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Semipreparative reversed-phase liquid chromatographic fractionation of aroma extracts from wine and other alcoholic beverages

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Abstract

The suitability of reversed-phase HPLC for the semi-preparative fractionation of aroma extracts from wine and other alcoholic beverages has been explored. Aroma extracts are separated in a 250×10-mm Kromasil-C_{18} column using a water–ethanol gradient system as mobile phase. It has been demonstrated that the chromatographic separation does not induce any chemical change in the sample components. The maximum volume that can be injected without altering efficiency is as high as 2 ml if ethanolic extracts are injected, and slightly less in the case of less polar extracts. Aroma extracts are injected directly without the need of any pretreatment. As major compounds elute first, it is possible to fractionate all the volatiles contained in a 1–1.5-l sample without peak distortion or mass overload problems. The usefulness of the method has been demonstrated by fractionating an extract from a Chardonnay wine to get 15 fractions that showed different aromas. The GC analysis with olfactometric and MS detection of those fractions has allowed us to identify more than 70 aroma compounds and to signal some of them as potential key aromas of Chardonnay wine. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Aroma analysis; Wine; Beverages; Food analysis

1. Introduction

Like many other natural products, beer, wine and natural spirits contain highly active aroma components present at concentrations as low as several ng per liter [1–10]. The analysis of these compounds demands highly selective and efficient enrichment steps, such as preparative liquid or gas chromatography, or multidimensional GC. In the case of alcoholic beverages, an additional difficulty is found, because those ultratrace aroma compounds are 'hidden' beneath a deep curtain of major volatile components formed mainly during yeast metabolism, and which can be present at concentrations higher than 100 mg/l. The presence of these compounds, particularly fusel alcohols and the ethyl esters of several organic acids, limits the concentration factor that can be reached during the normal previous isolation steps, and can make impractical the use of high-performance systems due to its low sample capacity. This can explain why the most frequently used technique to fractionate the extracts is, yet, column liquid chromatography over silica gel [7–9,11]. Although in no way do we question the validity of this technique, there are several reasons why other alternatives should be searched. Namely, the high

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consumption of time and (toxic and odorous) solvents, the risk of the formation of artifacts, and the convenience of having at hand different and complementary fractionation techniques.

In this paper, the usefulness of reversed-phase HPLC systems to fractionate aroma extracts from alcoholic beverages is explored, which, as far as we know, has not been studied in depth previously. The main goal of this work is to get an easy-to-use, robust, efficient, and fast fractionation method especially devoted to qualitative analysis. Since in qualitative aroma analysis it is necessary to determine the sensory properties of the isolated fractions, in order to check if the target aroma component is there and/or to have a previous estimation of its importance, only non-toxic solvents have been considered in the method development. Particular attention has also been paid to check if the chromatographic process could induce some change in the aromatic composition of the samples. Once the aforementioned questions were addressed, the conditions leading to a correct separation of aroma compounds and to the introduction of maximum masses into the HPLC system have been investigated. The results have been finally applied to the fractionation and analysis of an extract from a Chardonnay wine.

2. Material and methods

2.1. Reagents, samples and standards

All the reagents used were of analytical-grade quality. Ethanol (HPLC quality) was from Merck (Darmstadt, Germany). Reference compounds were purchased from Aldrich (Gillingham, UK), Sigma (St. Louis, MO, USA), and Fluka (Buchs, Switzerland). Water was obtained from a Milli-Q Plus purification system by Millipore (Bedford, MA, USA). All the samples used in this study were purchased directly from a retailer. Red wine: 'Viñas del Vero', a 1-year-old red wine from Somontano (Spain). White wine: 100% Chardonnay also from 'Viñas del Vero' (Somontano, Spain). Oxidized white wine: obtained by storing under pure oxygen the Chardonnay wine for a 2-week period. Whisky: a 20-year-old Scotch single malt 'Cardhu' (Scotland, UK). Brandy: a French Brandy Napoleon from Bowman distilleries.

2.2. HPLC fractionation

The HPLC system was from Waters (Milford, MA, USA), and was integrated by two 510 pumps, an automated gradient controller, a manual injector U6K, and and a diode array 990 UV detector. Column, Kromasil 5 μm, 25 cm×10 mm I.D. from Análisis Vínicos (Tomelloso, Spain). The column is protected by a 2-cm precolumn of the same phase, which is replaced after 10 injections. Chromatographic conditions: flow-rate, 2 ml/min; detection at 220, 254 and 350 nm; injection volume, between 200 and 8000 μl (typical volume 1 ml). Program gradient: phase A, water; phase B, ethanol. (A) Standard: min 0, 100% A, linear program until 20% B at min 8, 50% B at min 28, and 100% B at min 40. (B) Fast: min 0, 100% A, linear program until 20% B at min 8, 50% B at min 28, and 100% B at min 33. All the samples were filtered through a 0.45-μm filter before injection.

2.3. Aroma fractions re-extraction

The fractions eluted from the HPLC were diluted with water to adjust their alcoholic content to 12–14% (v/v), were then salted with 0.3 g of ammonium sulfate per ml, and finally extracted with 2 vol of dichloromethane (organic–aqueous phase ratio, 1:10). The dichloromethane volumes were mixed and concentrated under a nitrogen stream until the required volume.

2.4. High-resolution GC–MS–olfactometry

A Star 3400CX (Varian) gas chromatograph fitted with a Saturn 4 electronic impact MS detector and equipped with a sniffing port (open split interface; make-up flow-rate, 4 ml/min He). Columns: Carbowax 20 *M* (J&W, Folsom, USA): 30 m×0.32 mm I.D., and 0.5-μm film thickness. Chromatographic conditions: carrier He at 1.2 ml min^{-1}. A 1-μl sample was injected into a 1093 septum-equipped programmable injector held 6 s at 20°C, and then raised to 190°C at 200°C/min. Initial column temperature 40°C, held for 5 min, and then raised to

190°C at 2°C/min. Olfactometry was performed by three different trained sniffers.

2.5. Sensory analysis

The test panel was composed of 12 experienced individuals (five females and seven males, between 23 and 50 years of age). All the tests were carried out in standardized booths with tulip glasses containing 30 ml of solution.

2.6. Aroma extraction

Alcoholic extracts were prepared by salting out 250 ml of the beverage adjusted at 13% (v/v) alcohol with 33 g of $NaH_2PO_4 \cdot H_2O$ and 135 g of NH_4SO_4. The demixed ethanolic phase (about 13 ml) was collected and further reconcentrated until a final volume of about 1–2 ml by washing with a brine composed of 13.2 g of $NaH_2PO_4 \cdot H_2O$, 54 g of NH_4SO_4 and 87 ml of water. Diethyl ether–pentane extracts were prepared by extracting 1000 ml of product (alcoholic degree adjusted to 13%, v/v) salted with 100 g NaCl with 3×300-ml fractions of diethyl ether–pentane (1:1, v/v) for 3 h each. The three fractions were collected, dried with anhydrous $NaSO_4$ and concentrated by distillation first under a 60-cm Vigreaux column until 25 ml and later in a micro-Kuderna–Danish concentrator with a three-ball Snyder column until 1 ml. Dichloromethane extracts were prepared in a similar way, but the three volumes of extractant were 100 ml. Freon 11 extracts were obtained by continuous liquid–1iquid extraction of 1-l volumes of sample with 2×250-ml volumes of Freon 11 for 24 h each. The collected extract was concentrated as before.

2.7. Integrity of sample components

Experiment 1: chromatographic retention factors for a selected group of aroma compounds were determined by using ethanol 100% as mobile phase. Experiment 2: three 1-ml alcoholic extracts were prepared from each: whisky, cognac, red wine, white wine and oxidized white wine, as described before. One of the 1-ml extracts (test extract) was fractionated in the HPLC system (fast program) and the whole eluate collected until the end of the chromatogram (about 84 ml). The second and third extracts (control extracts) were directly added to the two 84-ml volumes eluted from two blank runs. A duo-trio sensory test was performed to determine the existence of significant differences between separated and non-separated extracts. In this duo-trio test [12,13] each judge was presented with three cups. The first cup, identified as reference, contained one of the control extracts. The other two were coded, and contained the other control and the test extract. The judges were asked to indicate which product is most similar to the reference. Twenty ml from both extracts were re-extracted as indicated before, concentrated until a final volume of 0.2 ml and analyzed by GC–MS to check for chemical differences.

2.8. Injection volume and sample solvent

Experiment 1: different volumes of ethanol (from 0.2 to 8 ml) containing constant masses of furfural, sotolon, vanillin, guaiacol, 2-phenylethanol and γ-nonalactone (0.2 mg each) were injected in the HPLC system (fast program) to measure the peak broadening caused by volume saturation. Experiment 2: a 20-ml alcoholic extract was prepared from 3000 ml of red wine as indicated before. Volumes of this extract between 0.5 and 8 ml were injected and fractionated in the HPLC system. Experiment 3: 0.5 ml of alcoholic extract from a chardonnay wine was injected into the HPLC. A second 0.5-ml volume of extract was diluted to 2 ml with ethanol, injected and compared with the first injection. Experiment 4: different volumes (0.5–4 ml) of diethyl ether–pentane (1:1, v/v), dichloromethane, Freon 11 and water–ethanol (4:6, v/v) containing fixed amounts (0.2 mg) of vanillin, 2-phenylethanol, 2-ethylphenyl acetate and β-ionone were injected in the HPLC (fast program) to determine the influence of the sample solvent on the peak broadening.

2.9. Chardonnay wine analysis

A pentane–ether extract from 1000 ml of Chardonnay wine was obtained and fractionated as described before (standard program). The aroma of the fractions was described by the test panel. Each fraction was later reextracted and analyzed by GC with simultaneous MS and olfactometric detection.

Table 1
Basic chromatographic characteristics of a selected group of aroma compounds: retention factors with ethanol 100% as mobile phase, and composition of the mobile phase at which the compound is eluted in a linear gradient (0–100% ethanol in 40 min)

Compound	k' in ethanol	Mobile phase ethanol content (%)
Acetic acid	0	2.1
Butanedione	0	3.3
Acetaldehyde	0	10.4
Furfural	0	21.6
Sotolon	0	24.1
Ethyl acetate	$\ll 0.1$	27.6
Vanillin	$\ll 0.1$	31.8
Guaiacol	<0.1	37.8
Isoamyl alcohol	<0.1	41.0
2-Phenylethanol	<0.1	43.0
Linalool	<0.1	44.5
γ-Nonalactone	0.1	85.8
Eugenol	0.1	85.8
4-Vinylguaiacol	0.1	90.0
2-Phenyl-ethyl acetate	0.1	93.2
Ethyl 2-methylbutyrate	0.1	98.2
Ethyl cinnamate	0.15	99.8
β-ionone	0.20	100
Isobutyl methoxypyrazine	0.25	100
Ethyl octanoate	0.30	100
Geraniol	0.36	100

3. Results and discussion

3.1. Integrity of sample components

Two basic prerequisites to ensure the applicability of the chromatographic method in flavor chemistry are that, in the working conditions, no aroma component of the original product should be lost or transformed during the process, and that all the aroma components eluted from the chromatographic column come really from the original product. In order to check that all these requirements were met, several experiments were carried out. Firstly, the chromatographic retention factors of a number of different aroma compounds were determined using 100% ethanol as mobile phase. This set of results is shown in Table 1. As all the retention factors are below unity, it can be concluded that ethanol is a sufficiently strong solvent to elute all aroma compounds from the column. This result guarantees that the water–ethanol gradient allows fractionation for an easy and safe sensory analysis.

Secondly, and in order to check if the chromatographic operation can induce the chemical decomposition of some aroma, or the generation of some other aroma not originally present in the sample, several alcoholic extracts from different products were injected in the HPLC system and separated following the standard procedure. The corresponding eluates were collected and sensorily compared with the original extracts through duo-trio tests (see Section 2). Results are shown in Table 2, and clearly demonstrate that the chemical changes affecting aroma components introduced by the chromatographic fractionation are, if any, of minor importance, since the test panel could in no case clearly distinguish between the original extract and the one collected after the chromatography. The MS chro-

Table 2
Results of the sensory discriminant tests (duo-trio) carried out to check if the chromatography on the reversed-phase HPLC column induces chemical changes in the aroma

Product	Number of responses	Number of correct responses	Probability of the result[a]
Whisky extract	18	8	0.760
Chardonnay wine extract	17	7	0.834
Oxidized white wine extract	18	8	0.760
Red wine extract	21	12	0.332
Cognac extract	19	11	0.324

[a] Refers to the probability of obtaining the number of correct responses given in the previous column, if the two tested products were just the same. A high probability indicates that it is very likely that the two products are really equal (or at least that the difference cannot be really perceived), a low probability would indicate the existence of a real sensory difference between the two products tested [13].

matographic traces of the extracts after and before the chromatographic process were also indistinguishable.

3.2. Chromatography

The elution order of some aroma components is also shown in Table 1. As expected, acetaldehyde, acetic acid, furfural and ethyl acetate, the most polar and water-soluble aroma compounds, are eluted in the first part of the chromatogram with a mobile phase containing less than 30% ethanol. Fusel alcohols, together with vanillin, some fatty acids and volatile phenols are eluted in a second part of the chromatogram, with mobile phase ethanol content less than 50%. The rest of compounds are eluted with mobile phases containing more than 80% ethanol. A great advantage of this elution profile is that the major aroma compounds of alcoholic beverages are just those eluted in the first part of the chromatogram and, as can be seen in the table, very well separated from the rest of aroma compounds. This effect will make it possible to introduce relatively large volumes and masses of extract without peak broadening or other chromatographic distortion effects, which constitutes the main advantage of this type of chromatography over the normal-phase modes.

3.3. Volume of injection

The injected volume of extract becomes a critical parameter, since this HPLC method is part of a semipreparative isolation scheme rather than a quantitative method. In order to measure the intensity of the reconcentration effect that the gradient exerts on the different analytes, increasing volumes of synthetic ethanolic solutions containing a fixed mass of several compounds were injected in the HPLC. The results of this experiment are shown in Table 3 and Fig. 1. The table gives the peak widths as a function of the injected volume. It can be seen that only the peaks that, as in the case of furfural, are eluted in the first part of the chromatogram are distorted promptly when the injection volume increases. The reconcentration effect of gradient becomes apparent for vanillin, and becomes really active for the last-eluted compounds, which are, fortunately, the major part of aroma compounds. All this means that it is possible to introduce 2–4 ml of extract in the column without serious peak distortion. Major volumes can be injected, but assuming strong peak distortion for the less retained compounds. With respect to mass overloading, it has not been observed in injections of extracts coming from up to 2000 ml of product. This fact is demonstrated in Fig. 2, which shows the chromatograms obtained from the injection of different volumes of a red wine alcoholic extract. The extract was deeply colored because anthocyanins and other wine phenols are also extracted by that extraction procedure, and thus, it represents a very 'dirty' and 'highly loaded' sample, useful to check the robustness of the method. The first peak contains monomeric anthocyanins that elute in an area almost free from aroma components. The area between 10 and 20 min corresponds to the elution of polar aroma compounds and to the elution of dimeric anthocyanins and flavanols, and most of the UV signal is due to these components. Most of the odorants elute after 2-phenylethanol (peak at 27 min). The figure

Table 3
Peak widths (in min) as a function of the volume of injection for a group of aromas (detection, UV at 220 nm)

Compound	Injected volume (ml)					
	Analytical injection (0.2)	0.5	1	2	5	8
Furfural	0.4	0.7	2.14	3.6	Split	Split
Vanillin	0.4	0.7	0.8	1.6	3.0	5.3
Guaiacol	0.4	0.4	0.7	1.0	1.6	2.5
2-Phenylethanol	0.4	0.4	0.4	0.7	1.2	1.8
γ-Nonalactone	0.3	0.3	0.3	0.4	0.4	0.5
2-Phenylethyl acetate	0.3	0.3	0.3	0.3	0.4	0.4

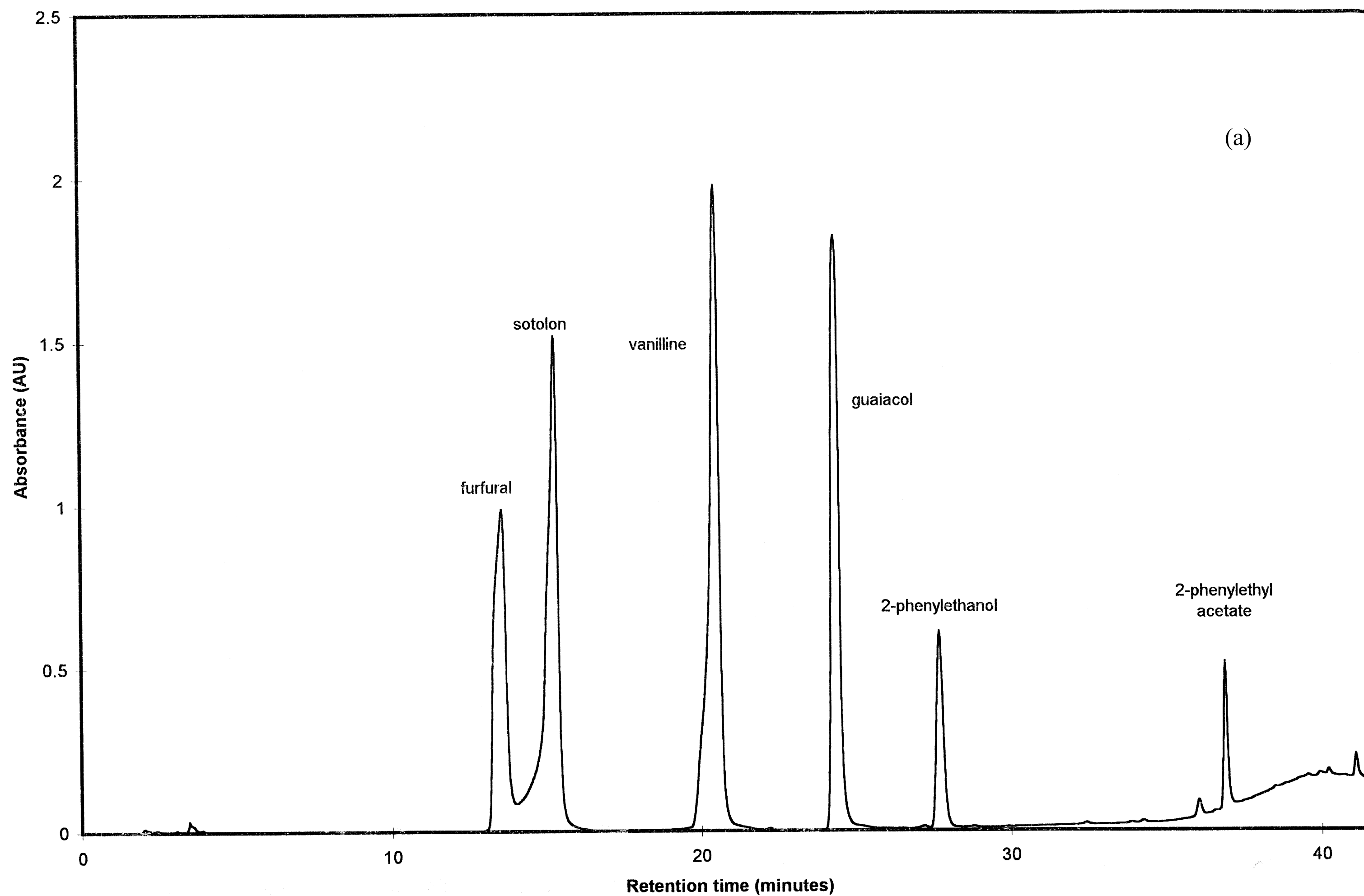

Fig. 1. HPLC chromatogram corresponding to the injection of (a) 0.5 ml, (b) 8 ml of a synthetic ethanolic solution containing the same mass of furfural, sotolon, vanillin, guaiacol, 2-phenylethanol and 2-phenylethyl acetate.

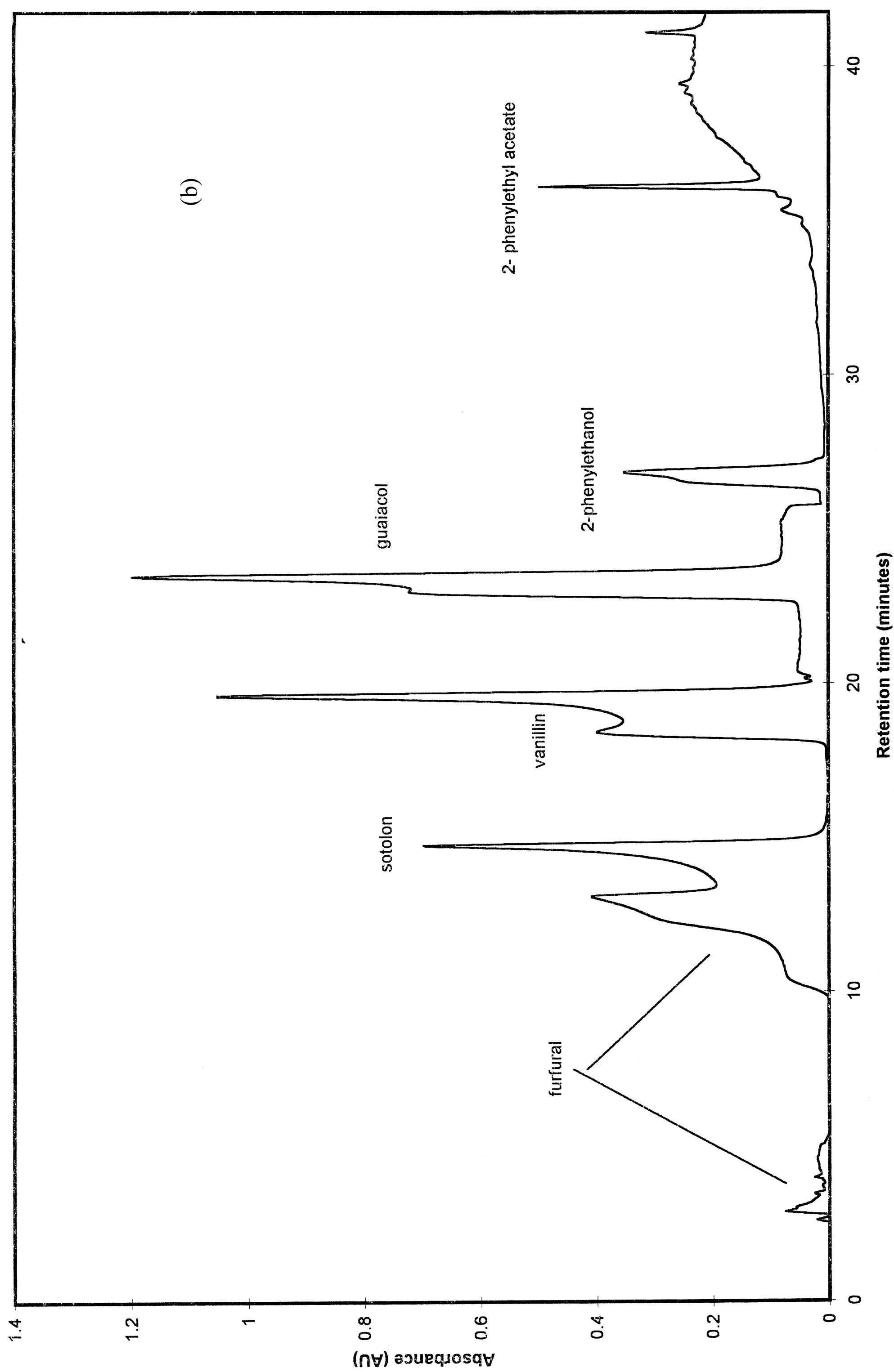

Fig. 1 (*continued*).

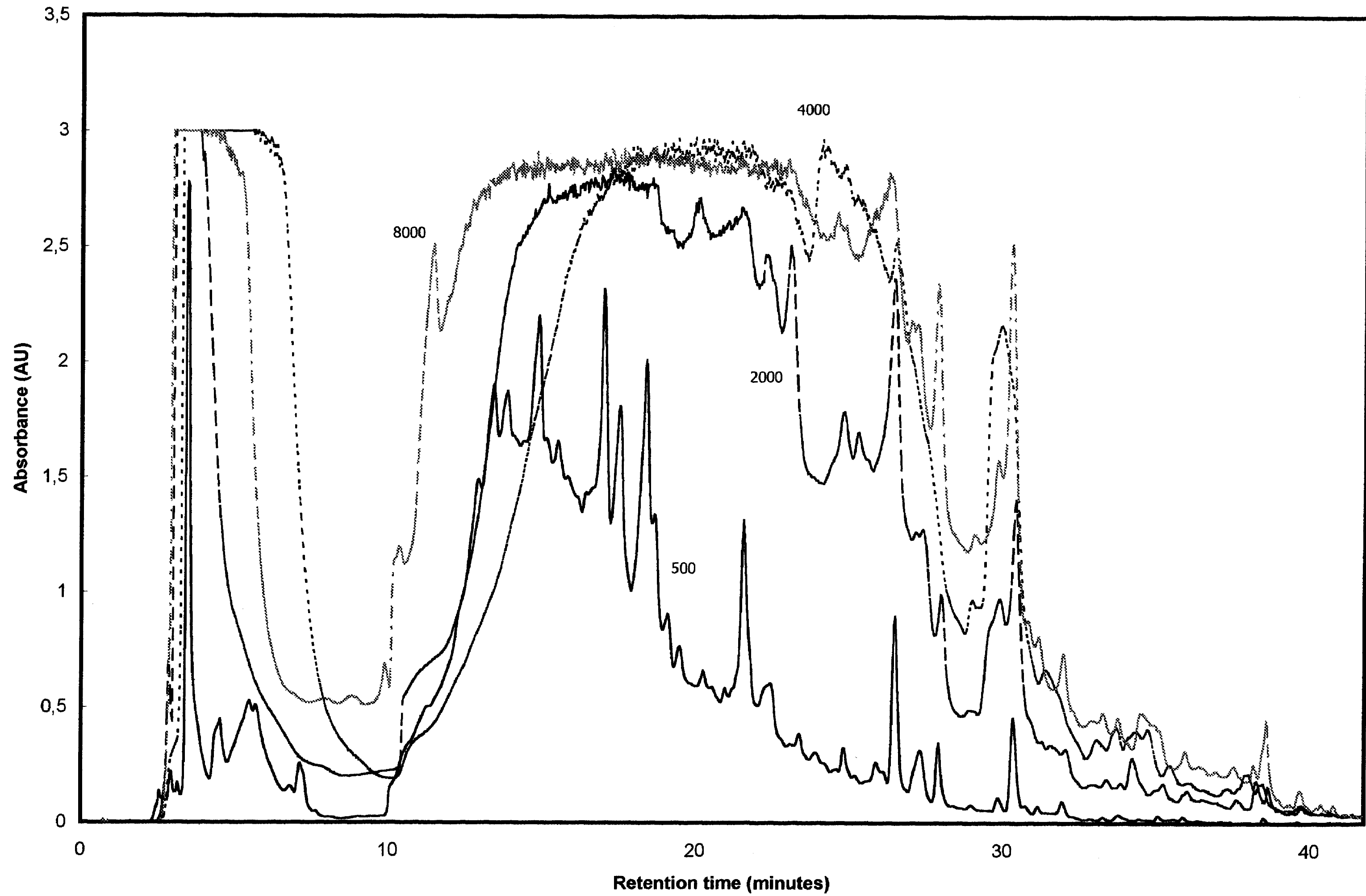

Fig. 2. HPLC chromatogram (UV detection at 340 nm) corresponding to the injection of 0.5–8-ml volumes of a deeply colored red wine ethanolic extract.

shows that although chromatographic efficiency seriously worsens with the highest injected volume, retention times remain fairly constant, which ensures a good reproducibility and robustness.

3.4. Sample solvent and aroma extraction

A problem associated with the use of reversed-phase chromatography in aroma analysis is the fact that most of the solvents used for aroma isolation are poorly soluble in highly aqueous mobile phases, which limits the reconcentration effect produced by the gradient. Because of this, the results previously shown in Table 3 cannot be directly extrapolated to solvents less polar than ethanol, and an additional study to determine the maximum volume that can be injected was carried out with three typical extraction solvents. Results can be seen in Table 4. The first column indicates the maximum volume of solvent that can be injected without noticeable peak broadening. The second one corresponds to the volume that causes a ‘modest’ peak broadening (not higher than 20% in all the peaks eluted after 2-phenylethanol), while the third column gives the maximum volume that causes a ‘still tolerable’ peak broadening (not higher than 20% for the latest eluted compound). It can be seen that, from a chromatographic point of view, the best solvent is ethanol or the mixture water–ethanol (40:60). These solvent systems have very high extraction efficiency and very low selectivity, and they are the best choice if polar compounds are involved in the targeted aroma. It must be said, however, that since they cannot be easily concentrated by evaporation, the concentration factor that they can reach is not very high and, thereby, does not make it possible to introduce very high masses into the system. On the other hand, dichloromethane and diethyl ether–pentane extracts cause a higher peak distortion, which limits their maximum injectable volume. Yet, as they can be easily concentrated, they make the introduction of higher masses of extract into the chromatographic system possible. The injection of up to 1-ml volumes of dichloromethane or diethyl ether–pentane extracts makes it possible to fractionate the odorants contained in 1–1.5 l of product in one run while keeping the analytical chromatographic efficiency.

3.5. Wine aroma fractionation

Fig. 3 corresponds to the chromatogram obtained in the fractionation of a Chardonnay wine extract. In the case shown in the figure, a diethyl ether–pentane extract coming from a 1-l wine sample was introduced into the column without any pretreatment. The chromatographic separation allows for an easy recovery of several fractions containing aroma compounds of equivalent polarities. The apparently mass saturated area eluting between the 30th and 40th minute of the chromatogram corresponds to the elution of several wine phenols with very high UV absorption coefficients, rather than to a real mass overload. In this experiment, 15 fractions were recovered, reextracted and analyzed by gas chromatography with simultaneous olfactometric and mass spectrometric detection. The aroma composition of each of the 15 fractions, together with the sensory

Table 4
Influence of the sample solvent on the maximum volume of injection and on the total volume of sample (given in ml of wine) that can be introduced into the column

Solvent	Maximum injection volume (μl)			Total volume of sample[a] (ml)
	No broadening	‘Soft’ broadening	‘Still tolerable’ broadening	
Dichloromethane	250	900	2100	900
Diethyl ether–pentane (50:50)	350	1300	2800	1300
Freon 11	200	700	1400	700
Ethanol–water (60:40)	500	2000	8000	300

[a] Refers to the volume of wine whose aromas can be introduced into the column (under soft broadening conditions), supposing that the extraction recovery is 100% and that the concentration achievable through solvent evaporation is 1000 (except in alcoholic extracts, where a demixture approach can yield concentration factors near 150).

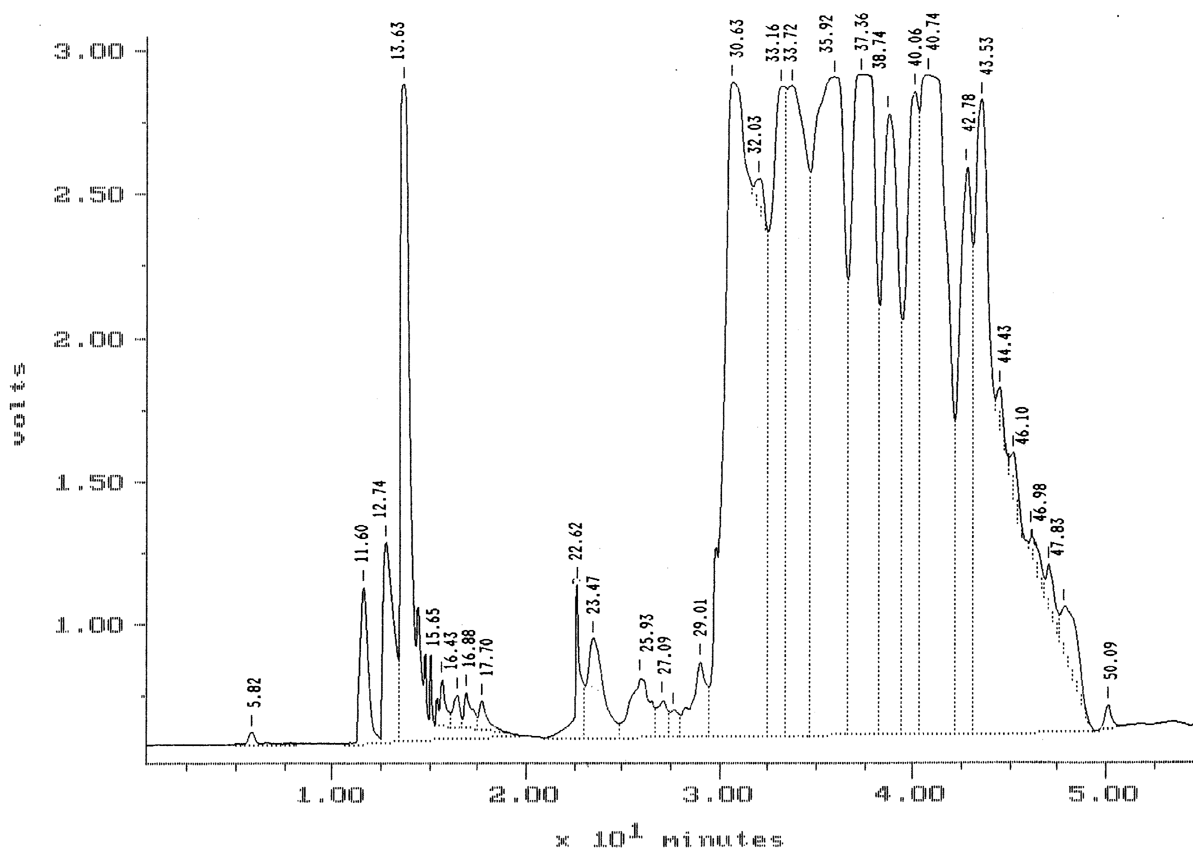

Fig. 3. HPLC chromatogram (UV detection at 254 nm) of 1 ml of a Chardonnay wine, diethyl ether–pentane extract concentrated 1000-fold.

descriptors that the panel used to describe them, are shown in Table 5. A total number of 70 aroma compounds could be identified. Most of them were found only in one, or in as many as two, fractions of quite simple composition, which greatly simplifies the task of correlating the aromas observed in the olfactometric port with the chromatographic peaks. At the same time, it becomes easier to correlate the aroma of the fraction with some of the aroma compounds it contains. For instance, the sensory descriptors of fraction 5 clearly indicate that furaneol is the key aroma of that fraction, and suggest that this compound may be a really important aroma of Chardonnay wine. These observations are not so obvious if the olfactometric experiment is performed on the unfractionated extract where 70 aromas are simultaneously present.

4. Conclusions

One of the most important advantages of the proposed HPLC method over the classical normal-phase approaches is that the isolated fractions can be sensorily tested without the problems of toxic and odorous solvents. Other additional advantages are its simplicity, since the extract does not need to be pretreated, its speed, the high mass output in a high-performance system, and the robustness and reproducibility associated with C_{18} systems. The major drawback is that the odorants need to be re-extracted again to be analyzed by GC–MS but, all in all, the proposed strategy can be a useful tool in the aroma chemistry laboratory and can have a role complementary to that of the normal-phase approaches.

Table 5
Aromatic descriptors and aromatic composition of the different fractions isolated from a Chardonnay wine extract

Fraction	Time (min)[a]	Aromatic descriptors	Odorants present in the fractions
1	10–17	Wet Very weak	Acetaldehyde, acetic acid
2	17–21	Yeast extract, wet Weak	Acetaldehyde, ethyl lactate, furfural, ethyl 3-hydroxybutyrate, methionol, 1,4-butanediol diacetate, diethyl malate
3	21–24	Bakery Moderate	Ethyl lactate, ethyl 3-hydroxybutyrate, isobutyric acid
4	24–27	Sweet. Very weak	Benzyl alcohol, vanillin
5	27–30	Caramel, burnt straw, sweet cotton, peach Very strong	Butyric acid, diethyl malonate, furaneol, vanillin
6	30–33 33–36	Sweet, anisse, fusel Weak	Isoamyl alcohol, guaiacol, 2-phenylethanol, vanillic acid methyl esther
7	33–36	Fusel, flowery Moderate	Diethyl succinate, 2-phenylethanol, benzaldehyde, ethyl 2-furoate
8	36–39	Alcohol, acid, cheese Very strong	2-Methyl and 3-methylbutyric acids, *cis*-3-hexenol, *cis*-2-hexenol, *trans*-3-hexenol, diethyl pentanodiate, *m*-cresol, vanillic acid, vanillic acid ethyl ester
9	39–42	Fruity, toothpaste, liquorice, cinnamon Moderate	Ethyl butyrate; ethyl 2-butenoate, isobutyl acetate, *trans*-2-hexenol, *cis*- and *trans*-whisky lactone (methyl-γ-octalactone), hexanol, eugenol, hexanoic acid, 4-vinylguaiacol
10	42–43	Citric, peach Moderate	2-Phenylethyl acetate, δ-octalactone, *trans*-whisky lactone, γ-nonalactone, eugenol, δ-decalactone
11	43–44	Fruity, banana, sweet Strong	Isoamyl acetate, ethyl cinnamate, ethyl dihydrocinnamate, β-ionone
12	44–45	Fruity, green, citric Very strong	Isoamyl acetate, *cis*-3-hexenil acetate, *tert*.-3-hexenil acetate, *cis*-octen-3-ol, camphor, linalool, 1-octanol, ethyl benzoate, α-terpineol, *tert*.-geraniol, octanoic acid
13	45–46	Apple, terpenic, rose Strong	Ethyl hexanoate, hexyl acetate, 2-ethyl-1-hexanol, octanal, *cis*- and *trans*-vitispiranes, linalool, 1-octanol, α-terpineol, β-citronellol, β-damascenone, nerol, octanoic acid
14	46–47	Pungent, acid, green Moderate	Ethyl hexanoate, hexyl acetate, decanoic acid
15	47–48	Alcohol, acid, citric Weak	Ethyl octanoate, decanal, ethyl decanoate, isoamyl octanoate, ethyl laurate, decanoic acid, lauric acid

[a] Refers to the chromatogram shown in Fig. 3.

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