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Optimization of solid-phase microextraction methods for GC-MS determination of terpenes in wine

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Abstract: Solid-phase microextraction using a 100 μm poly(dimethylsiloxane) fiber, followed by gas chromatography–mass spectrometry determination, has been optimized for the analysis of some terpenes in wine samples. The best results were obtained by direct immersion of the fiber using a sampling period of 15 min with constant magnetic stirring (1100 rpm) and an extraction temperature of 20 °C. The sample volume was 7 ml with 25% NaCl, in a 15 ml capped vial. Desorption was performed directly in the gas chromatograph injector port over 5 min at 250 °C using the splitless mode. The method is sensitive, with detection limits between 11 and 25 μg l^{-1}, precise, with variation coefficients in the range 1.28–3.71%, and linear over more than one order of magnitude. The related conditions were used for wine sample analyses with recoveries between 71.8 and 90.9%. Solid-phase microextraction remains an attractive alternative technique due to its rapidity and because it is a solvent-free extraction.

Keywords: solid-phase microextraction; gas chromatography–mass spectrometry; terpenes; wine

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.

Determination of aroma compounds is one of the most important steps in the evaluation of wine quality. Minor components of the aroma, such as the terpenic compounds, have a great importance because these compounds (especially the monoterpene alcohols) play a key role in the differentiation of the viniferous varieties.[1–3] Among these monoterpene alcohols, geraniol, nerol, α-terpineol and linalool are important to the aroma and flavor of wines.

Obtaining a 'terpenic profile' is extremely useful for differentiating the genuinely monovarietals wines from those made by a mixture of some other varieties. Therefore, the availability of the mentioned profiles is a valuable tool for fraud detection.

Some monoterpenic compounds are not modified considerably during the fermentation processes, so, their presence in wine is correlated with their presence in must and, consequently, if they are characteristic of the grape variety, they imply a guaranteed origin.[4,5]

The sample preparation for the analysis of flavor and fragrance compounds usually involves a concentration of the analytes using headspace technique,[6] steam distillation and supercritical fluid extraction,[7] trapping over a porous polymer,[8] solid–liquid extraction over resins,[9] purge–extraction techniques,[10] simultaneous distillation–extraction,[11] batch and continuous solvent extraction.[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 low sensitivity with respect to low volatile compounds and trace components. Sensitivity may be increased by purge-and-trap techniques. Simultaneous distillation–extraction is not time-consuming, but artifact formation due to thermally induced changes is possible. Likewise, distillation and liquid–liquid extraction are well fitted for monitoring the components responsible for aroma, but are time-consuming procedures. In recent years, analysis of monoterpenes in wine has mainly been performed by liquid–liquid extraction and requires multistage time-consuming procedures for extraction from must or wines. Ultrasound-assisted extraction was used for extraction methods with liquid solvents. This extraction procedure is fast in comparison with the traditional methods. The use of solvent-free systems such dynamic headspace, with or without cryofocusing, has been proposed only in a few papers.[13,14]

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Solid-phase microextraction (SPME) was developed in 1989 by Pawliszyn in order to facilitate a rapid sample preparation. SPME is a solventless extraction technique based on the exposure of an immobilized stationary phase in the matrix containing the analytes, which could be liquid, solid or gaseous, followed by thermal desorption of the analytes in the injector of a gas chromatograph.[15] Compared with traditional techniques, especially solid–liquid and liquid–liquid extraction, SPME shows significant advantages: high sensitivity and reproducibility, low cost, solvent-free extraction, no need for previous sample preparation, and the possibility of automation.[16]

Owing to its simplicity, speed, low cost, and lack of solvent requirements, SPME is potentially a very 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.[17–20]

The objective of this work was the systematic optimization of a solid-phase microextraction method for the analysis of terpenes in wine as well as the evaluation of the possibility of using this technique for the characterization of Ribeira Sacra CBO wines.[20–23]

EXPERIMENTAL

Wine samples

The wines used in this work are from the Certified Brand of Origin (CBO) Ribeira Sacra, a restricted wine-growing region of Galicia. To achieve a CBO, wines must be made with the varieties authorized by the CBO Council and grapes must be grown in vineyards located in a controlled geographical area. Wines are subject to rules related to the alcoholic content and the acidity; finally, wines are judged by their organoleptic properties by an expert tasting panel. Often, they are young red wines produced with a high percentage (more than 70%) of *Mencía* grape variety. *Brancellao* and *Caíño* are other traditional Galician grape varieties that may be used in minor proportions. All the analyzed wines are monovarietal Ribeira Sacra commercial wines, 2000 harvest. These wines comprised more than 70% *Mencia* grape variety. Samples were collected in 750 ml glass bottles and stored at 3–4 °C before analysis.

Apparatus

Gas chromatographic system

A Hewlett Packard 6890 series gas chromatograph equipped with a mass spectrometric detector (MSD) model 5973 was employed. The capillary column used was a HP-Innowax (Hewlett Packard; 30 m × 0.25 mm id, film thickness 0.25 μm).

Data acquisition

The chromatographic data were analyzed on an HP-Chemstation (Hewlett-Packard).

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 film thickness of 100 μm. This fiber was conditioned before use 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.

Reagents

The monoterpene compounds, linalool, α-terpineol, citronellol, nerol and geraniol, were supplied by Aldrich Flavor and Fragrances (Alcobendas, Madrid, Spain). Sodium chloride, used to control the ionic strength, was supplied by Panreac (Barcelona, Spain). Absolute ethanol (Panreac, Barcelona, Spain) and ultra-pure Milli-Q water (Millipore Co, Bedford, MA, USA) were used as solvents. All solvents and reagents used were analytical grade.

Stock standard solutions of 100 mg l^{-1} of each monoterpene were prepared by dissolution of monoterpenes in 0.2% ethanol and stored at 4 °C. Working standard solutions of each compound were prepared daily by mixing an aliquot of each individual solution and diluting with Milli-Q water.

Solid-phase microextraction procedure

All analyses were performed by direct immersion of a 100 μm PDMS fiber into 7 ml of the standard solution mixture containing 25% NaCl and placed in 15 ml PTFE coated septum-closed vials. The extraction time was 15 min using continuous magnetic stirring at 1100 rpm. After the extraction, the fiber was rinsed with Milli-Q water and dried prior to insertion into the GC injector port and the chromatographic analysis was carried out. Optimum desorption time and temperature were 5 min and 250 °C, respectively.

All experiments were performed in triplicate and the average values were calculated.

Chromatographic conditions

Operation conditions were as follows: injector and detector temperature, 250 °C; carrier gas, helium; constant flow rate, 1 ml min^{-1}; oven temperature program, 5 min at 40 °C then 5 °C min^{-1} up to 200 °C and finally 2 min at 200 °C. The injection was made in splitless mode for 5 min using a 0.75 mm id liner to improve the GC resolution.

The mass spectrometer was operated in the electron impact mode with a source temperature of 230 °C, quadrupole temperature, 150 °C, mass range m/z 25–500, scan rate 3.09 scans s^{-1}, and EM voltage, 400.

Compounds were identified based on NIST mass spectra library search and were further confirmed by comparing their mass spectra and retention times with those obtained for standards.

RESULTS AND DISCUSSION

Optimization of solid-phase microextraction

In order to optimize the adsorption and desorption processes, all factors influencing the equilibrium between the analytes in the sample and on the fiber were taken into account. All experiments were performed onto a PDMS 100 μm fiber owing to its demonstrated suitability for the terpene analysis.[24] The standard solution employed contained 1 $\mu g\,ml^{-1}$ of each terpene studied.

Selection of the extraction technique (headspace vs direct immersion)

Analytes that exhibit a vapor pressure can be extracted by immersing the fiber into the sample, or by sampling the headspace above the sample. Analytes which exhibit no vapor pressure must be extracted by immersion.

Although headspace has 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 solution of the monoterpenes. The results obtained for both techniques using the same solution of analytes and 30 min as extraction time are presented in Fig 1.

As can be seen, all extracted compounds showed greater peak areas in the direct immersion technique, so post analysis direct immersion was employed as the extraction technique.

Each fiber was used for about 50 extractions (average). All analyses were performed in triplicate and the average and the relative standard deviation were calculated. A standard mixture were extracted and injected daily in order to verify the extraction efficiency. Where the peak areas of the standards diminished drastically or no peaks were observed, the fiber was replaced.

Study of the adsorption conditions

In order to optimize the adsorption of the PDMS fiber, the factors influencing the solution equilibrium (agitation, extraction time, ionic strength) were considered. The influence of the alcoholic content was not systematically studied according to the results obtained by other authors.[25,26] A single experiment was performed using two standard solutions prepared in 0.2 and 12% ethanol (v/v) respectively. These solutions were extracted in the same conditions and after the desorption no differences were observed in the extraction efficiency.

Influence of agitation speed

Sample agitation enhances extraction and reduces extraction time, especially for higher molecular weight analytes with high diffusion coefficients. However, inconsistent stirring causes poor precision and is worse than no stirring. Sonication promotes analyte adsorption, but adds heat to the sample, which may improve by producing analyte vaporization for headspace extraction.

The influence of the agitation speed was also studied performing the extraction using no agitation, 500 and 1100 rpm. The amount of terpenes adsorpted increased using 1100 rpm. Therefore, this speed was maintained for posterior analyses. The results were presented in Fig 2 for all monoterpenes studied.

Influence of extraction time

The influence of the extraction time on the yield of microextraction is presented in Fig 3. This parameter was evaluated by immersing the fiber into the sample between 2 and 30 min. In all cases, 7 ml of the

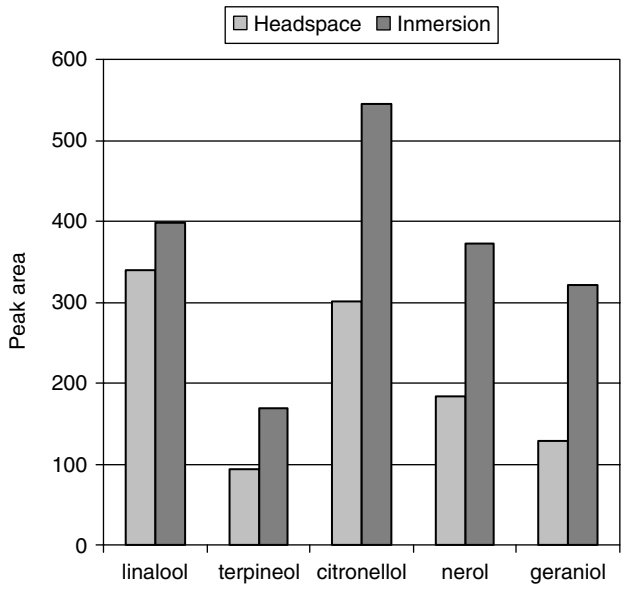

Figure 1. SPME adsorption peak areas for the different analytes according to the extraction technique.

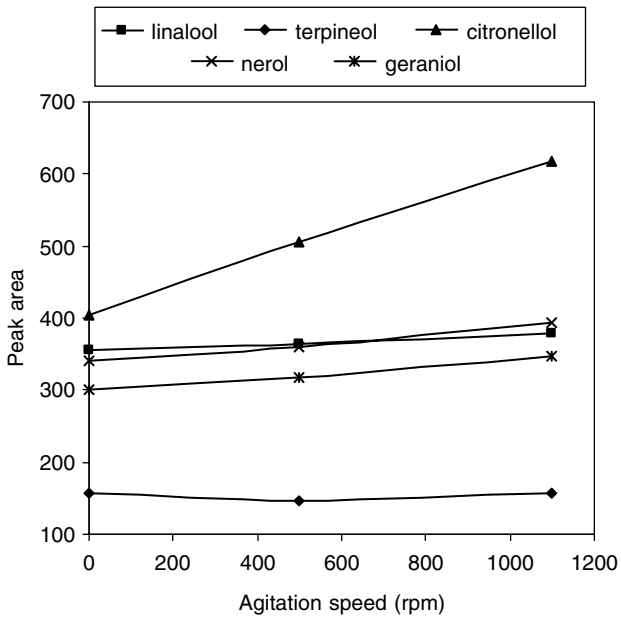

Figure 2. Peak areas vs speed of agitation.

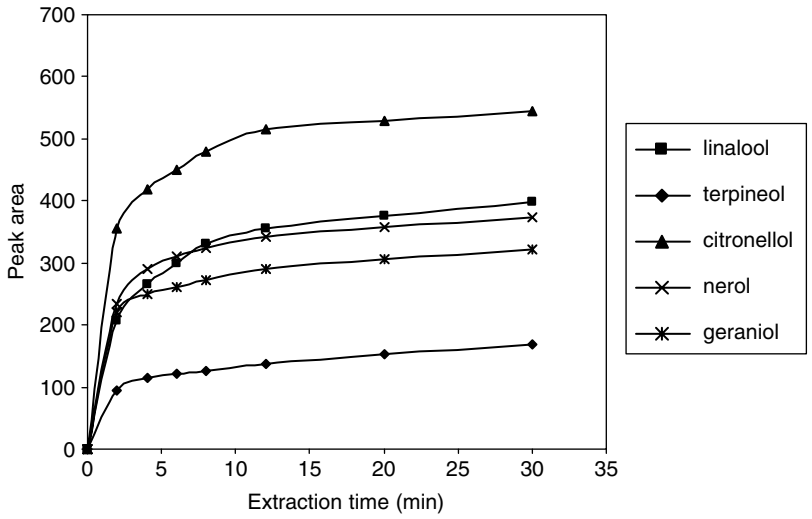

Figure 3. Peak areas vs extraction time.

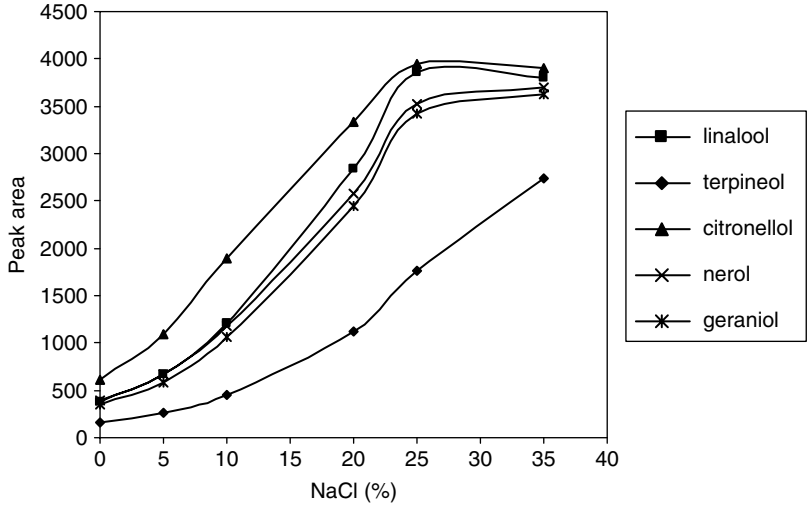

Figure 4. Peak areas vs salt concentration.

standard solution saturated with 25% NaCl were used. For all monoterpenes studied, the kinetic curves show that equilibrium between sample and fiber was essentially achieved within 15 min. This exposure time was enough to obtain a quantitative extraction with a good reproducibility.

Influence of ionic strength

In SPME methods, the amount of analyte adsorpted onto the fiber can be affected by the composition of the sample. Addition of 25–30% (wt/vol) of sodium chloride to the sample or adjusting the sample pH before extraction increases the ionic strength of the solution and, in turn, reduces the solubility of some analytes. The addition of salt to a sample greatly increases the extraction efficiency for many analytes, particularly polar compounds, and volatiles. Salt should be added for trace analyses. Salt is not needed to enhance extraction of analytes with high distribution constants, however, and may introduce interfering peaks. Changing the pH can minimize the solubility of some analytes.

The influence of the concentration of sodium chloride in the solution was studied using different amounts of NaCl ranged between 0 and 35%. The results obtained for this parameter are presented in Fig 4. It is observed that peak areas increase with increasing salt concentration until saturation of the sample is reached. For posterior analyses a 25% NaCl concentration was added to the sample in order to improve the extraction.

Optimization of desorption conditions

The optimization of thermal desorption has an important influence on precision and sensitivity. Thus parameters such as desorption time and injection port temperature must also be optimized for the analytes involved.

Some experiments using different desorption times were performed. The results are presented in Fig 5. For three terpenes, the desorption from the fiber was raised slightly with time while it decreased for the other two. For posterior analysis we considered that

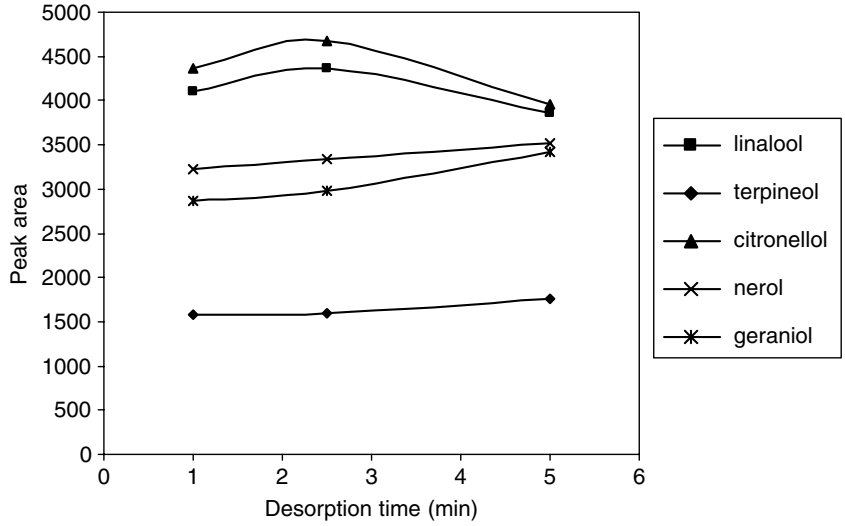

Figure 5. Peak areas vs desorption time.

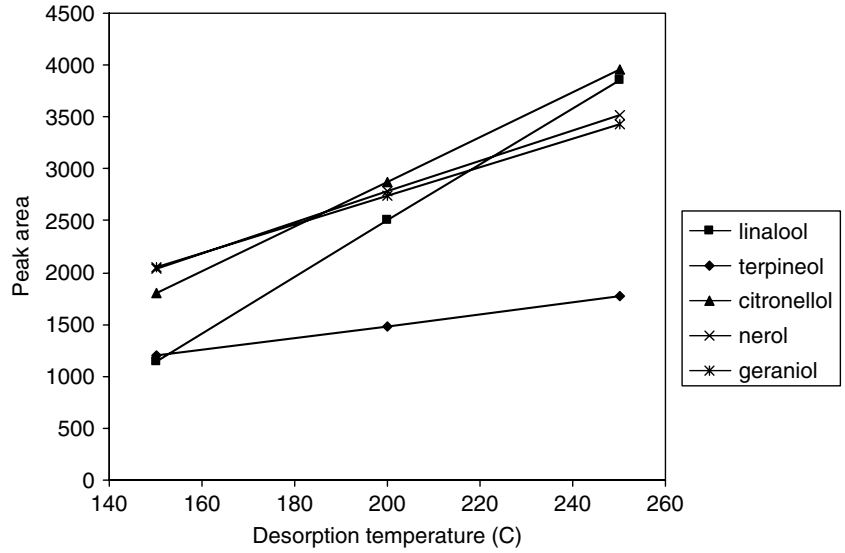

Figure 6. Peak areas vs desorption temperature.

thermal desorption of the analytes was completed using the splitless mode and 5 min of desorption time.

The study of the influence of the injector port temperature was performed using a terpene standard solution under the same conditions. Three different temperatures (150, 200 and 250 °C) were investigated. The results are presented in Fig 6. The amount of terpenes desorpted from the fiber increased with the desorption temperature, so 250 °C was set as optimum temperature. In order to evaluate the grade of desorption, a blank run was performed before each run. The results show that the terpenes were completely desorpted from the fiber at 250 °C

Performance evaluation of the SPME method

The linearity of the method was evaluated by injecting various concentrations for all the terpenes studied. The PDMS fiber exhibited a directly proportional relationship between the extracted amount of monoterpenes and its initial concentration in the sample, as can be seen in Table 1. The calibration lines obtained by plotting peak area vs the concentration of the test monoterpenes produced correlation coefficients (r^2) in the range of 0.9994–0.9999.

The precision of the experimental procedure was also evaluated. A series of six consecutive SPME gave a relative standard deviation (RSD) ranging from 1.20 and 3.71%, as can be seen in the results summarized in Table 2. The detection limits (signal-to-noise ratio of 3) calculated in $\mu g\,l^{-1}$ were linalool, 24; α-terpineol, 21; citronellol, 23; nerol, 25; and geraniol, 11. Recovery of the proposed method was studied using a wine sample spiked with different quantities of the monoterpenes under analysis. The recoveries, showed in Table 2, were satisfactory, ranging from 71.8 to 90.9%.

Table 1. Linearity data

Compound	Linalool	α-Terpineol	Citronellol	Nerol	Geraniol
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[a] ($\mu g\,ml^{-1}$)	0–1	0–1	0–1	0–1	0–1
Linear range ($\mu g\,ml^{-1}$)	Up to 5	Up to 5	Up to 5	Up to 5	Up to 5

[a] Each calibration level (six) is the mean of three determinations.

Table 2. Analytical figures of merit

Compound	Linalool	α-Terpineol	Citronellol	Nerol	Geraniol
Precision ($n = 6$) (RSD) (%)	1.28	1.31	1.46	2.91	3.71
LOD ($\mu g\,l^{-1}$)	24	21	23	25	11
Recovery (%)	71.8	80.08	90.9	89.9	79.7

Analysis of real samples

The applicability of SPME technique was evaluated using 10 red wines with CBO Ribeira Sacra. 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, following the procedure described in the Chromatographic conditions section. The obtained values were compared with those reported in the literature.[27–29] Compounds were identified based on NIST mass spectra library search. All of them were further confirmed by their mass spectra (match quality better than 90%), by their linear retention indices (LRI) or by injection of authentic standards. A total of 51 signals were identified and five of these compounds were terpenes: linalool, α-terpineol, citronellyl formate, citronellol and β-damascenone.

The compounds detected in wine samples by using SPME are listed in Table 3, and Fig 7 shows a representative total ion chromatograms for one of the analyzed samples. The results of the terpene determination for 10 wines with *Ribeira Sacra* CBO are presented in Table 4. For all the analyzed wines, linalool was the predominant monoterpenol (mean value $99\,\mu g\,l^{-1}$) followed by α-terpineol and citronellol with the same mean value of $61\,\mu g\,l^{-1}$. Only five wines presented detectable contents of nerol and none of them presented geraniol contents up to the limit of detection obtained for this method. These contents can be explained because linalool and α-terpineol are the predominant monoterpene alcohols in wine and their content increased with time during the wine storage in the bottle, while the geraniol and nerol contents decreased.

CONCLUSIONS

A method for the determination of monoterpenes in wine samples has been optimized using direct immersion SPME combined with gas chromatography–mass spectrometry. Different parameters that influence the extraction have been optimized. Direct immersion technique, 15 min extraction time, magnetic stirring and saturation with sodium chloride were selected.

Under the optimized extraction conditions, terpenes can be extracted with quantitative recoveries (71.8–90.9%) using a small volume of sample (7 ml) in only 15 min. The method has high repeatability with coefficients of variation between 1.28 and 3.71%. This extraction procedure followed by gas chromatography–mass spectrometry determination was successfully applied to several samples of Ribeira

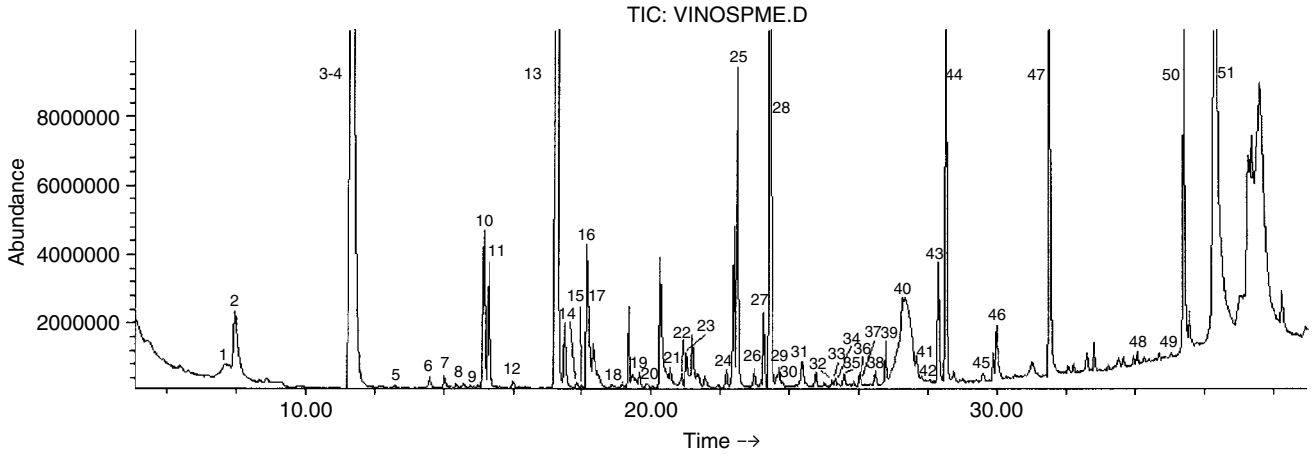

Figure 7. Typical chromatogram (total ion current) obtained from direct immersion SPME-GC-MS. Identified analytes are listed in Table 3.

Table 3. Compounds detected in wine samples using SPME

Peak	t_R(min)	LRI_{calc}	Compound	Id
1	7.47	1109	Acetaldehyde ethyl amyl acetal	MS
2	7.99	1125	Isoamyl acetate	MS, LRI
3	11.34	1231	Ethyl hexanoate	MS, LRI
4	11.36	1232	1-Pentanol	MS, LRI
5	12.60	1271	Hexyl acetate	MS, LRI
6	13.61	1303	Acetoine	MS, LRI
7	14.04	1318	2-Propanone 1-hydroxy	MS, LRI
8	14.35	1329	Ethyl heptanoate	MS, LRI
9	14.78	1343	Ethyl 2-hexenoate	MS
10	15.16	1356	Ethyl (*S*)-(−)-lactate	MS, LRI
11	15.31	1362	1-Hexanol	MS, LRI
12	16.01	1386	Methyl octanoate	MS, LRI
13	17.28	1432	Ethyl octanoate	MS, LRI
14	17.83	1453	Isoamyl hexanoate	MS
15	17.99	1459	1-Heptanol	MS, LRI
16	18.15	1465	Acetic acid	MS, LRI
17	18.33	1471	2-Furaldehyde	MS, LRI
18	18.87	1492	Octyl formate	MS
19	19.66	1523	Benzaldehyde	MS, LRI
20	19.87	1531	Ethyl nonanoate	MS
21	20.32	1549	Linalool	MS, t_R, LRI
22	20.55	1559	1-Octanol	MS, LRI
23	21.00	1577	5-Methyl furfural	MS, LRI
24	22.32	1623	γ-Butirolactone	MS, LRI
25	22.39	1625	Ethyl decanoate	MS, LRI
26	22.98	1643	1-Nonanol	MS, LRI
27	23.18	1649	3-Furanmethanol + 2-furanmethanol	MS, LRI
28	23.45	1658	Succinic acid diethyl ester	MS, LRI
29	23.65	1664	Ethyl 9-decenoate	MS, LRI
30	23.81	1669	α-Terpineol	MS, t_R, LRI
31	24.39	1687	α-Methyl crotonolactone	MS
32	25.23	1712	Crotonolactone	MS
33	25.31	1714	1-Decanol	MS, LRI
34	25.41	1717	Citronellyl formate	MS
35	25.42	1717	Citronellol	MS, t_R, LRI
36	25.83	1729	Phenethyl acetate	MS, LRI
37	26.46	1746	Propanoic acid 2-methyl-2-phenethyl ester	MS
38	26.48	1747	β-Damascenone	MS, LRI
39	27.06	1763	Ethyl laurate	MS
40	27.32	1771	HMF	MS
41	27.48	1775	o-Guaiacol	MS
42	27.54	1777	*N*-(3-methylbutyl) acetamide	MS
43	27.82	1785	Benzenemethanol	MS
44	28.52	1809	Benzeneethanol	MS
45	29.58	1868	Maltol	MS
46	29.98	1889	Orcinol	MS
47	31.51	1912	Octanoic acid	MS
48	34.06	1962	5-Acetoxy methyl 2-furaldehyde	MS
49	35.34	1982	4-H pyran-4-one, 2,3 dihydro, 3,5 dihydroxy-6 methyl	MS
50	35.40	1983	Decanoic acid	MS
51	36.34	1998	Glycerin	MS

LRI_{calc}, linear retention index calculated; Id identification: t_R, identification by comparison with retention time with authentic reference compounds recorder under the same conditions; MS, identification by comparison with mass spectrum stored in NIST library; LRI, identification by comparison with literature data.[27–29]

Sacra red wines produced in Galicia (northwest Spain).

SPME stands as a suitable extraction alternative for monoterpenes in GC-MS determination since organic solvents are not necessary. Other important advantages are the low analysis time and small sample volume. The results obtained indicated that the proposed method could be a useful alternative for the quantitative analysis of monoterpenes in wine. The evaluation of terpenic profile could be an important tool in order to develop characterization systems to control the Ribeira Sacra CBO wines.

Table 4. Monoterpene content in 10 Ribeira Sacra wines (the results are expressed $\mu g\,l^{-1}$ and each value is the mean of three determinations)

Wine	Linalool	α-Terpineol	Citronellol	Nerol	Geraniol
1	97	63	67	<LOD	<LOD
2	95	67	62	<LOD	<LOD
3	102	87	60	27	<LOD
4	102	48	42	25	<LOD
5	103	63	60	<LOD	<LOD
6	96	65	60	27	<LOD
7	102	60	60	29	<LOD
8	104	50	62	25	<LOD
9	97	59	72	<LOD	<LOD
10	93	53	70	<LOD	<LOD

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