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Determination of terpenoids in wines by solid phase extraction and gas chromatography

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Abstract

A new method for determination of volatile terpenoids in wine is proposed. An off-line solid phase extraction—gas chromatographic method has been used for the determination. The influence of several extraction variables was studied, including the solid phase employed (C-18 versus divinylbenzene-based), eluting solvent (*n*-pentane, dichloromethane, ethanol and methanol), volume of eluting solvent (1–4 ml) and drying time (0–20 min). Complete recovery of volatile terpenoids from several kinds of wines was obtained under the optimized conditions.

Keywords: Terpenoids; Wine; Solid phase extraction; Gas chromatography

1. Introduction

The grape variety greatly influences the final aroma of the resulting wine, especially for young wines. There are several volatile compounds present in grapes or are derived from compounds found in them which are related to wine aroma. In particular, it is well established that terpenoids are responsible for the Muscat aroma and are related to the aroma of other aromatic grape varieties such as Gewürtztraminer [1].

Several kinds of terpenoid derivatives have been identified in grapes, most of them monoterpenic derivatives, including alcohols and aldehydes [2]. Many wines show terpenoids above the threshold levels; therefore, in many cases they are an active component of the wine aroma [3].

Gas chromatography (GC) is the separation technique used for the determination of compounds related to wine aroma; however, a prior suitable extraction/concentration step is usually needed because of the low levels of the aromatic compounds in wines. Techniques like purge-and-trap, which is very attractive for analysis of volatile compounds in beverages, have a low sensitivity as their main drawback. Instead of classical methods [4], several extraction techniques have been used recently, e.g. microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and solid phase extraction (SPE). Monoterpenic alcohols present in must have been determined by MAE-GC [5], and fusel oils by SFE-GC [6]. However, these two techniques are not usually applied for aroma analyses.

SPE is a good choice for the extraction/concentration of aromatic compounds since several solid phases are available which allows for increasing selectivity in the extraction process. Moreover, great enrichment of the aromatic compounds in the extract can be achieved by using a small amount of organic solvent during the elution step from the solid phase. SPE has previously been used for the extraction of aromatic compounds from different samples [7,8]. Selection of the most adequate solid phase is the most time-consuming part of the method development. For volatile compounds in wine, SPE has been used for determining pesticides by SPE-GC-mass spectrometry (MS) [9] using a C-18 solid phase. Recently, minor and trace volatile compounds in wine have also been determined by SPE-GC-MS using a styrene–divinylbenzene solid phase [10]. An extensive study of the ability of different solid phases to retain aroma compounds from wines has been made [11], but styrene–divinylbenzene phases were not evaluated.

The main goal of this work was the development of a SPE-GC-flame ionization detection (FID) method for the quantitation of terpenoids in wines. Different solid phases

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had to be studied and different extraction variables optimized to guarantee complete recovery of terpenoids from wines.

2. Material and methods

2.1. Reagents and standards

Dichloromethane (HPLC grade) and methanol (Lichrosolv grade) were purchased from Merck (Darmstadt, Germany), ethanol (ACS grade) and *n*-pentane (ACS grade) were purchased from Panreac (Barcelona, Spain). Water (HPLC grade) was obtained from a Milli-Q system (Millipore Bedford, MA).

Linalool, γ-terpinene, terpineol and tartaric acid were from Sigma–Aldrich Chemie GmbH (Steinheim, Germany), geraniol, nerol, (+)-limonene, D-citronellol, and α-terpinene were from Extrasynthèse (Genay, France). During the method development, terpenoid standards were dissolved in a synthetic wine: 12% (v/v) ethanol in water, tartaric acid ($4\,g\,l^{-1}$) and pH 3.2 fixed with sulfuric acid. Octan-2-ol from Merck was used as internal standard.

2.2. Solid phase cartridges

Eight solid phase cartridges were evaluated for the extraction of terpenoids from wines. Table 1 shows their main characteristics.

2.3. SPE procedure

The SPE stage was performed in a Visiprep SPE vacuum manifold 12-port model from Supelco (Supelco Park, Bellefonte, PA), in which there are 12 positions available for conducting the SPE simultaneously.

During the method development, prior to use, cartridges were conditioned by rinsing with 4 ml of dichloromethane, 4 ml of methanol and finally 4 ml of an ethanol–water mixture (12%, v/v) [10]. Then, 50 ml of sample (wine or synthetic wine) was rinsed through the cartridge by vacuum suction (different vacuum levels were checked). Clean-up was obtained by flushing the cartridge with water (different volumes of water were studied). The cartridge was then dried under vacuum. Finally, terpenoids were eluted from the solid phase using an organic solvent (four solvents were evaluated: dichloromethane, *n*-pentane, methanol and ethanol).

Table 1
Main characteristics of solid phase cartridges

	Solid phase					
	C-18			Styrene–divinylbenzene		
	Bond Elut C-18	Discovery DSC-18	Strata C-18E	Chromabond EASY	Lichrolut EN	Strata SDB-L
Supplier	Varian	Supelco	Phenomenex	Macherey-Nagel	Merck	Phenomenex
Amount of solid phase (mg)	500	500	500	500	200, 500	200, 500
Volume (ml)	3	3	3	6	3, 6	3, 6

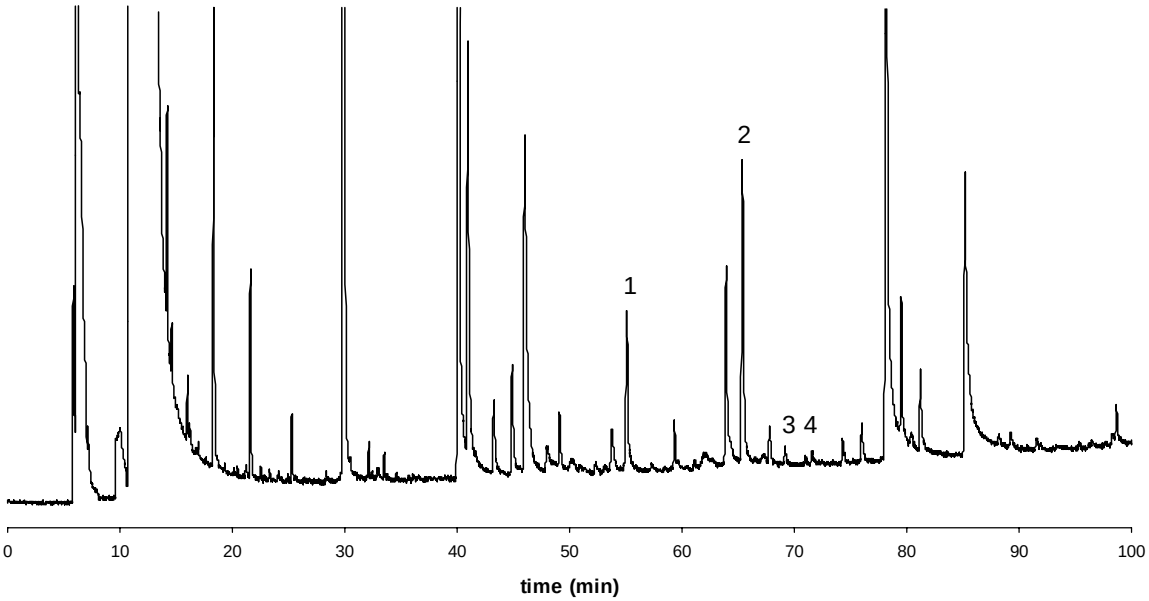

Fig. 1. Typical chromatogram of a Muscat wine: (1) linalool, (2) terpineol, (3) D-citronellol, (4) nerol.

2.4. GC analysis

Separation and quantification were achieved on a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph equipped with a 60 m × 0.32 mm ID fused silica capillary column coated with DB-WAX (J&W Scientific, Folsom, CA), and FID. Identification was achieved by mass spectrometric analysis. In these analyses, a GC 8000 coupled to a MD 800 mass detector (Fisons Instruments, Milan, Italy) was used. The mass detector operated in EI+ mode at 70 eV in the range 40–400 a.m.u.

For both instruments, separation conditions were as follows: injector temperature 200 °C, GC column temperature: 40 °C (5 min), at 2 °C min^{-1} to a final temperature of 230 °C (20 min); carrier gas He at 40 kPa. Fig. 1 shows a typical chromatogram obtained by treatment of a Muscat wine.

3. Results and discussion

Eight terpenoids were used as target compounds during the method development: α-terpinene, (+)-limonene, γ-terpinene, linalool, terpineol, D-citronellol, nerol and geraniol.

3.1. Solid phase and eluting solvent selection

Two kinds of solid phases in six different commercial solid phase extraction cartridges were studied, three of them with a C-18 solid phase and the other three with a styrene–divinylbenzene solid phase.

Cartridges were conditioned as described in Section 2. Two fractions of 2 ml of each eluting solvent were used to recover the standards from the solid phase.

Table 2 shows the average recovery for the various eluting solvents and solid phases used in the study. Percentages of compounds recovered in the final 2 ml last 2 ml are also shown.

n-Pentane was the worst eluting solvent. It produced recoveries which were always <57%. Even considering the last 2 ml, *n*-pentane did not produce good recoveries. It was due not to the solid phase but to the solvent, since other solvents produced much higher recoveries. Ethanol and methanol showed similar results with recoveries >60% in most cases.

Table 3
Recoveries (%) obtained for the terpenoids using Strata SDB-L and Lichrolut EN cartridges and dichloromethane as eluting solvent

Compound	Strata SDB-L	Lichrolut EN
α-Terpinene	92.8	98.2
(+)-Limonene	89.2	92.4
γ-Terpinene	88.7	92.0
Linalool	101.8	101.6
Terpineol	102.8	102.4
D-Citronellol	103.8	104.2
Nerol	106.6	103.1
Geraniol	91.7	99.23

All analyses were run in duplicate.

Dichloromethane produced the highest recoveries, especially when styrene–divinylbenzene-based phases were used. Recoveries > 72% were obtained for all the solid phases used. The best results were obtained for both Lichrolut EN and Strata SDB-L, using these cartridges complete recoveries of the assayed compounds were obtained. Therefore, dichloromethane was selected as the best eluting solvent for terpenoids elution from the cartridge.

Among the C-18 based solid phases, only Strata C-18E recovered >95% of target compounds using methanol as extracting solvent, although good results (recoveries >80%) were also obtained using either dichloromethane or ethanol with Strata C-18E, Bond Elut C-18 and Discovery DSC-18.

Based on the individual recoveries obtained for Strata SDB-L and Lichrolut EN using dichloromethane as extracting solvent (Table 3), the latter was chosen as the most adequate solid phase cartridge, even though there were no big differences from Strata SDB-L.

3.2. Optimization of extraction variables

Five extraction variables which could greatly influence the extraction process were studied: volume of washing water, drying time, volume of extracting solvent, vacuum level in the extraction system and the amount of the solid phase in the extraction cartridge. All analyses were run in duplicate.

A step using water for clean-up is very interesting since it can eliminate most polar compounds such as a sugar, phenols and several kinds of glucosides. However, it is necessary to determine if washing the cartridge with water can produce losses of terpenoids retained in the solid phase. Three

Table 2
Average ($n = 2$) recoveries (%) for the total terpenoids using different solid phase cartridges and solvents

	C-18-based			Styrene–divinylbenzene-based		
	Bond Elut C-18	Discovery DSC-18	Strata C-18E	Chromabond EASY	Lichrolut EN	Strata SDB-L
n-Pentane	40.4 (16.6)	29.1 (0.7)	23.7 (5.8)	22.0 (3.3)	29.4 (7.5)	56.4 (12.6)
Methanol	67.0 (3.4)	70.9 (2.4)	95.5 (5.3)	38.7 (15.5)	74.9 (11.9)	76.7 (8.3)
Dichloromethane	84.5 (0.2)	91.1 (0.6)	85.4 (0.1)	72.8 (3.2)	104.0 (2.5)	101.2 (0.1)
Ethanol	86.0 (0.7)	73.0 (0.8)	85.4 (0.5)	43.2 (11.4)	63.4 (13.2)	72.5 (1.4)

The terpenoids were recovered using 2 + 2 ml of solvent.

Table 4
Recoveries (%) obtained from Lichrolut EN using dichloromethane (1 + 1 + 1 + 1 ml) as extracting solvent

Compound	First fraction	Second fraction	Third fraction	Fourth fraction
α-Terpinene	102.7	4.4	1.1	0.6
(+)-Limonene	96.3	4.1	n.d.[a]	n.d.
γ-Terpinene	96.0	3.3	n.d.	n.d.
Linalool	100.2	1.4	n.d.	n.d.
Terpineol	101.6	1.4	n.d.	n.d.
D-Citronellol	99.8	0.9	n.d.	n.d.
Nerol	104.9	1.2	n.d.	n.d.
Geraniol	118.9	n.d.	n.d.	n.d.

[a] Not detected.

volumes of water were assayed: 0, 5 and 10 ml and the average recoveries for total terpenoids were 100.9, 99.0 and 100.1%, respectively. So, any volume between 0 and 10 ml could be used in the extraction method.

After the clean-up step, a drying step is needed in order to avoid introduction of water into the GC column. Three drying times were checked: 10, 20 and 30 min. The average recoveries for the total assayed terpenoids were: 99.8, 99.9 and 96.9%, respectively. Thus, no influence on the recoveries was observed for this variable.

To determine the volume of extracting solvent needed for a total recovery, 4 ml of dichloromethane were used. It was passed through the solid phase in four successive steps (4× 1 ml), without drying between them. Recoveries for the assayed terpenoids in each fraction are shown in Table 4. As can be seen, most compounds are almost totally recovered in the first fraction; however, most of them are also detected in the second fraction. So, 2 ml is needed to obtain quantitative recoveries for all the compounds assayed.

After determining the extraction volume, it is necessary to check if the vacuum applied during the eluting step influences the recovery. Three levels were assayed (−0.33, −0.50 and −0.67 atm). The average recoveries were 99.8, 93.1 and 102.9%, respectively. Therefore, there was no clear influence of vacuum level on the recovery. As the higher the vacuum, the shorter the extraction time, a vacuum of −0.67 atm was chosen as the most satisfactory vacuum level.

Table 5
Average recoveries (%) of terpenoids obtained using different amounts of Lichrolut EN

Compound	Lichrolut EN (200 mg)	Lichrolut EN (500 mg)
α-Terpinene	101.1	91.2
(+)-Limonene	100.7	98.5
γ-Terpinene	94.4	102.1
Linalool	107.3	86.9
Terpineol	99.6	78.7
D-Citronellol	105.4	80.9
Nerol	109.4	79.7
Geraniol	103.6	76.2

Finally, two amounts of solid phase in the extraction cartridge were checked in duplicate: 200 and 500 mg. The recoveries obtained for individual terpenoids are shown in Table 5. As can be seen slightly higher recoveries were found for the 200 mg cartridges.

Therefore, the optimized method is as follows:

1. *Condition step*: A sequence of 4 ml of dichloromethane, 4 ml of methanol, 4 ml of ethanol (12%, v/v) in water.
2. *Retention step*: 50 ml of sample on Lichrolut EN (200 mg).
3. *Clean-up and drying step*: 10 ml of water, 10 min under −0.67 atm.
4. *Elution step*: 2 ml of dichloromethane.

3.3. Analytical properties of the optimized method

Table 6 shows the analytical properties of the devised method. Alamin package [12] was used for determining limits of detection (LOD) and limits of quantification (LOQ). The resulting LOQ were lower than the levels found usually in young wines. Good repeatability and reproducibility were obtained for the optimized method.

3.4. Application to real samples

Twelve commercial wines were analyzed using the optimized method (Table 7). Both identification by retention time and by mass spectrum were used to identify the terpenoids in real samples. For all the samples, 2 + 2 ml of

Table 6
Analytical properties of the optimized method

Compound	Concentration range (μg l^{-1})	r^2	Linearity curve (%)	LOD (μg l^{-1})	LOQ (μg l^{-1})	Recovery (%)	Repeatability (RSD)[a] ($n = 5$)	Reproducibility (RSD) ($n = 5$)
α-Terpinene	4–501	0.9998	99.441	1.13	3.73	99.9	4.87	7.66
(+)-Limonene	4–501	0.9996	99.148	2.10	7.00	97.0	5.86	5.20
γ-Terpinene	4–500	0.9998	99.398	2.13	7.13	96.8	4.41	7.98
Linalool	5.3–678	1.0000	99.683	0.43	1.40	97.9	4.92	5.58
Terpineol	5.7–728	0.9999	99.612	0.40	1.27	96.8	6.54	6.27
D-Citronellol	7.0–864	0.9992	98.603	3.37	11.17	100.8	2.61	5.97
Nerol	6.7–846	0.9990	98.212	0.73	2.47	97.1	6.31	5.97
Geraniol	8.0–66.6	0.9994	98.281	0.33	1.07	97.6	6.20	6.63

[a] Relative standard deviation.

Table 6
Concentrations ($\mu g\,l^{-1}$) of terpenoids found in wines

Compound	Castillo Irache[a]	Original Muscatb[b]	Marques de Riscal[c]	Viña Esmeralda[b]	Gran Feudo[b]	Viña Mireia[b]	Cresta Real[d]	Moscatel Dorado[b]	Tokaji Furmint[e]	Castillo Aguaron[f]	Castillo de Liria[b]	Ribera del Puerto[g]
α-Terpinene	n.d.[h]	n.d.	13.6	n.d.	n.d.	n.d.	8.8	n.d.	tr[i]	8.92	n.d.	4.5
Limonene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
γ-Terpinene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Linalool	21.4	15.6	4.9	102.9	26.8	14.7	6.6	59.5	15.5	9.0	87.1	5.6
Terpineol	15.5	107.0	6.0	146.1	102.2	60.3	9.0	115.4	16.6	13.8	75.0	7.0
ɒ-Citronellol	n.d.	28.5	n.d.	22.8	22.5	20.1	19.1	22.2	18.4	17.1	27.4	18.0
Nerol	30.4	n.d.	29.2	25.8	31.9	16.7	27.8	15.8	34.1	23.0	18.5	85.1
Geraniol	n.d.	n.d.	n.d.	n.d.	77.1	38.0	n.d.	n.d.	n.d.	64.2	n.d.	n.d.
Total	67.3	151.0	53.7	297.7	260.5	149.9	71.3	212.8	93.3	135.9	208.1	120.2
Percentage of terpineol	23.0	70.8	11.2	49.1	39.2	40.2	12.6	54.2	17.8	10.1	36.0	5.8

[a] Chardonnay.
[b] Muscat.
[c] Sauvignon Blanc.
[d] Verdejo.
[e] Furmint.
[f] Grenache.
[g] Palomino-Macabeo.
[h] Not detected.
[i] Traces below LOQ.

dichloromethane were used in the extraction and no terpenoids were detected in the second extracts. Moreover, re-extraction of the 50 ml of eluting sample was performed to determine if the solid phase was able to retain all the terpenoids in the samples. No terpenoids were detected in any of the re-extracts.

The highest levels of terpenoids were found in wines derived from Muscat grapes, mainly due to the high levels of terpineol which represents >50% of total terpenoids in some wines. Linalool was the only terpenoid found in all the analyzed wines whereas both terpineol and nerol were detected in 11 samples.

4. Conclusions

A fast off-line SPE-GC method has been developed for quantitative analysis for terpenoids in wines. After evaluation of several eluting solvents and solid phases for the SPE, dichloromethane as eluting solvent and styrene–divinylbenzene as solid phase were selected as the best combination. After optimization of the extracting variables, full recovery of terpenoids from 12 real samples was guaranteed as terpenoids were not detected in any re-extraction of the samples. All the samples showed significant levels of linalool; however, the highest levels were found for terpineol in Muscat-derived wines.

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