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Analytical Characterization of the Flavor of Oxygen-Spoiled Wines through the Gas Chromatography–Ion-Trap Mass Spectrometry of Ultratrace Odorants: Optimization of Conditions

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Abstract

A method for the direct determination of the most important aromas generated in oxygen-spoiled wines is developed. The method allows the simultaneous determination of some key ultratrace carbonyls, such as t-2-hexenal, t-2-octenal, and t-2-nonenal; other carbonyls such as furfural, 5-methyl furfural, hexanal, and benzaldehyde; some alcohols, such as 1-octen-3-ol, 2-buthoxyethanol, and furfurol; and a volatile phenol, such as eugenol. Only one internal standard is used, and chemical derivatization of carbonyls is not required. The method combines a powerful preconcentration based on the demixture of an alcoholic fraction by salting out the wine with a liquid–liquid microextraction of an ether–pentane (10:90) mixture. The first step guarantees sensitivity, and the second step ensures selectivity and cleanliness. The extract, which is enriched between 200- and 2000-fold in the analytes, is directly injected into the gas chromatography–ion-trap mass spectrometry system. The preconcentration, injection, and mass-selective detection analysis steps have been optimized with both synthetic and real extracts in order to reach a reasonable compromise among sensitivity, selectivity, and cleanliness. The method is free from matrix effects, and linearity is satisfactory. The global method reproducibility ranges from 3 to 7% for most of the analytes. Detection limits range from 10 to 600 ng/L.

Introduction

Flavor stability is a very important concern in all branches of the food industry, and it is therefore understandable that this aspect should be the subject of a large amount of research. In wine, flavor stability has been mainly associated with polyphenolic polymerization, a problem which has received a lot of attention and that, in fact, can be satisfactorily measured today (1–3). In contrast, there has not been any consistent research on the interpretation of the changes in aroma. This discrepancy must be attributed to the fact that it is commonly believed among wine professionals (4,5) that the changes taking place in wine aroma during oxidation are mainly caused, directly or indirectly, by acetaldehyde (in fact, the process is often named as acetalization). Because Singleton (6) magistrally demonstrated that acetaldehyde is concomitantly generated during polyphenolic polymerization, it is generally believed that both color and aroma degradation are two results of the same problem.

This is not true, however, because it has been demonstrated (7,8) that oxidative wine spoilage involves the generation of some odorants irrespective of polyphenolic polymerization and acetaldehyde formation. Because the presence of very small amounts of some odorants can change the flavor profile of a foodstuff completely, there is a key interest in the development of fast analytical methods for the determination of these oxidation-generated odorants. The particular nature of wine makes the number of chemical compounds and chemical functionalities involved in the process fairly large. Actually, among the compounds involved, there are aldehydes formed in lipid oxidation, such as t-2-hexenal and t-2-nonenal, as there are in beer (9,10), but there are also some alcohols or volatile phenols, such as 1-octen-3-ol and eugenol, in contrast with beer. This means that the normal strategies used in beer flavor control, such as the analysis of carbonyls after their derivatization so as to form a high-molecular-mass/high-halogen-content derivative easier to detect with mass spectrometry (MS) or electron capture detection (ECD), or a highly colored derivative that can be determined through high-performance liquid chromatography (HPLC) with ultraviolet detection (11–14), do not provide all the information necessary to evaluate wine flavor oxidation.

With regard to detection selectivity, there are two options to consider: the use of mass-selective detection or the use of an atomic emission detector working with the oxygen emission lines

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coupled to the gas chromatography (GC) system, as proposed by Schirle-Keller and Reineccius (15). Considering both instrumental availability and the need of additional qualitative information, a first logical choice is the use of high-resolution GC (HRGC) with mass-selective detection, although its requirements for sample cleanliness and selectivity are higher and its sensitivity is poorer. In this sense, the use of an ion-trap detector presents a great advantage over the usual benchtop MS, although it still imposes additional requirements of sample cleanliness and selectivity. On the one hand, the ion trap makes it possible to get sensitivities similar to those obtained in single-ion monitoring (SIM) mode in normal scan operation, or even better sensitivities if it works in narrow-range scanning mode. In both cases, the qualitative information obtained is far more meaningful than that provided in the normal SIM mode. On the other hand, the possible disadvantage of the ion trap is that of accuracy, because the mass spectra of the analytes may become distorted because of the presence of variable amounts of ions in the trap (16,17). This problem has been partially overcome with the introduction of the automatic gain control (AGC) system, but it still presents a problem if the analyte coelutes with a major compound. In these cases, a more selective sample preparation scheme has to be introduced, or more conservative calibration approaches, such as isotopic dilution techniques, have to be considered.

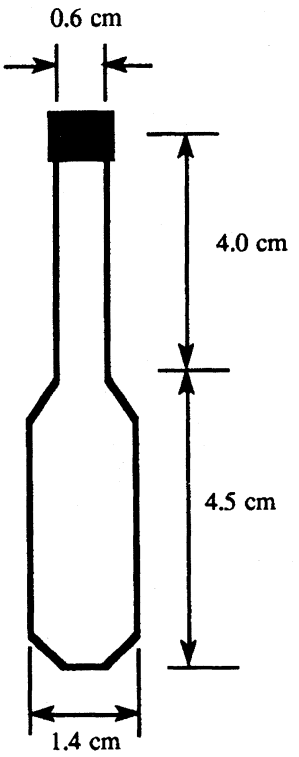

Figure 1. Extraction tube used in the liquid–liquid microextraction.

In any case, the sample preparation scheme must fulfill all the requirements of the detector, and these requirements should be determined first. We followed a strategy based on the previous determination of those detector requirements. Then, we designed and optimized the different sample preparation phases step-by-step. The result is a method that allows us to simultaneously analyze the most important odorants generated in wine during its oxidation. This method shows some remarkable features, such us a very low solvent consumption (150 µL), the need for only one internal standard, and very low detection limits without the use of chemical derivatization.

Experimental

Reagents and solutions

All reagents were analytical quality. Freon 11 was from Merck (Schuchardt, Germany); 1,2-propanediol was from Aldrich (Gillingham, UK); dichloromethane, hexane, and diethyl ether

Table I. Analytes Considered in This Study: Olfactory Threshold, Approximate Concentrations, and Quantitative Mass Fragments

Odorant	Reagent	Olfactory threshold (µg/L)*	Approximate concentration (µg/L)†	Quantitative mass fragments
Hexanal	Sigma, 99%	20	< 2	56 + 57 + 82
t-2-Hexenal	Aldrich, 99%	40	< 2	80 + 83
4-Hidroxy-4-methyl-2-pentanone	Aldrich, 99%	n/a‡	< 1	59 + 101
2-Nonanone	PolyScience, 99.5%	190	< 1	58
2-Butoxyethanol	Chemservice, 98%	n/a	< 1	87 + 100
t-2-Octenal	Aldrich, 94%	3	< 5	93
1-Octen-3-ol	Aldrich, 98%	200	< 5	57
Furfural	Chemservice, 99%	14000	2–5000	95 + 96§
Benzaldehyde	PolyScience, 99%	2000	2–500	77 + 105 + 106
t-2-Nonenal	Aldrich, 97%	0.1	< 1	70 + 83 + 96
5-Methylfurfural	Fluka, 97%	2000	< 2	53 + 109 + 110
Furfurol	Fluka, 98%	n/a	10–125	81 + 98
Eugenol	Aldrich, 99%	11	< 80	164

* Reference 23.
† References 7, 8, 23, and 24.
‡ n/a, data not available.
§ MS–MS conditions: excitation time, 20 ms; excitation amplitude, 0.00 V.

were HPLC quality from Lab-Scan (Dublin, Ireland); and ethanol was from Panreac (Barcelona, Spain). With the exception of 1,2-propylglicol and ethanol, all the solvents were redistilled before use. The pure chemical standards were purchased from Aldrich (Gillingham, UK), PolyScience (Niles, IL), Chemservice (West Chester, PA), Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO), as listed in Table I.

The standards were directly weighed with precision better than 1% and dissolved in known weights of ethanol or hexane to form standard solutions of at least 1 mg/g. These solutions were weight-controlled and were used in method optimization, characterization, and the building of the calibration graphs. The internal standard solution was approximately 0.3 mg/g 2-octanol–ethanol.

Synthetic wines were prepared by adjusting their pH to 3.4 in a hydroalcoholic solution containing 12.3% alcohol (v/v) and 5 g/L tartaric acid solution with 5% NaOH (w/v). Saline solution consisted of 35 g $(NH_4)_2SO_4$ dissolved in 100 mL water. All wines used in this study were 1-year-old Spanish dry white wines. Oxidized wines were prepared by storing a wine under pure oxygen for a period of 1–4 weeks.

Apparatus and conditions

GC–MS

The system consisted of a Star 3400CX GC fitted to a Saturn 4 electronic impact MS from Varian (Walnut Creek, CA). For identification purposes, the GC–MS system was additionally equipped with a sniffing port (open split interface; make-up flow, 4 mL/min He) which allowed for simultaneous MS scanning and sniffing of GC effluents.

The analytical column (60 m × 0.32 mm; 0.5-µm film thickness) was a DB-WAX from J&W Scientific (Folsom, CA) preceded by a 2-m × 0.32-mm retention gap deactivated with methyl phenyl siloxane. The carrier gas was He at 1 mL/min. The oven temperature program was 40°C (5 min) to 190°C at 2°C/min.

The injector was a 1093 septum-equipped programmable injector (SPI) from Varian. The injector temperature was 30°C (6 s) to 190°C at 200°C/min. The injection volume was 1 µL.

MS detection

The mass range was *m/z* 35–200 at 1 scan per second. The AGC system was on. The transfer line temperature was 220°C. The signal was registered without attenuation for 93 min and stored and processed on a 4/50 Compaq Prolinea computer (Houston, TX) equipped with Saturn 5.0 software (Varian) and the Wiley 5.0 (Wiley & Sons, New York, NY) and NIST92 (National Institute of Standards and Technology, Gaithersburg, MD) MS libraries. The mass fragments used in the quantitative determination are given in Table I. The different relative areas to that of 2-octanol (total ion current peak area) were interpolated in the corresponding calibration graphs built with synthetic solutions. Furfural was determined after isolation of its 95 and 96 MS fragments in the trap.

HPLC prefractionation

The HPLC apparatus consisted of a Waters 510 pump, a Waters U6K manual injector, and a Waters RI detector. The column was 250 × 4.6 mm (5-µm silica) from Knauer (Berlin, Germany). The mobile phase consisted of diethyl ether, dichloromethane, and hexane (7.8:2.0:90.2), all freshly distilled. The flow rate was 1 mL/min. The injection volume was 75 µL.

Methods

Proposed method

A 385-mL aliquot of wine was poured into a 500-mL graduated cylinder. To a dry 500-mL volumetric flask were added 43.0 g H_2NaPO_4–H_2O and 176.7 g $(NH_4)_2SO_4$. Over the salt, approximately 100 mL of the wine to be extracted was added, and the required amount of ethanol (or water) was added to adjust the alcoholic content of 385 mL wine to 12.3% (v/v). For instance, if the wine was 11.5% (v/v) in ethanol, it was necessary to add 3.1 mL of ethanol ([12.3 – 11.5] × 385/100 = 3.1). A 50-µL aliquot of the internal standard solution was added, and more wine was added to ensure that the internal standard solution fully dissolved in the liquid. The rest of the wine contained in the graduated cylinder was added, and the solution was mixed with a magnetic stirrer until all the salt had dissolved. The mixture was left to stand for approximately 3 h to ensure phase separation. Two milliliters of the separated organic phase was pipetted and transferred to the screw-capped test tube described in Figure 1. A 0.15-mL aliquot of an ether–pentane (10:90) mixture was added, and the solution was diluted with 5 mL of the saline solution. The tube was shaken for approximately 1 h and centrifuged at 2500 rpm for 5 min. The organic layer was recovered with a syringe and injected directly into the GC–MS under the described conditions.

GC–olfactometry detection (sniffing analysis)

Volumes (250 mL) of oxidized and non-oxidized wines were continuously extracted for 48 h with two 125-mL Freon 11 fractions. The freon extracts were concentrated first under a 60-cm Vigreaux column, then in a microKuderna-Danish concentrator (water bath temperature, 28°C), until there was a final volume of aproximately 250 µL. A 1-µL aliquot of the concentrated extracts were analyzed in triplicate by GC–MS with simultaneous sniffing detection (two different judges) to detect those points of the chromatogram showing clear odor differences between the oxidized samples and the corresponding controls.

Isolation of the odorants

A 200-µL amount of the freon extracts coming from oxidized wines were washed first with 3 × 200 µL 5% (m/v) aqueous $NaHCO_3$ to remove fatty acids, then with 3 × 200 µL 1,2-propylglycol to remove fusel alcohols, and were further concentrated under a stream of N_2 to remove the freon completely. The essence was diluted with the mobile phase to a final volume of 75 µL. This extract was injected into the HPLC under the described conditions. Eighteen fractions were collected, taking the signal of the IR detector as reference. The fractions were carefully concentrated under N_2 to a final volume of 100 µL and analyzed by GC–MS with simultaneous sniffing detection to identify, using the cleaner chromatograms now obtained, the same odorants detected in the sniffing analysis.

Method optimization and analytical characterization

Injection. Mass transfer in the SPI injection was first checked by monitoring the signal produced by the injection of 1 µL of pentane–ether under different injector temperature programs

(from –30°C for 3 min to 40°C for 0.1 min). Second, 1 µL of a pentane–ether standard solution containing 5 ng/µL of the analytes was injected repeatedly under the same conditions. Peak areas were measured in order to get an estimation of analyte mass transfer efficiency and reproducibility and to check for possible non-linear behaviors.

Detection. The masses between 40 and 220 amu of the targeted analytes which provided the maximum signal-to-noise ratios were chosen (Table I). Detection reproducibility and the effect of interferences were checked by repetitive injection (n = 6) of three sets of solutions: A, clean standard solutions (0.5 ng/µL), and B and C, two spiked non-oxidized wine extracts (0.5 ng/µL). These spiked wine extracts were similar to those used in the sniffing analysis but were concentrated to a final volume of 250 µL (1000-fold concentration, solution B) or 800 µL (300-fold concentration, solution C). Linearity was studied on solution C containing variable amounts of analytes and a fixed amount of interferences. At least six concentration points were tested, ranging from 0.1 to 5 ng/µL. Detection limits were estimated as concentrations of analyte with a signal-to-noise ratio of 3.

Preconcentration by salting-out. In a preliminary experiment, increasing amounts of $(H_2NaPO_4)H_2O$–$(NH_4)_2SO_4$ salt (1:4.11, w/w) were added to a set of synthetic wines (adjusted to 11–13% alcohol) until demixture of the organic layer was achieved, and the organic layer volume was measured. Final readjustments were made on real wine (12.3% alcohol, v/v), which was the maximum alcoholic content that still yielded consistent recoveries of a small volume of organic phase (phase ratio, approximately 200).

Liquid–liquid microextraction. Five 380-mL volumes of spiked wine (10 µg/L) were demixtured by salting out as described in the proposed method. The organic phases were collected and combined to be used in a study of the liquid–liquid microextraction. Then, 2-mL volumes of the combined extract (approximately 56.5% in ethanol, v/v) were diluted with 10, 8, 6, 4, and 2 mL of saline solution and extracted with 0.15 mL ether–pentane. The extracts were injected in duplicate under the conditions of the proposed method to study the effect of the matrix composition on the absolute noise and signal-to-noise ratios.

Precision. Three different wines were used in the precision experiment. Each of them was spiked at two different levels (1 and 10 µg/L) and analyzed in triplicate using the proposed method. A relative standard deviation (RSD) was calculated for each trio of similar samples, and a global RSD was then calculated by averaging the corresponding variances.

Method linearity. Synthetic wines containing different amounts of the analytes were prepared and analyzed using the proposed procedure. At least 6 concentration points below the maximum concentration were studied per analyte. These solutions were used to build the calibration graphs.

Method accuracy. The signal increment measured in the precision experiment was interpolated in the calibration graphs built with synthetic solutions, and the concentration figure was divided by the actual added amount to get a value defined as efficiency. An analysis of variance (ANOVA) experiment was carried out, taking the different wines as factors to see if there were any significant differences in the efficiency values found in the different wines.

Results and Discussion

Analytical objectives

A preliminary step in the analytical method development process is to clearly decide which analytes should be determined and what detection limits are required in the analysis. In this case, the desired analytes were odorants that were generated during the oxidation process. In order to determine which analytes were generated, a series of GC-olfactometry experiments were carried out to compare the aromograms of both oxidized and non-oxidized wine samples. Those experiments, combined with the HPLC fractionation of the extract to get cleaner fractions, helped us to discover the presence of several odorants in the oxidized samples that could not be detected or that were present in smaller amounts in the non-oxidized control samples (7). Most of those odorants could be identified and are presented in Table I.

In regards to the detection limits, we considered that they should be well below (at least 10-fold) the olfactory detection threshold of the compound (if known) or the concentration level at which the compound can be found in an oxidized wine. Those data are given in Table I as well. There are 13 compounds in the table: 7 aldehydes, 2 ketones, 3 alcohols, and 1 volatile phenol. As data in Table I suggest, the method should be able to quantitate from less than 0.01 µg/L (t-2-nonenal) to nearly 15 mg/L (furfural). It should be noted that, in general, the more polar the compound, the less flavor active it becomes. This means that maximum sensitivity should be achieved only for unsaturated aldehydes, whereas more polar compounds, such as furfural or eugenol, will require more modest detection limits. This observation was very useful in designing the optimal sample preparation scheme.

GC–ion-trap-MS analysis

The next step in the optimization process was to select the mass fragments which provided the best quantitative performance. To determine this, we first studied the behavior of wine extracts spiked with the analytes on three different chromatographic phases. As expected, we found that the best selectivity was obtained in the carbowax-based phases. Typical chromatograms are given in Figure 2. Selective masses were then chosen, attending mainly to the optimization of the analyte signal/interference signal ratios and analyte signal/noise signal ratios. The masses chosen are presented in Table I. All the analytes required selective detection because it was impossible to get enough chromatographic resolution at the levels that were studied. In general, however, the use of MS–MS conditions did not significantly improve the signal characteristics (except in the case of furfural, where it was not the use of second-generation mass fragments but the isolation and storage of some of the first-generation mass fragments in the trap that allowed us to improve the signal-to-noise ratio nearly one order of magnitude).

The analytical behavior of the GC–MS analysis was studied with both real and synthetic non-oxidized wine flavor extracts. Wine extracts were obtained through a two-step continuous extraction that has proven to be highly efficient (18), and no selective enrichment in any compound was expected to have

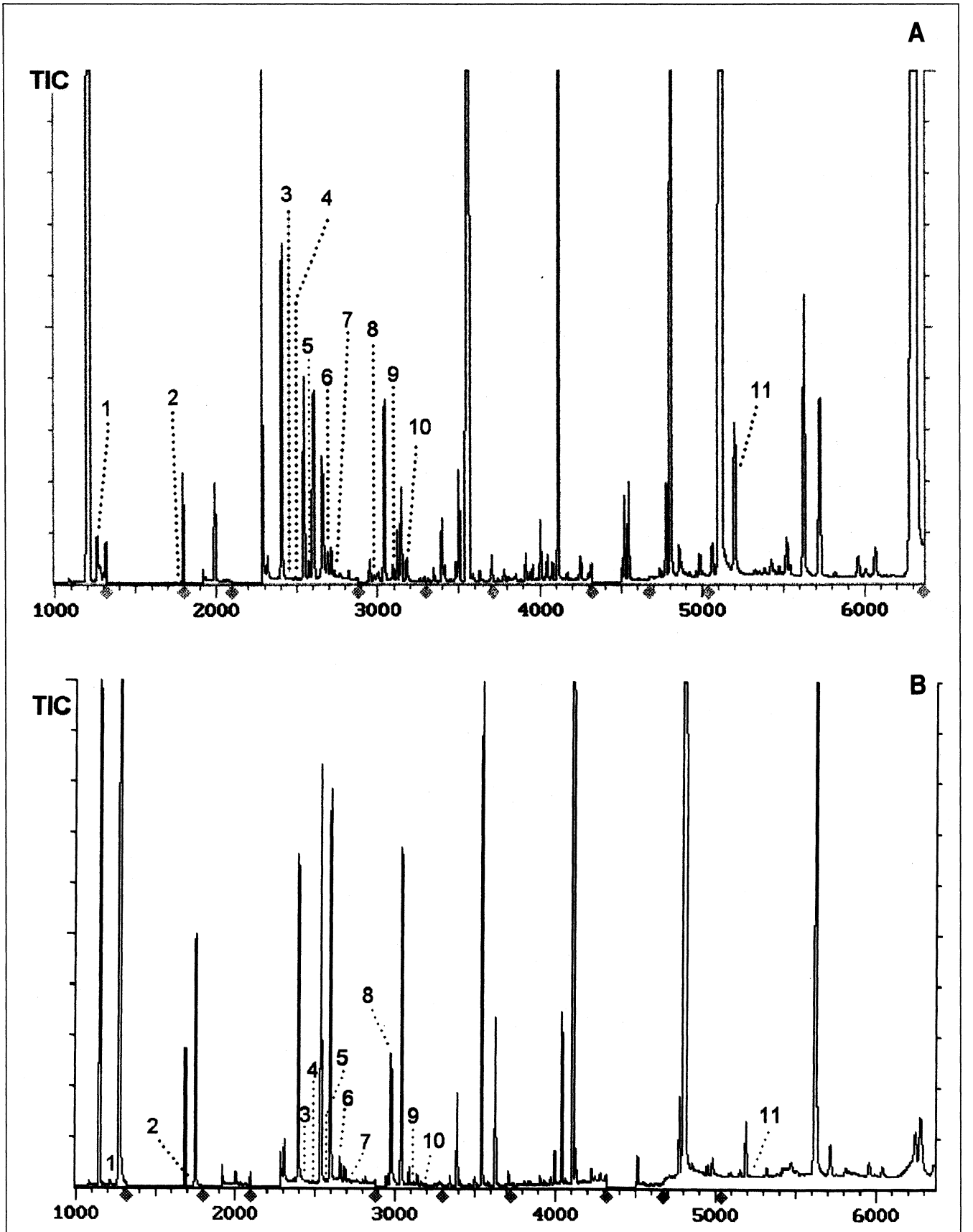

Figure 2. A, chromatogram (TIC) of an oxidized white wine fermented in an oak cask (Wine A); B, chromatogram (TIC) of an oxidized white wine fermented in stainless steel vats (Wine B). Peaks: 1, hexanal; 2, t-2-hexanal; 3, 2-nonanone; 4, 2-butoxyethanol; 5, t-2-octenal; 6, 1-octen-3-ol; 7, furfural; 8, benzaldehyde; 9, t-2-nonenal; 10, 5-methylfurfural; 11, eugenol.

taken place. In this manner, these extracts allowed us to determine the effect of the interference compounds on the signal of our analytes and to measure the degree of selectivity that the sample preparation steps should provide. In order to do that, a standard addition experiment was carried out with a fixed amount of analytes (about 0.5 mg/L) and various amounts of interferences (between 0- and 1000-times their concentration in wine). The results are in Table II. The first set of three columns are the results obtained when working with clean solutions with no interfering compounds. The second and third sets of columns correspond to the solutions that contained interferences concentrated about 300- and 1000-fold, respectively.

As shown in Table II, the level of interferences exerts a main effect on the limits of detection, and the more concentrated the interferences, the poorer the limits of detection. Fortunately, the response factors were only slightly affected, which means that the level of 1000-fold interfering compounds did not significantly alter the pattern of fragmentation of the analytes and, consequently, the GC–MS quantitation did not depend on matrix composition. There are, however, three components on which the interferences exerted a critical influence. These most critical components were t-2-hexenal, 1-octen-3-ol, and t-2-octenal, which coeluted with isoamyl alcohol, acetic acid, and ethyl octanoate, respectively. These interferences are major compounds that can be present at relatively high levels in wine extracts and, as demonstrated in Table II, the selectivity provided by the MS may not be enough to obtain a good signal in certain wines. This problem will be reconsidered later. Three other analytes (4-OH-4-methyl pentanone, 2-nonanone, and t-2-nonenal) were also influenced by the concentration level of the interferences, but only to a minor extent.

The reproducibility values shown in Table II are for a 0.5-mg/L level, and they are quite satisfactory. The average RSD is below 4% in the spiked samples and below 3% in the standard solutions. Of course, the worst results were obtained with the three critically affected compounds. It is worth mentioning that the high reproducibility obtained in the experiment, together with the stability of the response factors, allowed the use of a single internal standard during quantitation. Several components were tested, but the best results were obtained with 2-octanol. The linearity of the GC–MS analysis was also studied using a real extract (concentrated 300-fold in interference compounds). The linear regression coefficients of the calibration graphs are presented in Table III and are quite satisfactory as well. Regression coefficients better than 0.999 were obtained in almost all cases, with the single exception of t-2-octenal. These results show not only that the injection was linear in the working interval, but also that there were no significant matrix effects caused by the presence of variable amounts of analytes and coeluting peaks entering the ion trap. Detection limits of the GC–MS analysis ranged from 2 to 47 µg/L (clean solutions), which demonstrates that the trap surpasses FID sensitivity for nearly two orders of magnitude. The worst results were obtained with those compounds whose electronic-impact mass spectra are less selective (hexanal, t-2-hexenal, 2-nonanone, 2-buthoxyethanol, t-2-octenal, and 1-octen-3-ol) but were still far better than the results obtained with an FID.

Sample preparation

The study revealed that the sample preconcentration step would have to provide concentration factors ranging from 2 (furfural) to more than 1000 (t-2-nonenal), with an average value near 100, as shown in Table III. In addition, because the selectivity of the GC–MS system did not suffice in six cases, it would be convenient if the sample preparation strategy provided additional selectivity. On the other hand, it would also be

Table II. Analytical Characteristics of the GC–MS Quantitation: Detection Limits, Sensitivity, and Repeatability. Influence of the Level of Interfering Compounds

	Standard solutions			Wine matrix 300*			Wine matrix 1000†		
Odorant	RSD‡ (%)	S§	LOD** (µg/L)	RSD (%)	S	LOD (µg/L)	RSD (%)	S	LOD (µg/L)
Hexanal	3.3	2.90	47	5.2	2.96	70	5.0	3.03	78
t-2-Hexenal	2.1	12.3	23	4.9	11.7	58	> 20		
4-OH-4-Methyl-2-pentanone	3.1	24.2	13	3.4	24.3	11	5.2	22.9	31
2-Nonanone	0.9	12.0	26	4.8	12.3	32	6.3	12.8	78
2-Butoxyethanol	2.5	11.3	27	2.7	10.9	31	3.9	11.5	33
t-2-Octenal	4.6	7.29	12	5.1	6.88	39	> 20		
1-Octen-3-ol	4.3	6.16	18	5.0	6.20	47	> 20		
Furfural	3.5	2.93	5	4.1	3.05	8	3.8	3.02	8
Benzaldehyde	2.8	12.8	10	3.9	12.1	11	2.7	12.7	13
t-2-Nonenal	2.8	10.4	14	3.2	10.4	14	5.1	10.9	38
5-Methylfurfural	2.9	3.61	2	3.5	3.50	3	3.8	3.52	7
Furfurol	3.1	17.7	22	4.0	17.2	27	5.8	18.0	52
Eugenol	2.2	1.12	2	2.7	1.14	4	3.1	1.13	6

* This set of data was obtained from the analysis of Freon 11 wine extracts concentrated 300-fold (0.8–250 mL) and spiked with 0.5 ng/mL of analytes.
† Obtained from the analysis of Freon 11 wine extracts concentrated 1000-fold (0.25–250 mL) and spiked with 0.5 ng/mL of analytes.
‡ Relative Standard Deviation (n = 6).
§ Sensitivity, given as units of normalized relative area per ng of analyte.
** Detection limit, defined as the concentration of analyte giving a signal-to-noise ratio of 3.

better to avoid the use of large sample evaporation steps, since some of the analytes were fairly volatile and it was known that they can affect method reproducibility and accuracy (19). The strategies that best satisfied all the requirements were those based on microextractions; in this case, liquid–liquid microextraction was chosen because it was not possible to perform solid microextraction directly in wine. However, it should be pointed out that the concentration factor achieved with a liquid–liquid system should not be greater than 100, otherwise the recovery of the liquid phase turns out to be very difficult and imprecise. This means that it is necesary to couple two microextractions to get the concentration factors shown in Table III. In this case, we used two liquid–liquid partition processes.

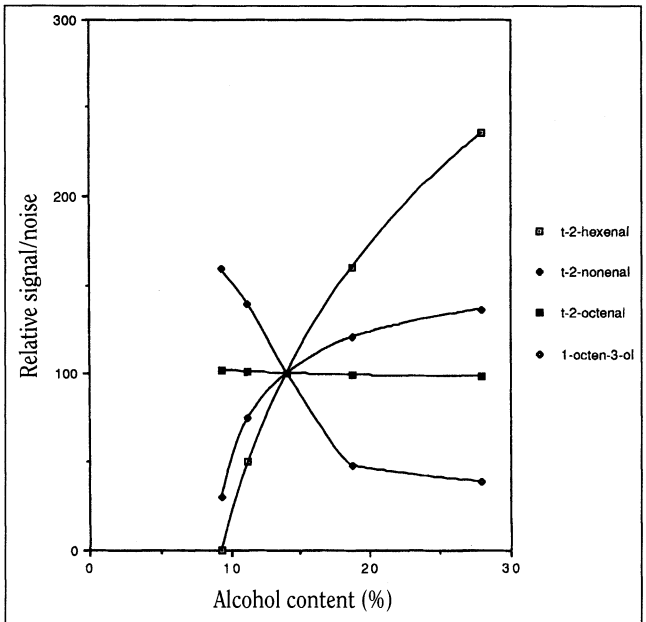

Figure 3. Influence of the alcohol content of the hydroalcoholic phase in the signal-to-noise ratio of some of the analytes.

The first step consisted of the addition of a large amount of salt to a 12.3% alcohol wine (v/v, previously adjusted) to provoke the separation of an alcoholic fraction containing most of the aroma compounds and other less polar wine components (20,21). In this step, the recovery of volatile components was almost total (data not shown), but the remaining alcoholic fraction was dirty and contained a high amount of non-volatile materials (mostly polyphenols). Following the proposed procedure (which was developed after many trials and which has been successfully applied to the analysis of a large set of wines) it was possible to achieve a concentration factor near 200. In the second step, the alcoholic phase was rediluted with an aqueous saline solution and further extracted with a small volume of a pentane–ether mixture. In previous studies, Freon 113 was used as the solvent because of its safety (it is odorless, neither toxic nor irritant, and non-flammable), its low solubility in ethanol, and its good overall behavior (20,22). However, it is becoming almost impossible to acquire because of the legal restrictions on its storage and commerce. This solvent was replaced by a pentane–ether mixture (90:10) whose behavior and selectivity was quite similar.

The preconcentration by demixture played a dual role in the method. First, it allowed a low-selective preconcentration of the wine, and second, but not less important, it had a leveling effect on the matrix composition. In fact, a good number of compounds that affect the wine's tendency to form emulsions (such as certain proteins, polysaccharides, glycerine, etc.) were retained in the aqueous layer, and the second step of the sample treatment was carried out with higher reproducibility and cleanliness. The combination of these two steps had an additional advantage: it was possible to control the dilution of the organic layer by demixture, and it was thereby possible to get the additional selectivity we were trying to

Table III: Linearity of the GC–MS System and Requisites of the Sample Preparation Strategy

Odorant	Linear range (mg/L)	r^2 (n = 6)*	Desired M.Q.L. (µg/L)†	Required concentration factor‡	Additional selectivity required§
Hexanal	up to 4.5	0.9994	2	120	None
t-2-Hexenal	up to 4.9	0.9996	4	45	High
4-Hidroxy-4-methyl-2-pentanone	up to 5.0	0.9993	1	45	Medium
2-Nonanone	up to 4.0	0.9993	10	40	Medium
2-Butoxyethanol	up to 4.8	0.9995	1	100	None
t-2-Octenal	up to 4.5	0.9988	0.3	300	High
1-Octen-3-ol	up to 7.8	0.9997	1	150	High
Furfural	up to 6.2	0.9999	15	2	None
Benzaldehyde	up to 9.3	0.9993	2	20	None
t-2-Nonenal	up to 5.5	0.9998	0.01	>1000	High
5-Methylfurfural	up to 6.0	0.9996	1	21	None
Furfurol	up to 8.2	0.9998	10	150	None
Eugenol	up to 5.3	0.9999	1	20	None

* Correlation coefficient.
† Minimum Quantitative Level refers to the minimum concentration that should be quantified. Determined from data in Table I and the quantitation limits of the GC–MS system (detection limit × 3).
‡ Concentration Factor refers to the concentration that must be achieved in the sample preparation scheme (e.g., hexanal must be concentrated 120-fold).
§ Refers to the degree of separation that should be achieved in the sample preparation scheme between the analyte and its interferences (coeluting compounds).

achieve. The effects that the degree of alcohol in the diluted organic phase had on the relative signal-to-noise ratio are presented in Figure 3. The signal-to-noise ratio found in the extraction of a 14% alcohol (v/v) solution (from the dilution of 2 mL of ethanolic phase with 6 mL of saline solution) was used as reference.

The alcohol level exerted a critical influence. In general, the lower the alcoholic degree, the higher the extraction efficiency and the lower the extraction selectivity. Consequently, the signal-to-noise ratio decreased in some cases. The most marked effects are those observed in t-2-hexenal and 1-octen-3-ol. In the first case, the signal-to-noise ratio became zero at low alcoholic contents because of the high extraction efficiency of isoamyl alcohol, which is the main interference of t-2-hexenal. On the other hand, 1-octen-3-ol is itself an alcohol, and it was extracted more easily from low alcoholic solutions. Figure 3 shows that a reasonable compromise can be obtained at alcoholic degrees near 15% (v/v), which was selected as optimum.

Analytical characteristics of the proposed method

The analytical precision of the overall method was studied in three different wines spiked at two levels of concentration (1 and 10 µg/L). The results of these sets of experiments, given as an average percentual RSD, are presented in Table IV. At the low level of addition, values ranged from 5 to 9%, while the worst results were obtained for t-2-octenal (too near the limit of detection) and furfurol (below its limit of detection). At the higher level, the values ranged from 3 to 5%, which was considered to be quite satisfactory. Linearity data were also satisfactory in most cases and are presented in Table IV. Linearity was maintained for at least two orders of magnitude, and we did not detect non-linear behavior in any case.

The increment of signal produced by the addition of known amounts of analytes to the three different wines was compared to that obtained in the analysis of synthetic ones. Results are given in Table IV. Efficiency was the average percentual quotient between the amount of analyte added (as determined by interpolation of the increments of relative area between spiked and non-spiked samples in the calibration graphs built with synthetic solutions) and the actual amount added. The confidence interval attached to the efficiency value corresponds to the standard deviation of the efficiency figures found in the different wines. An analysis of variance was applied to check if those values were caused by the existence of matrix effects, but the ANOVA showed that those differences can be explained by experimental uncertainty. In addition, the average efficiency values did not significantly differ from 100% in any case, which means that the calibration graph can be built with synthetic solutions made with hydroalcoholic media, and only one internal standard is needed to get accurate results.

Finally, detection limits are provided in Table IV. They range from 10 ng/L to 600 ng/L (except for furfurol). Moreover, these limits fulfill the method requirements for the control of the oxidation of the wine. The only exception is that of t-2-octenal, which cannot be quantified at its lowest amounts in wines that contain relatively high amounts of ethyl octanoate.

Conclusion

The proposed method uses a relatively fast and inexpensive operation to quantitate several wine flavor compounds responsible for the stale flavor of some oxidized wines. The success of the method depends on the sensitivity and selectivity provided by the ion-trap mass-selective detection and the analytical simplicity of the sample treatment.

Table IV: Analytical Characteristics of the Overall Method

Odorant	1-µg/L spike RSD* (%)	10-µg/L spike RSD (%)	Linear range (µg/L)	r^2 (n = 6)†	LOD‡ (µg/L)	Efficiency§ (%)	Wine A** (ng/L)	Wine B (ng/L)
Hexanal	7.6	4.8	up to 47	0.9995	0.3	102 ± 8	4731	1915
t-2-Hexenal	9.0	5.3	56	0.9990	0.3	102 ± 11	< LOD	< LOD
4-Hidroxy-4-methyl-2-pentanone	6.3	3.2	48	0.9979	0.3	97 ± 8	< LOD	< LOD
2-Nonanone	6.9	4.4	27	0.9995	0.01	95 ± 7	28	20
2-Butoxyethanol	3.8	4.1	60	0.9970	0.4	100 ± 8	2046	400
t-2-Octenal	11.7	5.0	52	0.9966	0.6	106 ± 15	3989	< LOD
1-Octen-3-ol	5.0	2.7	44	0.9990	0.02	100 ± 11	4101	3583
Furfural	5.5	4.9	250	0.9984	0.5	98 ± 12	81875	1598
Benzaldehyde	4.9	2.4	49	0.9992	0.02	96 ± 8	3083	45056
t-2-Nonenal	5.7	3.1	45	0.9995	0.01	97 ± 9	843	75
5-Methylfurfural	4.7	3.9	58	0.9995	0.3	98 ± 8	200475	3792
Furfurol		4.7	65		1.3	90 ± 11	< LOD	< LOD
Eugenol	3.3	3.1	39	1.000	0.02	99 ± 6	9969	245

* The average percentual RSD of three different wines spiked at two levels of concentrations (1 and 10 µg/L).
† Correlation coefficient.
‡ Limit of detection.
§ Efficiency is the quotient between the determined amount of analyte added in a standard addition experiment (through the interpolation in a calibration graph of the increment of signal) and the real amount added. An efficiency quotient of 100 means that there are not differences in recovery between the synthetic solutions and the wines.
** Compositional data of the two wines whose chromatograms are shown in Figure 2. Wine A was made from Macabeo grapes in Aragón (Spain); wine B was made from Airen grapes in La Mancha (Spain).

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