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Comparison of ultrasound-assisted extraction and direct immersion solid-phase microextraction methods for the analysis of monoterpenoids in wine

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Abstract

Ultrasound-assisted extraction (UAE) and direct immersion solid-phase microextraction (DI-SPME) were evaluated for the monoterpenic compounds determination in wine samples. The wine extracts obtained were analyzed by gas chromatography–mass spectrometry (GC–MS). The optimization of the variables affecting UAE and SPME methods was carried out in order to achieve the best extraction efficiency. Both UAE and SPME are quantitative (recoveries in the range 93–97% and 71.8–90.9%, respectively), precise (coefficients of variation below 5.5%), sensitive (limits of detection between 30–39 $\mu g\,L^{-1}$ and 11–25 $\mu g\,L^{-1}$, respectively) and linear over one order of magnitude. The application of both methods to red wine samples showed that UAE provided higher extraction of monoterpenic compounds than SPME. Although SPME remains an attractive alternative technique due to its speed, low sample volume requirements and solvent free character.

Keywords: Ultrasound-assisted extraction; Direct immersion solid-phase microextraction; Gas chromatography–mass spectrometry; Monoterpenoids; Wine

1. Introduction

The composition of wine depends on many factors, some of which are related to the specific production area: grape varieties, soil and climate, culture, yeasts, and wine making practices. Different type of wine compounds were used as variety markers, however, the most promising results were obtained from the volatile fraction [1–3]. Several hundred chemically different aroma compounds such as alcohols, esters, organic acids, aldehydes, ketones, terpenes and others, have been found in wines at different concentration levels. Therefore, certain compounds could be analyzed by direct injection gas chromatography while others need to be extracted and concentrated before chromatographic analysis. The sample pre-treatment for flavor and fragrance compound analysis usually involves the analyte concentration using headspace technique [4], steam distillation and supercritical fluid extraction [5], trapping over porous polymer [6], solid–liquid extraction over resins [7], purge-extraction techniques [8], simultaneous distillation–extraction [9] or batch and continuous solvent extraction [10]. The use of solvent-free systems such as dynamic headspace with or without cryofocusing has been proposed only in a few papers [11,12]. These methods have various drawbacks including excessive preparation time and the use of organic solvents. The primary disadvantage of static headspace technique is its poor sensitivity for low volatile compounds and traces. Instead, it may be increased by purge and trap techniques. Simultaneous distillation–extraction is not time-consuming, but presents the inconvenience of artifacts formation due to thermally induced changes. Likewise, distillation and liquid–liquid extraction are well-fitted practices for monitoring aroma compounds. In the case of monoterpenoid analysis in wine samples, the latter was the most used technique but it requires multistage time-consuming procedures for the quantitative extraction of monoterpenes from must or wines.

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Ultrasound-assisted extraction is used for the isolation of volatile compounds from natural products at room temperature with organic solvents. Ultrasonic radiation is a type of energy that aids the sample pre-treatment facilitating and accelerating operations such as the extraction of organic and inorganic compounds from solid and liquid samples. Ultrasonic-assisted extraction methods proved to be useful and rapid procedures for wine analysis in comparison to the traditional methods [13–17].

Solid-phase microextraction (SPME) was developed in 1989 by Pawliszyn in order to facilitate a rapid sample preparation. Solid-phase microextraction is a solventless extraction technique based on the exposure of an immobilized stationary phase into the matrix containing the analytes (which could be liquid, solid, or gas) followed by their thermal desorption in the injector of a gas chromatograph [18]. Compared to traditional techniques, especially solid–liquid and liquid–liquid extraction, SPME shows significant advantages: high sensitivity and reproducibility, low cost, solvent-free extraction, no previous sample preparation, and the possibility of automatization [19]. Due to these issues, SPME is considered a promising useful technique for the analysis of flavor compounds in solid and liquid samples. This technique has been successfully used for the analysis of volatile flavor compounds in several matrices [20–22] and wine [23–30].

Taking into account that UAE and SPME methods are successful extraction procedures for aroma compounds, the objective of this work was to evaluate the performance of both techniques for the analysis of certain monoterpenoids in wine.

2. Experimental

2.1. Wine samples

Ten samples of Galician (NW Spain) red wine were used in this study. All of them are monovarietal, 2000 harvest, *Ribeira Sacra* Certified Brand of Origin (CBO) wines. Wines were elaborated using more than 70% of *Mencía* grape variety and following the wine making practices established by the *Ribeira Sacra* CBO Council. Samples were collected in 750 mL glass bottles and stored in darkness at 3–4 °C before analysis.

The enological characteristics of *Ribeira Sacra* CBO wines are: minimum alcoholic content 11%, total acidity between 4.5 and 6.5 g L^{-1} of tartaric acid, maximum volatile acidity 0.65 g L^{-1}, maximum total sulphurous dioxide level 120 mg L^{-1}, minimum free sulphurous dioxide 15 mg L^{-1} and maximum residual sugar 3 g L^{-1} [31].

2.2. Apparatus

2.2.1. Ultrasound device

An ultrasonic bath Ultrasons-H 3000838 P-Selecta (J.P. Selecta, Barcelona, Spain) equipped with a 2 L vessel and temperature control was used.

2.2.2. Rotary evaporator

The organic extracts obtained in UAE method were concentrated using a rotary evaporator Labo-Rota C-311, Resona Technics, Buchs, Switzerland.

2.2.3. Gas Chromatographic system

An Agilent 6890 gas chromatograph coupled to 5973N quadrupole mass spectrometer (Agilent Technologies Deutschland Gmbh, Waldbronn, Germany) was employed. The capillary column used was a HP-Innowax (Agilent Technologies) (30 m × 0.25 mm i.d., film thickness 0.25 μm).

2.2.4. Data acquisition

The chromatographic data were processed on a HP-Chemstation version D.00.00.38 (Agilent Technologies).

2.3. SPME fibers

The SPME manual holders and fibers were obtained from Supelco (Bellefonte, PA, USA). In this work, all analyses were performed using a polydimethylsiloxane (PDMS) fiber with a 100 μm film thickness. This fiber was conditioned before being used by inserting it into the GC injector port for 1 h at 250 °C. Between injections, the fiber was desorpted during 10 min at 250 °C in split mode in order to prevent any contamination.

2.4. Reagents

Monoterpenoids (linalool, α-terpineol, citronellol, nerol, and geraniol) were supplied by Aldrich Flavor and Fragrances (Alcobendas, Madrid, Spain). Methyl hexanoate (internal standard), and sodium chloride (ionic strength buffer) were supplied by Panreac (Barcelona, Spain). The solvents employed were absolute ethanol (Panreac, Barcelona, Spain) and Milli-Q ultra-pure water (Millipore Co., Bedford, USA). All solvents and reagents used were analytical grade. For ultrasound-assisted extraction dichloromethane and ethanol were obtained from Panreac (Barcelona, Spain) and anhydrous sodium sulphate was obtained from Merck (Darmstadt, Germany).

Stock standard solutions (100 mg L^{-1}) were prepared for each monoterpene by solving the appropriate amount in 10% ethanol. Standard solutions were stored at 4 °C in darkness. Working solutions were prepared daily.

2.5. Ultrasound extraction

The ultrasound-assisted extraction procedure applied in this work is based on the method described by Cocito et al. [14] with some modifications. One hundred milliliters of a standard solution (or the wine sample) containing 5 mg L^{-1} of each monoterpene was placed into a 200 mL spherical flask and was extracted three times by means of ultrasounds for 10 min with 30, 10, and 10 mL of dichloromethane, respectively. The three extractions were performed at 20 °C. The

organic layers collected were mixed and dried with sodium sulphate anhydrous for 12 h to be sure that all traces of water are removed. The organic extract was transferred to a vacuum flask and concentrated to about 100 μL in a rotary evaporator at 40 °C. Once the volume was adjusted to 500 μL with dichloromethane, the extract was ready for gas chromatographic analyses under the conditions described in Section 2.7. All determinations were performed in triplicate.

2.6. Solid-phase microextraction procedure

SPME extractions were performed by direct immersion of a 100 μm PDMS fiber into 7 mL of the standard solution (or the wine sample) containing 1 mg L^{-1} of each monoterpene and 25% of NaCl in 15 mL PTFE coated septum-closed vials. Extraction time was 15 min using continuous magnetic stirring at 1100 rpm. After each extraction, the fiber was rinsed with distilled water to remove the excess of polar non-volatile compounds. It was dried with a lint free tissue by carefully dipping before inserting into the GC injector port. The chromatographic analysis was performed under the conditions described in Section 2.7. Desorption time and temperature were 5 min and 250 °C, respectively. All experiments were carried out in triplicate and the average values were calculated.

2.7. Chromatographic conditions

The gas chromatographic operation conditions were as follows. The injector and detector temperatures were 250 °C; the carrier gas employed was Helium at a 1 mL min^{-1} constant flow; the oven temperature program was 10 min at 40 °C, then 3.5 °C min^{-1} up to 210 °C and finally 1 min at 210 °C. The injection was made in splitless mode for 5 min (DI-SPME) and in split mode (UAE) split ratio 1:20 using a 0.75 mm i.d. liner in order to improve the GC resolution.

The mass spectrometer was operated in the electron impact mode with the following conditions. The source temperature of 230 °C, the quadrupole temperature selected was 150 °C, the mass range *m*/*z* between 35 and 500, the scan rate was 3.09 scans/s, and relative electron multiplier voltage (EM) applied was 400 V with a resulting voltage of 1553 V. Monoterpenoids were identified using the NIST98 version 2.0 mass spectra library. Each monoterpene was further confirmed by comparing its mass spectra, linear retention index (LRI) and, when possible, retention times with those obtained for standards. Linear retention indices were determined by injection of a solution containing homologous series of normal alkanes (C_{11}–C_{20}) in a temperature-programmed run, as described above. The obtained values were compared with those reported in literature [32–34].

3. Results and discussion

3.1. Optimization of ultrasound-assisted extraction

The optimization of the UAE procedure was performed using a wine sample spiked with 5 mg L^{-1} of each terpene as well as the internal standard. The variables optimized were sample volume, solvent type and solvent volume, extraction time and temperature. Different experiments were carried out in order to obtain the best conditions for the extraction process. The distinct experimental conditions assayed are summarized in Table 1. It was observed that the relative abundance of the terpenes studied increases as the sample volume increases up to 100 mL. Sample volumes higher than 100 mL showed lower extraction efficiency. This result agrees with those obtained by other authors [13], so 100 mL was considered as the optimum value for sample volume.

Once the sample volume was fixed (100 mL), the solvent volume was studied. Different solvent volumes were assayed for the three extraction steps. The achieved results showed a rise of the extraction efficiency for solvent volumes up to 30 mL; for higher volumes, an appreciable improvement of the extraction efficiency and chromatographic resolution was not observed. Therefore, the optimum solvent volumes were considered 30, 10, and 10 mL for first, second and third extraction steps, respectively. The solvent volume was always up to 30% of the sample volume. These results confirm those published by Cocito et al. [14] remarking that an extractant volume equal to 30% of the sample volume is enough to allow the analyte extraction avoiding the formation of stable emulsions.

In order to obtain concentrate extracts, the optimization of the extraction time was taken into account. Different extraction times were evaluated: 10, 15, and 20 min. The results demonstrated that there are not significant differences in the extraction efficiency for both 10 and 15 min. However, for longer extraction time (20 min), a reduction of the chromatographic resolution was produced since the major compounds overlapped the minor ones, such as terpenes. Ten minutes was considered the optimum time for the three extraction steps.

The extraction temperature is another important parameter in order to attain the better extraction efficiency. Due

Table 1
Variables optimized for ultrasound-assisted extraction procedure

Sample volume (mL)	Solvent volume (mL)	Extraction time (min)	Temperature (°C)	Solvent
50	10	10	20	Pentane (A)
100	20	15	30	Dichloromethane (B)
200	30	20		A:B (60:40)
	40			A:B (50:50)
				A:B (40:60)

Table 2
Influence of studied parameters on UAE efficiency

	Sample volume (mL)			Solvent volume (mL)			Extraction time (min)			Extraction temperature (°C)	
	50	100	200	10	30	40	10	15	20	20	30
Linalool	0.085	0.131	up	nd	0.142	0.131	0.154	0.134	up	0.154	0.142
α-Terpineol	0.093	0.113	up	nd	0.132	0.127	0.171	0.125	up	0.172	0.155
Citronellol	0.074	0.159	up	nd	0.175	0.168	0.125	0.162	up	0.181	0.167
Nerol	0.091	0.172	up	nd	0.184	0.154	0.151	0.157	up	0.163	0.149
Geraniol	0.083	0.163	up	nd	0.153	0.126	0.166	0.128	up	0.190	0.172

Results are expressed as normalized area (A/A_{IS}). nd: not detected; up: unresolved peak.

the low boiling point of dichloromethane, only 20 and 30 °C were checked out. No significant differences were observed in the extraction efficiency for the two temperatures assayed. Therefore, for further analysis, the extraction temperature selected was the lower: 20 °C.

The last feature optimized was the solvent. Different experiments were performed using pentane and dichloromethane, as well as diverse mixtures of these solvents in several proportions. When pure pentane was used, the chromatograms presented minor number of peaks and the extraction efficiency was worse in comparison with the use of pure dichloromethane. Using mixtures in different proportions of both solvents, the extraction efficiency increased when the proportion of dichloromethane rose. The best results were found when 100% dichloromethane was used. Thus, pure dichloromethane was selected for further extractions. The influence of the parameters mentioned above on the ultrasound-assisted extraction efficiency is summarized in Table 2.

3.2. Optimization of solid-phase microextraction

In order to optimize the adsorption and desorption processes in solid-phase microextraction, all features influencing the analyte equilibrium between the sample and the fiber were taken into account. Due to the demonstrated suitability of the PDMS 100 μm fiber for the terpene analysis [24], all experiments were performed using this kind of fiber.

Analytes with a favorable vapor pressure can be extracted by immersing the fiber into the wine, or by sampling the headspace above the sample. Analytes, which exhibit unfavorable vapor pressure, must be extracted by immersion [35].

Although headspace shows the advantage of avoiding contamination and increasing the fiber lifetime, a comparison between both direct fiber immersion and headspace techniques was carried out in order to establish their efficiency. Different extraction times were evaluated using both techniques and the same standard monoterpene solution. The results obtained illustrated that all the extracted compounds showed greater peak areas when direct immersion technique was employed (Table 3). This conclusion agrees with those obtained by Demyttenaere et al. [30], which pointed out higher extraction efficiency for direct immersion in comparison with headspace procedure. Thus, direct immersion technique was chosen as extraction mode for further determinations.

The analyte adsorption onto the PDMS fiber was optimized taking into account the factors influencing the solution equilibrium: agitation, extraction time, sample volume and ionic strength. Sample agitation enhances extraction and reduces extraction time, especially for higher molecular weight analytes with high diffusion coefficients. However, inconsistent stirring could cause poor precision and is worse than no stirring. Sonication promotes analyte adsorption, but can add heat to the sample. This might be beneficial for vaporizing the analytes for headspace extraction [35]. The influence of the agitation speed was also studied in three experiments with no agitation, 500, and 1100 rpm, respectively (Table 3). The terpene adsorption augmented with the agitation speed up to 1100 rpm. Therefore, this speed was retained as optimal for later analyses.

The effect of the extraction time on the yield of microextraction is also evaluated. Different times in the range comprised between 2 and 30 min were assayed by immersion of the fiber into 7 mL of the sample solution satured with 25% of NaCl. For all monoterpenes considered, the kinetic curves showed that equilibrium between sample and fiber was

Table 3
Influence of studied parameters on SPME efficiency

	Extraction mode		Stirring (rpm)			Extraction time (min)			NaCl content (%)			Desorption time (min)			Desorption temperature (°C)		
	DI	HS	0	500	1100	10	15	20	0	25	35	1	2.5	5	150	200	250
Linalool	0.226	0.192	0.202	0.206	0.215	0.187	0.201	0.214	0.215	2.188	2.153	2.326	2.470	2.188	0.649	1.419	2.188
α-Terpineol	0.096	0.053	0.089	0.083	0.089	0.071	0.078	0.087	0.089	1.002	1.551	0.899	0.904	1.002	0.678	0.840	1.002
Citronellol	0.309	0.171	0.230	0.287	0.350	0.272	0.292	0.301	0.350	2.241	2.211	2.472	2.655	2.241	1.021	1.631	2.241
Nerol	0.211	0.104	0.193	0.204	0.223	0.184	0.194	0.203	0.223	1.996	2.097	1.830	1.894	1.996	1.157	1.576	1.996
Geraniol	0.182	0.072	0.171	0.180	0.197	0.155	0.165	0.174	0.197	1.941	2.059	1.625	1.996	1.941	1.163	1.552	1.941

Results are expressed as normalized area (A/A_{IS}).

Table 4
Analytical parameters for the monoterpenoid determination by the two proposed methods

	Compound				
	Linalool	α-Terpineol	Citronellol	Nerol	Geraniol
SPME method					
Precision ($n = 5$) (R.S.D.) (%)	1.28	1.31	1.46	2.91	3.71
LOD ($\mu g\,L^{-1}$)	24	21	23	25	11
Recovery (%)	71.8	80.1	90.9	89.9	79.7
UAE method					
Precision ($n = 5$) (R.S.D.) (%)	1.87	3.41	3.56	4.69	5.43
LOD ($\mu g\,L^{-1}$)	36	37	33	39	30
Recovery (%)	94.5	94.7	97.5	95.0	93.6

essentially achieved within 15 min. This exposure time was enough to obtain a quantitative extraction with a good reproducibility.

In SPME methods, the efficiency of the analyte adsorption onto the fiber can be affected by the sample composition. Addition of 25–30% (w/v) of sodium chloride to the sample or adjusting the pH before extraction increases the ionic strength of the solution and, in turn, reduces the solubility of some analytes. Salt addition increases extraction efficiency significantly for polar and volatile compounds [35]. Thus, the influence of sodium chloride concentration in the solution was studied using different amounts of NaCl ranged between 0 and 35%. As it can be observed in Table 3, an enlargement of peak areas for higher NaCl concentration up to the saturation was reached. Twenty-five percent of NaCl was chosen as the optimum addition in order to improve the extraction.

Since thermal desorption has an important influence on precision and sensitivity, the related features such as desorption time and injection port temperature were also optimized. Several experiments using different desorption times between 1 and 5 min were carried out. As can be seen in Table 3, for three terpenes, the desorption from the fiber raised slightly with time while it decreased for the other two. The peak area decrease of linalool and citronellol for high desorption times is probably due to the partial decomposition of these compounds when the fiber exposition time (in the injector port at 250 °C) increases from 2.5 to 5 min. It was judged that the thermal desorption was completed for 5 min of desorption time using the splitless mode.

The study of the injector port temperature was carried out using a terpene standard solution under the same conditions. Three different temperatures (150, 200, and 250 °C) were investigated. The amount of terpenes desorpted from the fiber increased with the desorption temperature (Table 3). Two hundred and fifty degree centigrade was established as the optimal temperature. In order to verify the complete analyte desorption, a blank run was performed after each run. The results showed that the terpenes were totally desorpted from the fiber at 250 °C.

3.3. Performance evaluation of the UAE method

Due the low level expected for certain monoterpenoids in real wine samples and in order to be sure that the five compounds studied were detected, the precision study for UAE method was performed for five extractions using a wine sample spiked with 5 mg L^{-1} of each terpene and methyl hexanoate as internal standard (to minimize the deviations due to the injection). Once the organic layer was concentrated and redissolved, it was injected in the chromatographic device per triplicate. The results achieved for the precision of the extraction procedure are appropriate (Table 4), the relative standard deviation ranged between 1.87 and 5.43%. The recovery for the extraction method was studied by the spiked

Table 5
Linear regression ($y = a + bx$) for area vs. concentration

	Compound				
	Linalool	α-Terpineol	Citronellol	Nerol	Geraniol
SPME method					
Slope	30.99	14.18	31.68	28.42	27.77
Intercept	0.69	0.11	0.38	0.405	−0.08
Correlation coefficient	0.9992	0.9996	0.9995	0.9994	0.9999
Calibration range (mg L^{-1})	0–5	0–5	0–5	0–5	0–5
UAE method					
Slope	1.7542	1.8431	1.7394	1.8030	2.0649
Intercept	0.2013	0.1345	0.0957	0.4019	0.3334
Correlation coefficient	0.9981	0.9988	0.9985	0.9979	0.9987
Calibration range (mg L^{-1})	0–5	0–5	0–5	0–5	0–5

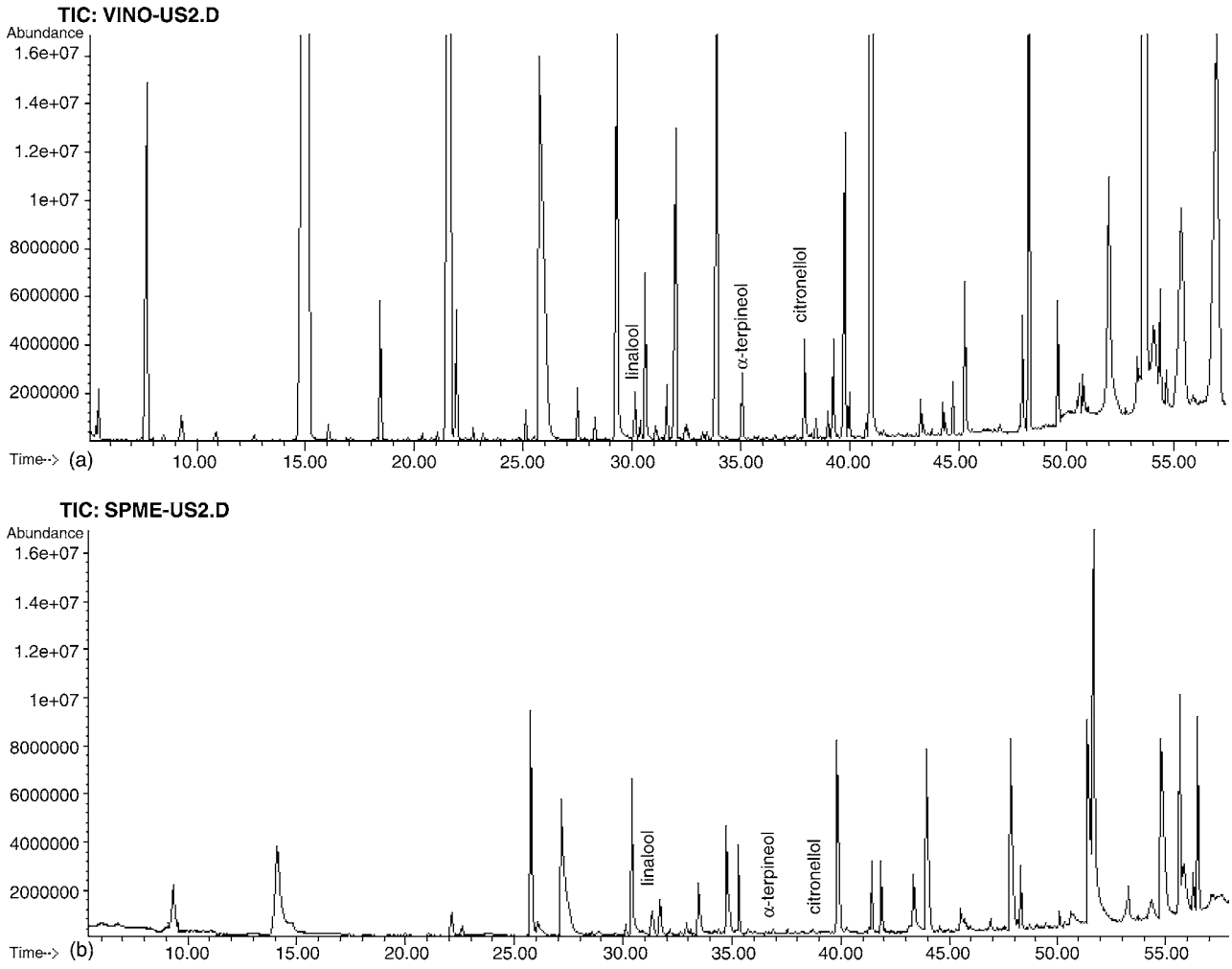

Fig. 1. Wine sample chromatogram (total ion current) obtained from (a) UAE-GC–MS method and (b) DI-SPME-GC–MS method.

wine sample described above. The recoveries obtained, which are summarized in Table 4, were satisfactory in the range comprised between 93 and 97%. The linearity of the method was evaluated by injecting different concentrations for all the monoterpenoids studied between 0 and 5 mg L^{-1} (Table 5). The calibration plots obtained presented correlation coefficients of 0.9979 or better in all cases. The detection limits, in μg L^{-1}, calculated by using a signal-to-noise ratio equal to 3, were: linalool, 36; α-terpineol, 37; citronellol, 33; nerol, 39 and geraniol, 30.

3.4. Performance evaluation of the SPME method

The precision of the experimental procedure was evaluated. Five different extractions, using the spiked wine sample described in Section 3.3, were carried out. The results achieved for the precision of the extraction procedure are appropriate, the relative standard deviation ranged between 1.28 and 3.71% as can be seen summarized in Table 4. The recovery of the proposed method was investigated using a wine sample spiked with fixed amount of 5 mg L^{-1} of each of the monoterpenes under analysis. The recoveries, showed in Table 4, were satisfactory, ranging from 71.8 to 90.9%. The linearity of the method was also evaluated in the range from 0 to 5 mg L^{-1} by injecting different concentrations for all the monoterpenes studied (Table 5). The PDMS fiber exhibited a directly proportional relationship between the extracted amount of monoterpenes and its initial concentration in the wine sample. The calibration lines obtained by plotting peak area versus monoterpenes concentration produced correlation coefficients (r^2) in the range of 0.9994–0.9999. The detection limits (signal-to-noise ratio: 3) calculated in μg L^{-1} were: linalool, 24; α-terpineol, 21; citronellol, 23; nerol, 25; and geraniol, 11.

3.5. Comparison between ultrasound-assisted extraction and SPME

In spite of the different performances showed for UAE and SPME, both extraction procedures were suitable to detect monoterpenic compounds which can be useful to develop chemometric systems in order to classify wine samples elaborated in different geographical zones with different CBO's.

Table 6
Determination of monoterpenoids in red wine samples from *Ribeira Sacra* CBO by means of ultrasound-assisted extraction method

Compound	Sample									
	1	2	3	4	5	6	7	8	9	10
Linalool	286	279	295	300	302	283	299	306	285	274
α-Terpineol	273	253	239	172	240	237	241	250	287	285
Citronellol	406	433	560	314	406	422	389	322	379	342
Nerol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Geraniol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd: not detected. Results are expressed in $\mu g\,L^{-1}$.

The extraction efficiencies for UAE and DI-SPME methods were compared. As can be seen in Table 4, both methods presented similar precision values (minor than 5.5%) and the sensitivity of both procedures was also comparable. The major difference between the two extraction methods studied was found in the recovery values: UAE method showed an average recovery value for the monoterpenoids evaluated of 95.1% while, in the same conditions, the DI-SPME method achieved 82.5%. In addition, as it can be seen in Fig. 1, the UAE method provides a richer qualitative–quantitative flavor profile than SPME. However, due to the fastness (15 min for SPME versus 30 min + 12 h for UAE), the low sample volume required (7 mL for SPME versus 100 mL for UAE) and because its solvent free character, SPME remains also as an attractive alternative technique for the analysis of monoterpenoids in wine samples.

In the present work, taking into account the best performance of the ultrasound-assisted extraction, this method was selected for measurement of monoterpenoids in the *Ribeira Sacra* wine samples. UAE demonstrated to be an appropriate extraction procedure for the chromatographic determination of the studied analytes in wine. The results of the monoterpene determination for 10 red wines with *Ribeira Sacra* guaranteed origin were presented in Table 6. For all the analyzed wines, citronellol was the predominant monoterpenol (mean value 397 $\mu g\,L^{-1}$), followed by linalool (291 $\mu g\,L^{-1}$) and α-terpineol (248 $\mu g\,L^{-1}$). Since the content of nerol and geraniol decreased during the wine storage in the bottle, none of the wines studied presented detectable contents of these two monoterpenoids.

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