compounds relatively to the other fibers.

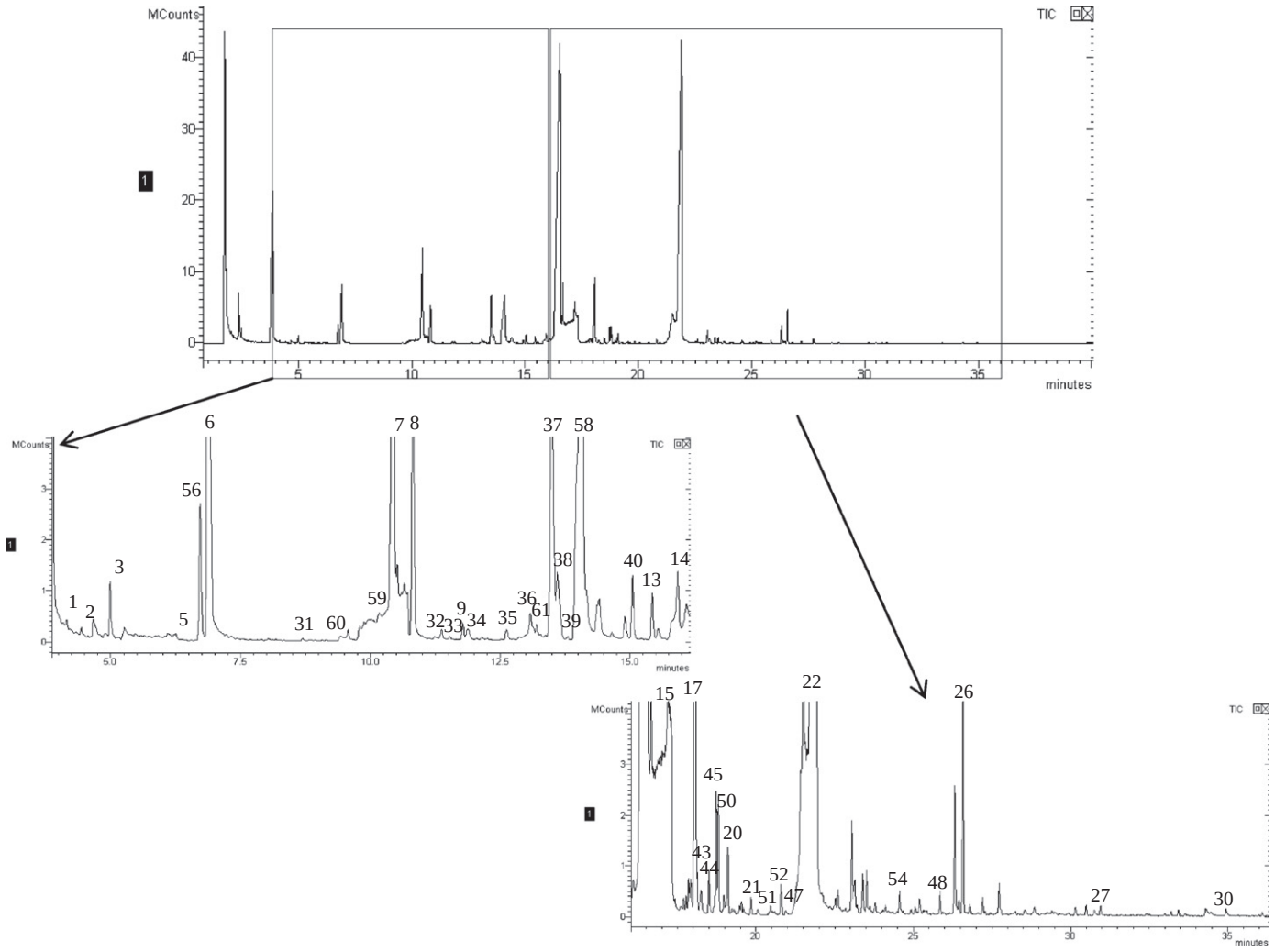

Fig. 1. Total ion current (TIC) chromatogram of the HS-SPME volatile compounds of Loureiro white wine. The main peaks were assigned as in Table 2.

Results obtained using the DVB/CAR/PDMS fiber were significantly different than the other four fibers and showed the highest sums of areas for esters, terpenes, sesquiterpen alcohol, norisoprenoids and alcohols, as well as detected more volatile compounds (39) among these functional groups. Furthermore, the DVB/CAR/PDMS repeatability was lower than 15% (data not shown). Despite the DVB/CAR/PDMS and PDMS/DVB being bi-polar fibers, covered with a porous solid coating, which suggests that the analyte extraction occurs via adsorption [6], the PDMS/DVB fiber showed worse qualitative and quantitative behavior than the DVB/CAR/PDMS, detecting 32 compounds. The PDMS/DVB fiber was also inefficient for the extraction of norisoprenoids.

The PDMS fiber, which represents a non-polar coating, identified 31 volatile compounds; however, the peak areas of these compounds presented the low values, showing that this fiber is not recommended for quantitative analysis. Indeed, the PDMS fiber repeatability, for ethyl heptanoate, methyl dodecanoate, ethyl tetradecanoate and ethyl pentadecanoate, geraniol and nerolidol, was higher than 15%. The combination of DVB and CAR increases both the porosity distribution and the polarity of the fiber, improving the retention of the analytes on the fiber as compared to a coating that only consists of PDMS [6].

No significant differences were obtained in peak areas/data from the polar PA fiber and the bi-polar CAR/PDMS fiber. Both fibers allowed the identification of 16 compounds; however, these presented the lowest extraction efficiency, and the worst repeatability (RSD > 15%) and selectivity values. Therefore, PDMS, PA and CAR/PDMS fibers showed lower efficiencies than the others fibers.

Under the same extraction conditions, the DVB/CAR/PDMS fiber was selected for HS-SPME optimization because it showed the highest extraction efficiency and repeatability of volatile compounds in Loureiro wine (RSD < 12%). Previous studies showed that the combination of the three stationary phases, DVB/CAR/PDMS, is the most appropriate due to its extraction ability over an expanded range of compounds (molecular mass 40–275) [1,24–27].

3.1.2. HS-SPME optimization

A CCD, using the DVB/CAR/PDMS fiber, was tested considering the extraction time and temperature, the incubation time and ionic strength (NaCl amount) as independent variables. Extraction time and temperature, as well as the incubation time may improve the HS-SPME extraction efficiency due to their strong influence on vapor pressure and on equilibrium of volatile compounds in the HS of the sample [6,7,21,28,29]. The extraction temperature is also important because the distribution coefficient between the sample and the HS and between the HS and the fiber is influenced by this parameter [21]. Moreover, the NaCl amount may also improve the extraction efficiency and sensitivity of

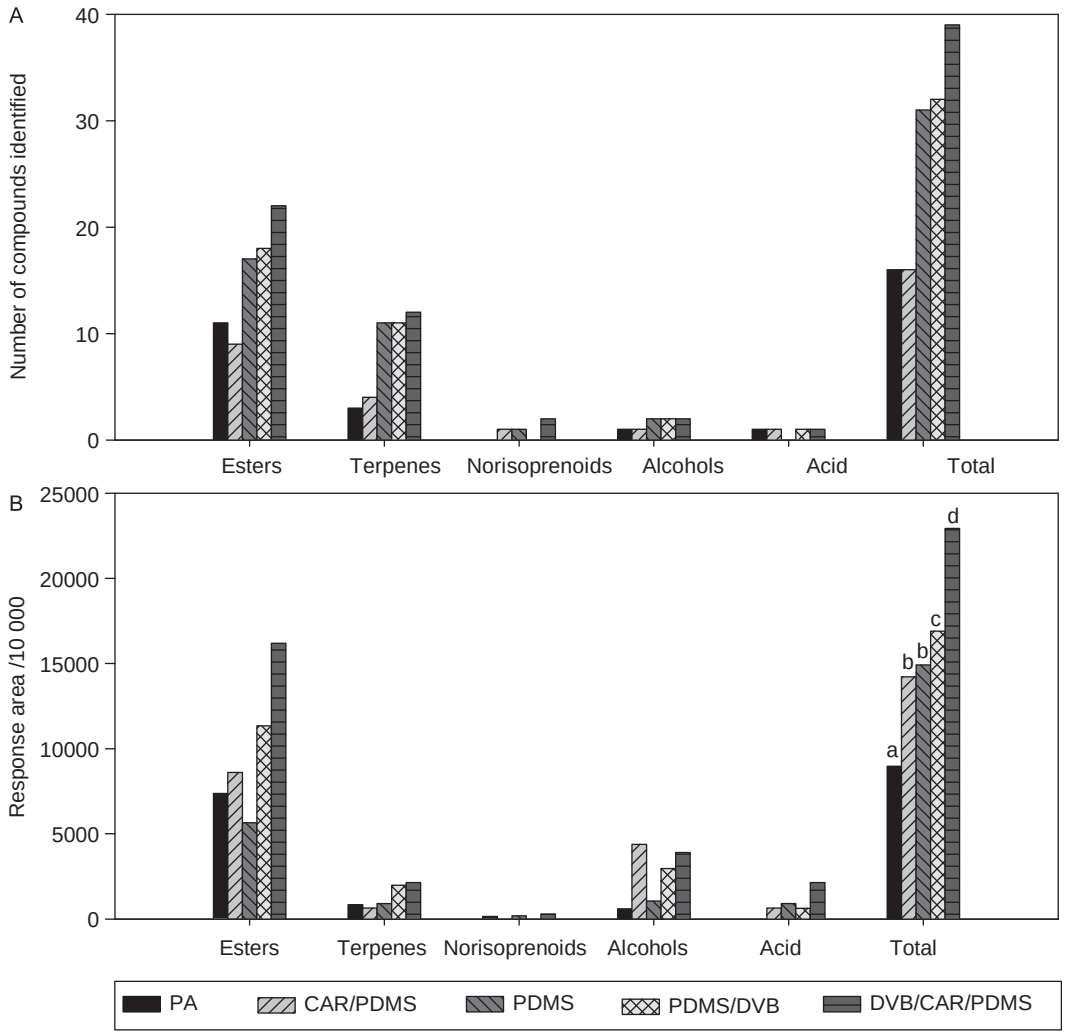

Fig. 2. (A) Number of volatile compounds and (B) response areas of different esters, terpenes, norisoprenoids, alcohols, and acid extracted with different fibers measured in the Loureiro wine. Values not sharing the same superscript letter (a, b, c, d) on top bar are different according to the Tukey test.

analytical methods. The salt modifies the analyte solubility which usually decreases with the ionic strength [19,30].

The CCD is used to evaluate the variables significancy and the interaction among them. In particular, close to an optimum, it is often necessary to fit a quadratic response surface model [31]. In this study, 29 runs of the experimental design using a Loureiro wine were randomly performed and the responses values (total area) are presented in Table 3. The response, based on the sum of the peak areas of all the 39 volatile compounds identified in the sample, is one of the most frequently parameters for the optimization of the SPME conditions [6,28]. The runs 17 and 19 of the experimental conditions of the CCD presented the lower response values, due to the lower concentration of volatile compounds on the HS. The salt effect (run 17) and the extraction time (run 19) were studied on their lower experimental domain values, showing the importance of both variables for HS-SPME optimization.

The experimental error was calculated using the replication values of the central point; the RSD was 5.6%, showing the good reproducibility of the method. An analysis of variance (ANOVA) was performed to determine the significance ($p < 0.05$) of the experimental factors on the performance of the HS-SPME optimization. The r^2 in the experimental model was 0.96. Fig. 3 shows the Pareto chart with the main effects and their interactions. The NaCl effect and the extraction time were the most significant parameters ($p < 0.05$), having a strong positive influence; the interaction between these variables were also significant. The negative values obtained for the interactions between the time extraction, the temperature extraction and the incubation time indicate an opposite effect on the analytical response. Results presented show that the salt effect during longer extraction times may increase the analytical method sensibility. This result is supported by the fact that the extraction time influences the equilibrium between the analyte concentration in the aqueous phase and that in the polymeric phase of the fiber [7]. Furthermore, the Pareto chart showed that the extraction temperature was not significant within the experimental domain studied. The extraction temperature can influence the partition coefficients of the compounds both between the sample and the headspace and between the headspace and the fiber, as well as the change in the vapor pressure of the compounds in the sample. According to Pellati et al. [32], the increase in sampling temperature increased the headspace concentration of the volatile compounds, favoring the extraction; however, SPME involves an exothermic process and the extraction of compounds decreases as the temperature increases. Similarly results were obtained for the pre-incubation time. Consequently, the extraction temperature was fixed at 45 °C and the incubation time for 5 min, conditions used for the selection of the fiber coating. According to Fig. 4, the effects of salt and the extraction time on the total area of volatile compounds can be visualized in the surface model. The analytical signal increases with the increase of the NaCl content and the

extraction time meaning that the best response is obtained for larger amounts of NaCl and longer time extractions.

At the optimum, the tangential plane of the response surface has a slope of zero in all the directions and a precise localization of the optimum conditions can be obtained by solving the equation system defined by setting the partial derivatives $\partial y/\partial x_i$, equal to zero [31]. As the pre-incubation time and the extraction temperature variables were not significant variables, they were not included in the equation. Results found that the optimum conditions are 2 g of NaCl and 30 min of extraction time.

Table 3
Experimental conditions and response values (total area) of the CCD used to optimize the extraction conditions of Loureiro wine.

Run	NaCl (g)	t_{ex} (min)[a]	t_{inc} (min)[b]	T (°C)[c]	Response value (total area)[d]
1	0.5	10.5	5.0	40.0	83941787
2	0.5	10.5	5.0	50.0	87612496
3	0.5	10.5	15.0	40.0	82987529
4	0.5	10.5	15.0	50.0	93386135
5	0.5	30.5	5.0	40.0	122106635
6	0.5	30.5	5.0	50.0	110667977
7	0.5	30.5	15.0	40.0	114183495
8	0.5	30.5	15.0	50.0	87278400
9	1.5	10.5	5.0	40.0	110001889
10	1.5	10.5	5.0	50.0	120281387
11	1.5	10.5	15.0	40.0	145669314
12	1.5	10.5	15.0	50.0	150697805
13	1.5	30.5	5.0	40.0	189828524
14	1.5	30.5	5.0	50.0	173768490
15	1.5	30.5	15.0	40.0	194280994
16	1.5	30.5	15.0	50.0	180868888
17	0.0	20.5	10.0	45.0	69882231
18	2.0	20.5	10.0	45.0	175528723
19	1.0	0.5	10.0	45.0	27824483
20	1.0	40.5	10.0	45.0	152522152
21	1.0	20.5	0.0	45.0	130522282
22	1.0	20.5	20.0	45.0	136414965
23	1.0	20.5	10.0	35.0	137711809
24	1.0	20.5	10.0	55.0	136196000
25	1.0	20.5	10.0	45.0	147320966
26	1.0	20.5	10.0	45.0	149957100
27	1.0	20.5	10.0	45.0	151001234
28	1.0	20.5	10.0	45.0	159786222
29	1.0	20.5	10.0	45.0	136680173

[a] t_{ex}—extraction time.
[b] t_{inc}—incubation time.
[c] T—extraction and incubation temperature.
[d] Total area is expressed in arbitrary units.

3.2. Method validation

A total of 64 compounds were extracted and identified in wines by DVB/CAR/PDMS fiber (Table 2). The proposed HS-SPME method was applied to determine the concentration of 20 volatile compounds in eight Portuguese white wines.

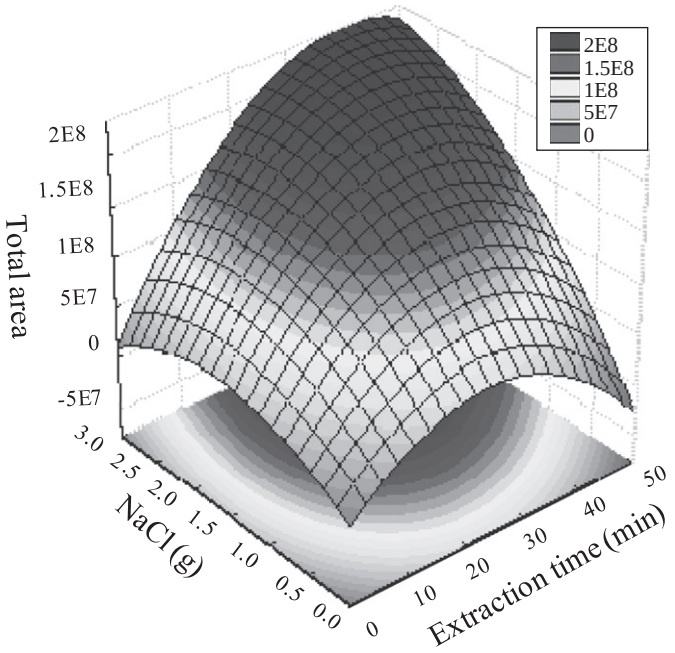

Fig. 4. Response surface model for total area of all volatile compounds vs extraction time and NaCl used for HS-SPME of Loureiro white wine.

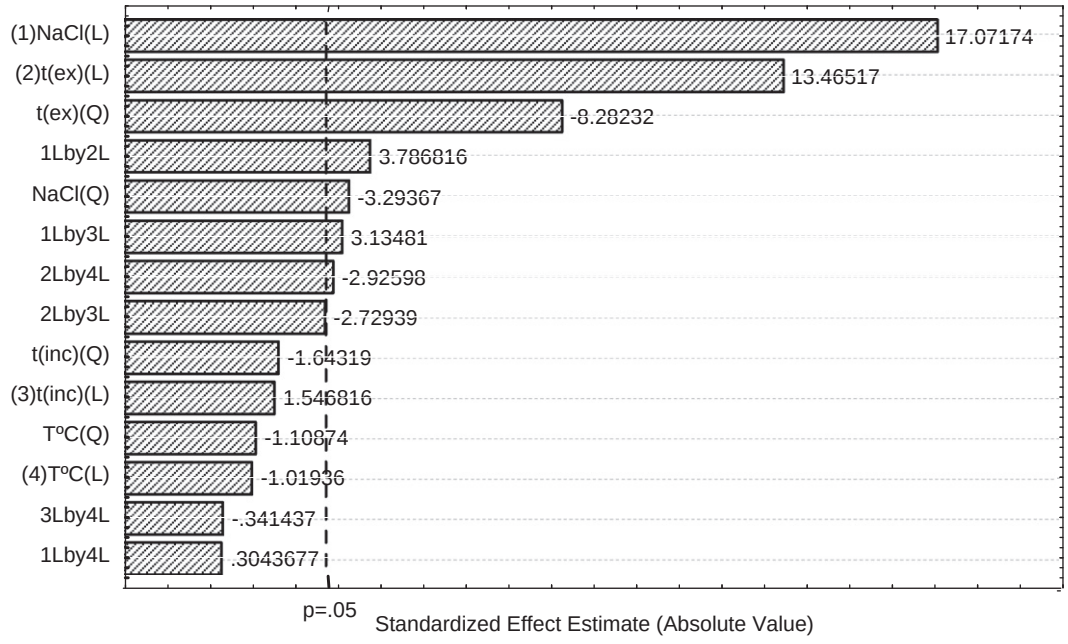

Fig. 3. Pareto chart for the total area of all volatile compounds of the GC-IT/MS analysis of the HS-SPME of Loureiro white wine.

Table 4
Linear range, determination coefficients, limits of quantification (LOQ) and limits of detection (LOD) of the proposed method ($n=3$).

Compounds	Linear range ($\mu g\,l^{-1}$)	Determination coefficient (r^2)	LOQ ($\mu g\,l^{-1}$)	LOD ($\mu g\,l^{-1}$)
Esters				
Ethyl butanoate	300–4320	0.9931	233	70.0
Ethyl hexanoate	100–4320	0.9925	24.0	7.20
Ethyl octanoate	50–4320	0.9949	19.3	5.80
Isoamyl acetate	100–4320	0.9895	190	57.0
Hexyl acetate	100–4320	0.9908	240	72.0
Phenylethyl acetate	300–4320	0.9909	163	49.0
Diethyl succinate	360–4320	0.9978	348	104
Alcohol				
Phenylethyl alcohol	4320–34530	0.9953	4300	1290
Terpenes				
Limonene	10–1000	0.9954	1.10	0.33
cis-Linalool oxide	5–1000	0.9945	3.50	0.97
Terpinolene	5–1000	0.9800	1.67	0.50
β-Linalool	8–1000	0.9848	6.86	2.06
β-Terpineol	15–1000	0.9991	13.6	4.06
α-Terpineol	10–1000	0.9970	8.33	2.50
Nerol	5–1000	0.9936	1.67	0.50
Geraniol	5–1000	0.9959	1.67	0.50
Neryl acetate	5–1000	0.9917	3.33	1.00
Sesquiterpen alcohol				
Nerolidol	1–1000	0.9898	0.33	0.10
Norisoprenoids				
α-Ionone	10–1000	0.9947	7.89	2.34
β-Ionone	5–1000	0.9865	1.27	0.35

3.2.1. Linearity, LOD and LOQ

The calibration curves for 7 esters, 1 alcohol, 9 terpenes, 1 sesquiterpen alcohol and 2 norisoprenoids, in a model synthetic solution were constructed using the optimized HS-SPME method. Linear curves were fitted onto the calibration points, and the concentration range and the correlation coefficient of the curves are given in Table 4 for each compound. Good linearity could be obtained for most compounds in large concentration ranges. LOQ obtained for the compounds analyzed varied in a large range: varying between 19.3 and 348 $\mu g\,l^{-1}$ for esters, 4300 $\mu g\,l^{-1}$ for phenylethyl alcohol, within 0.33 and 13.6 $\mu g\,l^{-1}$ for terpenes, 0.33 $\mu g\,l^{-1}$ for nerolidol, and 1.27 and 7.89 $\mu g\,l^{-1}$ for α- and β-ionone respectively.

3.2.2. Precision and accuracy

Precision and accuracy results are presented in Table 5. The precision of the method was evaluated studying intraday precision (repeatability) and interday precision (reproducibility) for all 20 volatile compounds at three different concentrations. The developed method is considered precise for the compounds studied, because the RSD values calculated for intra and interday precision did not exceed 15%. The accuracy values calculated for nearly all concentrations of the 20 volatile compounds were within 15% of the nominal value, which means that the method is considered accurate [18]. However, ethyl octanoate (1440 $\mu g\,l^{-1}$), nerol (10 $\mu g\,l^{-1}$) and α-ionone (10 $\mu g\,l^{-1}$) were, respectively, 17%, 17% and 18% of the nominal value.

3.3. Volatile profile of white wines

Significant differences were obtained in the concentration of volatile compounds in wines, according to the grape cultivars (Table 6).

Table 5
Precision and accuracy of esters, phenylethyl alcohol, terpenes, and norisoprenoids of the proposed method.

Compounds	Concentration ($\mu g\,l^{-1}$)	Intraday precision (%)	Interday precision (%)	Accuracy (%)
Esters				
Ethyl butanoate	360	6.11	8.56	97
	1440	5.54	2.80	88
	4320	3.91	3.46	98
Ethyl hexanoate	360	11.2	4.43	103
	1440	11.5	3.95	98
	4320	6.02	6.92	93
Ethyl octanoate	360	5.02	4.37	101
	1440	7.11	0.95	117
	4320	4.61	5.27	101
Isoamyl acetate	360	5.42	5.12	93
	1440	6.81	5.34	106
	4320	4.20	6.61	113
Hexyl acetate	360	7.32	3.59	94
	1440	3.59	2.67	86
	4320	3.53	7.11	93
Phenylethyl acetate	360	2.54	5.98	116
	1440	3.08	1.38	87
	4320	2.52	3.98	109
Diethyl succinate	360	3.55	5.01	109
	1440	9.59	5.79	104
	4320	6.87	9.52	115
Alcohol				
Phenylethyl alcohol	4320	4.31	3.72	105
	8640	2.03	9.39	111
	25980	3.25	8.60	115
Terpenes				
Limonene	10	4.31	11.3	95
	150	6.40	10.3	86
	500	1.54	12.6	104
cis-Linalool oxide	15	5.14	4.34	90
	75	7.66	5.36	98
	250	3.11	7.50	105
Terpinolene	10	9.87	8.89	102
	150	7.97	6.72	106
	500	8.73	2.24	103
β-Linalool	10	11.7	8.89	94
	150	5.51	6.72	96
	500	2.82	2.24	99
β-Terpineol	20	6.82	10.58	115
	150	7.69	6.18	108
	500	6.75	3.13	93
α-Terpineol	10	6.05	6.90	91
	150	13.6	6.17	91
	500	3.85	5.71	103
Nerol	10	10.1	8.49	117
	150	7.48	4.45	112
	500	2.06	13.1	88
Geraniol	10	8.42	12.1	97
	150	8.19	11.4	109
	500	6.47	11.9	91
Neryl acetate	10	5.94	9.57	107
	150	8.89	5.98	105
	500	10.2	6.95	99
Sesquiterpen alcohol				
Nerolidol	10	7.14	12.1	111
	150	5.26	11.5	108
	500	6.81	10.4	102
Norisoprenoids				
α-Ionone	10	4.74	13.8	118
	150	5.96	4.39	104
	500	11.5	7.13	95
β-Ionone	10	6.36	6.83	107
	150	9.88	12.0	104
	500	4.56	4.97	95

Alvarinho wine presented a highest concentration of ethyl butanoate and ethyl hexanoate. Arinto wine were characterized by high content of ethyl octanoate, whereas in other wines this

Table 6
Concentration of esters, phenylethyl alcohol, terpenes and norisoprenoids in Portuguese white wines.

Compounds	Loureiro	Alvarinho	Antão Vaz	Arinto	Fernão Pires	Sauvignon Blanc	Viosinho	Verdelho
Esters ($mg\,l^{-1}$)								
Ethyl butanoate	0.834 (0.063)^{b}	1.28 (0.05)^{a}	0.618 (0.034)^{c,d}	0.422 (0.021)^{f}	0.709 (0.023)^{c}	0.478 (0.076)^{e,f}	0.473 (0.017)^{e,f}	0.564 (0.01)^{d,e}
Ethyl hexanoate	0.227 (0.007)^{g}	2.33 (0.04)^{a}	1.79 (0.08)^{b}	1.06 (0.05)^{d}	1.40 (0.04)^{c}	0.875 (0.080)^{e}	0.449 (0.009)^{f}	1.11 (0.04)^{d}
Ethyl octanoate	nq	nd	nq	2.03 (0.15)^{a}	nd	nq	0.287 (0.015)^{b}	nd
Isoamyl acetate	3.61 (0.25)^{a}	2.61 (0.18)^{b}	1.31 (0.03)^{d}	1.15 (0.07)^{d}	2.06 (0.06)^{c}	1.12 (0.09)^{d}	0.207 (0.006)^{e}	1.21 (0.06)^{d}
Hexyl acetate	0.742 (0.067)^{a}	0.63 (0.03)^{b}	0.230 (0.015)^{c}	0.254 (0.006)^{c}	0.62 (0.04)^{b}	0.278 (0.013)^{c}	nq	0.24 (0.01)^{c}
Phenylethyl acetate	0.548 (0.015)^{a}	0.54 (0.01)^{a}	0.391 (0.010)^{c}	0.413(0.010)^{b,c}	0.43 (0.01)^{b}	0.382 (0.006)^{c}	0.349 (0.007)^{d}	0.396 (0.002)^{c}
Diethyl succinate	0.774 (0.020)^{e}	1.03 (0.03)^{d}	0.680 (0.017)^{f}	0.681 (0.025)^{f}	0.55 (0.01)^{g}	1.39 (0.04)^{b}	2.38 (0.06)^{a}	1.19 (0.01)^{c}
Alcohol ($mg\,l^{-1}$)								
Phenylethyl alcohol	52.1 (1.1)^{a}	33.9 (1.5)^{c}	15.2 (1.1)^{e}	26.0 (1.4)^{d}	17.1 (0.8)^{e}	15.2 (0.5)^{e}	40.0 (5.1)^{b}	25.5 (0.8)^{d}
Terpenes ($\mu g\,l^{-1}$)								
Limonene	10.9 (0.4)^{b}	16.1 (0.0)^{a}	nq	1.16 (0.09)^{c}	10.8 (0.4)^{b}	1.64 (0.07)^{c}	nq	nd
Terpinolene	40.3 (3.3)^{a}	37.43 (2.29)^{a}	nq	2.86 (0.14)^{c}	14.0 (0.4)^{b}	5.16 (0.05)^{c}	nq	nd
Geraniol	5.08 (0.19)^{a}	nd	5.05 (0.18)^{a}	5.03 (0.18)^{a}	5.10 (0.21)^{a}	nd	nd	nd
cis-Linalool oxide	21.2 (0.7)^{a}	9.62 (0.44)^{b}	nd	nd	7.80 (0.42)^{c}	nd	nd	nd
β-Linalool	57.2 (3.4)^{a}	11.2 (0.2)^{c}	26.4 (0.4)^{b}	nd	24.9 (0.2)^{b}	58.4 (2.3)^{a}	nd	nd
β-Terpineol	nd	nd	19.8 (0.6)^{b}	nd	26.3 (0.39)^{a}	nd	nd	nd
α-Terpineol	45.9 (1.3)^{a}	23.9 (0.6)^{b}	nd	nd	22.4 (0.7)^{b}	10.0 (0.7)^{c}	nd	nd
Neryl acetate	5.24 (0.49)	nd	nd	nd	nd	nd	nd	nd
Nerol	5.12 (0.07)	nd	nd	nd	nd	nd	nd	nd
Sesquiterpen alcohol ($\mu g\,l^{-1}$)								
Nerolidol	1.11 (0.10)^{a,b}	1.22 (0.07)^{a}	nd	1.01 (0.08)^{b}	1.04 (0.02)^{b}	0.551 (0.045)^{c}	1.20 (0.07)^{a}	1.05 (0.02)^{a,b}
Norisoprenoids ($\mu g\,l^{-1}$)								
α-Ionone	10.9 (0.6)^{a}	10.1 (0.2)^{a}	nd	nd	10.5 (0.4)^{a}	nd	nd	nd
β-Ionone	10.2 (0.3)^{b}	nq	17.7 (1.7)^{a}	nd	nd	nd	nd	nq

Values in parentheses are standard deviations from tree determinations; nq: not quantified; nd: not detected; values not sharing the same superscript letter (a–g) within the horizontal line are different according to the Tukey test.

compound was not detected or was found at lower amounts. The highest contents of isomyl acetate and phenylethyl acetate were found in Loureiro and Alvarinho wines. These wines also present significant amounts of hexyl acetate, as well as Antão Vaz wine. The ethyl esters are the most important group of the yeast-synthesized aroma substances of the fermentation bouquet. The acetates of higher alcohols contribute positively to wine aroma, presenting an intense fruity odor of banana, acid drops and apple. The ethyl esters of fatty acids also present pleasant odors of wax and honey [5,13]. The Viosinho wine showed the highest content in diethyl succinate.

The highest content in phenylethyl alcohol was obtained in Loureiro wine (52.1 $mg\,l^{-1}$), followed by Viosinho (40.0 $mg\,l^{-1}$). The lowest values were obtained in Antão Vaz and Sauvignon Blanc wines (15.2 $mg\,l^{-1}$). Phenylethyl alcohol, as well its acetate, presents a rose-like aroma [5,13].

Terpenes were obtained in significant amounts in Loureiro, followed by Alvarinho and Fernão Pires wines. Terpinolene, *cis*-linalool oxide, β-linalool and α-terpineol were the major terpenes found in Loureiro wine; whereas limonene and terpinolene in Alvarinho wine, and geraniol, β-linalool and β-terpineol in Fernão Pires wine. Similarly, Oliveira et al. [18] showed that Loureiro wine (318 $\mu g\,l^{-1}$) has a higher terpenic content than Alvarinho (54.9 $\mu g\,l^{-1}$) and Rocha et al. [12] found that a content in terpenes varying between 0.77 and 1.1 $mg\,l^{-1}$ in Fernão Pires wines. Sauvignon Blanc wine presented a high content in β-linalool similar to Loureiro. Antão Vaz presented at higher contents geraniol and β-terpineol. Viosinho and Verdelho wines presented the lowest contents in terpenes. Only nerolidol (sesquiterpen alcohol) was detected. Terpenes and norisoprenoids are responsible for the varietal aroma of wines.

α-Ionone was quantified in Loureiro, Alvarinho and Fernão Pires wines at similar amounts. β-Ionone was only present in Loureiro wine at 10.2 $\mu g\,l^{-1}$ and in Antão Vaz wine at 17.7 $\mu g\,l^{-1}$. The presence of these molecules contributes with violet notes to wine aroma [33].

4. Conclusion

The HS-SPME coupled with GC-IT/MS is a rapid, simple and solventless method. Chromatographic results after extraction with PDMS, PDMS/DVB, PA, DVB/CAR/PDMS and CAR/PDMS coatings fibers showed that DVB/CAR/PDMS was the most suitable for the SPME analysis of alcohols, esters, terpenes, sesquiterpen alcohols and norisoprenoids in white wines. The central composite design was used to optimize the extraction conditions, being NaCl and extraction time, the significant variables of the experimental design. The method allowed the extraction of 64 volatile compounds. Optimal extraction conditions were obtained using 5 ml of wine with 2 g of NaCl and 5 min of incubation time at 45 °C, followed by extraction with the fiber for 30 min at 45 °C. Afterwards, calibration and validation was performed for 20 volatile compounds considered as important aromatic contributors for the aroma of white wines and was demonstrated to be a linear, precise, accurate and sensitivity method.

Acknowledgments

The authors gratefully acknowledge CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the financial support to the Doctoral Sandwich Program, the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) as well as the Faculdade de Farmácia from Universidade do Porto (Portugal) and the Programa de Pós-graduação de Ciência de Alimentos, from Universidade Federal do Rio de Janeiro (Brazil).

This work was also supported by National Funds from FCT (Fundação para a Ciência e a Tecnologia) through project PEst-OE/EQB/LA0006/2011 and postgraduate fellowship ref. SFRH/BPD/63851/2009.

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