Quantitative determination of five hydroxy acids, precursors of relevant wine aroma compounds in wine and other alcoholic beverages

Elisa Gracia-Moreno [1]

Ricardo Lopez [1,*]

Email riclopez@unizar.es

Vicente Ferreira [1]

[1] Laboratorio de Análisis del Aroma y Enología, Departamento de Química Analítica, Facultad de Ciencias, Aragon Institute of Engineering Research (I3A), Universidad de Zaragoza, 50009 Zaragoza, Spain

Abstract

A method for the quantitative determination of 2-hydroxy-2-methylbutanoic (2OH2MB), 2-hydroxy-3-methylbutanoic (2OH3MB), 3-hydroxy-3-methylbutanoic (3OH3MB), 2-hydroxy-4-methylpentanoic (2OH4MP) and 3-hydroxybutanoic (3OHB) acids has been optimized, validated and applied to a set of wines and other alcoholic beverages. The analytes were preconcentrated in a solid phase extraction cartridge and derivatized with 2,3,4,5,6-pentafluorobenzyl bromide at room temperature for 30 min, followed by GC-MS analysis. Detection limits were between 0.5 and 29 μg L^{-1}, and linearity was maintained up to 3 or 12 mg L^{-1}, depending on the analyte. Recovery values were between 85 and 106 %, and reproducibility was better than 12 % RSD in most cases. The first specific study of these analytes in wine and other alcoholic beverages is herein reported. Concentrations ranged from the method detection limits to 7820, 519, 8510, 3470 and 2500 μg L^{-1} for 2OH2MB, 2OH3MB, 3OH3MB, 2OH4MP and 3OHB, respectively, which may have relevant

sensory effects. These products were not found in distillates (except 3OHB) but were all present in beer. 2OH2MB, 3OH3MB and 3OHB were found in unfermented grape derivatives. Sherry wines had the highest levels of all except for 3OHB.

Keywords

Hydroxy acid
Wine
Solid phase extraction
Gas chromatography-mass spectrometry
Cheese aroma
Derivatization

Electronic supplementary material

The online version of this article (doi: 10.1007/s00216-015-8959-9) contains supplementary material, which is available to authorized users.

Introduction

While short chain fatty acids display odours described as cheese, sweat, rancid or acid [1–3], their ethyl esters have pleasant fruity aromas [1, 3–5]. As both families of compounds are interrelated due to the esterification/hydrolysis equilibrium [6, 7], relevant aroma changes can take place during ageing if they are initially present at concentration ratios different to that of the corresponding esterification equilibrium [8]. This is certainly the case of ethyl 2-, 3- and 4-methylpentanoate and ethyl cyclohexanecarboxylate which were described by Campo et al. as responsible for powerful strawberry aromas in aged wines and spirits [9, 10]. These compounds are formed by slow esterification with ethanol of the corresponding acids [10] which are naturally present in different wines and alcoholic beverages [11]. This is most surely also the case of other fruity ethyl esters present in wine, such as ethyl 2-hydroxy-4-methylpentanoate and ethyl 2-hydroxy-3-methylbutanoate which were recently identified as powerful strawberry-smelling aroma compounds [10]. The former has been associated with “fresh blackberry aroma” in red wines [12] with reported

concentrations ranging from 48 μg L^{-1} in blackberry liqueur to 672 μg L^{-1} in one of the aged red wines. While these concentrations are close to but not higher than the perception threshold, the omission of ethyl 2-hydroxy-4-methylpentanoate has been detected in many of the omission tests, suggesting an important role in overall wine flavour. Furthermore, an additive effect with ethyl butanoate has been suggested because the omission was more clearly perceived when this included the two esters [12]. The same authors subsequently studied the enantiomeric composition of ethyl 2-hydroxy-4-methylpentanoate in a set of 42 red wines and 13 white wines finding mainly the R-enantiomer, with increasing levels of the S-enantiomer in older wines [13]. The sensory study demonstrated a synergic effect of both enantiomers to enhance fruity aromas in wine, in particular “blackberry” and “fresh fruit notes” [13, 14].

Ethyl 3-hydroxybutanoate was described in 2009 by Pineau et al. as one of the esters responsible for “redberry” and “blackberry” aromas in red wines. The fact that its concentration is most often under its individual olfactory threshold implies an indirect impact [5], further described as hyper-additive because its addition decreased the threshold of the total aromatic reconstitution by 2.09-fold, although it did not affect the aroma perceived [15]. Other compounds of the same family, such as ethyl 2-hydroxy-2-methylbutanoate, have been identified in Tokaji wines [16] and pineapple [17], while ethyl 3-hydroxy-3-methylbutanoate has been found in cashew apple juice [18] and other fruits [19–21].

Some of the corresponding hydroxy acids such as 2OH2MB, 2OH3MB and 2OH4MP have already been identified in wine or wine residues [22–26], although only 2OH4MP has been quantified [24]. These hydroxy acids are also present in other biological matrixes such as honey [27, 28]. Furthermore, 2OH4MP has been found in roasted cocoa [29], fermented soybean [30] and cyanobacteria [31]. 3OHBc is present in grape leaf tissue [32] and has been proposed as a biomarker of grape fungal resistance by Batovska et al. [33]. To the best of our knowledge, no specific studies have so far been carried out to quantify the hydroxy acid potential precursors of any of these important fruity esters in wine or any other product.

The high polarity and low volatility of hydroxy acids make headspace

techniques difficult to apply. In fact, an SPME method with a DVB/CAR/PDMS fiber found 2OH4MP below the method quantitation limit [25]. Solid-phase extraction (SPE) on polar sorbents is a good option to isolate polar analytes [34], although polar interferences will also be retained. Anionic mixed-mode sorbents would be interesting to take advantage of the acid properties of the analytes, although this kind of sorbent provided low breakthrough volumes in previous work [35]. Pure anionic sorbents were used in the past to isolate acidic compounds present in wine but only as an additional purification step after using a polyvinylpolypyrrolidone column [24].

The polarity of hydroxy acids also determines chromatographic behaviour. While, in general, acids are poorly soluble in apolar columns, the presence of an additional hydroxyl group will provide strong interactions in polar phases, potentially causing tailing effects. Because of this, derivatization is mostly preferred for hydroxy acid analysis [23, 24, 27, 28, 31]. In general, GC-MS has been used to analyse these molecules [23, 24, 27, 28, 31], although GCxGC/TOF-MS has also been used in the analysis of cigarette smoke samples [36].

Among the many derivatizing reagents, 2,3,4,5,6-pentafluorbenzylbromide (PFBBr) can help not just lowering the polarity of the compounds but also increasing their detectability by using the most sensitive and less fragmentation-prone negative chemical ionization (NCI) detection mode [35]. For of all these reasons, a method has been selected combining a fast non-specific SPE with apolar sorbents, chemical derivatization and further GC-NCI-MS analysis in order to provide the first data about the occurrence of the aforementioned hydroxy acids in wine and other alcoholic beverages.

Materials and methods

Reagents and standards

The standards of 2-hydroxy-2-methylbutanoic acid (2OH2MB), 2-hydroxy-3-methylbutanoic acid (2OH3MB), 2-hydroxy-4-methylpentanoic acid (2OH4MP), 3-hydroxybutanoic acid (3OHMB), 2-hydroxy-2-methylpropanoic acid (2OH2MPr) and 2-hydroxy-3,3-dimethylbutanoic acid (2OH33diMB) were purchased from Sigma-Aldrich and 3-hydroxy-3-

methylbutanoic acid (3OH3MB) from Alfa Aesar, with purity higher than 95 % in all cases. 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) and tetrabutylamonium chloride (NBu_4Cl) (>97 %) were also purchased from Aldrich.

The solvents used were: Unisolv quality hexane (Hx), Lichrosolv quality ethanol, Suprasolv quality methanol (MeOH) and dichloromethane (DCM) and diethyl~~ic~~ ether, all from Merck. Triethylamine (TEA) 95 % was from Aldrich. A milli-Q purification system (Millipore, Bedford, MA, USA) provided pure water.

Sorbents used were: LiChrolut EN resins pre-packed (200 mg, 3 mL reservoirs) and packed in house (200 mg in 1 mL reservoir) from Merck. SPE was performed with the help of a Vac Elut 20 system from Varian. Silica-gel 60 from Merck was used for the purification process.

Standard solutions of the hydroxy acids were prepared directly in ethanol because of their insolubility in apolar solvents.

Wines and alcoholic beverage samples

A variety of wines (white, rosé, young red, barrel aged red, natural sweet and Sherry) as well as other alcoholic beverages (beer, brandy and whisky) have been analysed. The objective was to test the performance of the method presented in this paper and to have an initial idea of the occurrence of the five hydroxy acids studied in wine and other alcoholic beverages. The whole list of samples analysed is presented in Table S1 of the Electronic Supplementary Material (ESM) with information of their type, brand, origin and grape variety.

SPE method optimization

The influence of the pH was studied, comparing two aliquots of the same wine. A red wine aged in wooden barrels for 18 months was spiked with 200 µg L^{-1} of each of the analytes. Two aliquots of 10 mL each of the spiked wine were analysed. One aliquot was at the natural pH of the wine (3.5) and the other at pH 1.5 (adjusted with HCl, 37 %). These aliquots were loaded into 200 mg LiChrolut EN cartridges (3 mL reservoir). After drying, elution

was done with 1 mL DCM, followed by derivatization and purification through silica gel as explained in the final method.

After that, the influence of several variables on the retention of the analytes was tested, as can be seen in Table 1. A red wine aged in wooden barrels for 18 months spiked with 2 mg L^{-1} of each of the analytes was used for all the subsequent experiments described in this section. A reference sample was prepared loading 10 mL of the spiked wine into a pre-packed 200 mg LiChrolut EN bed (3 mL cartridge), washing with 3 mL of acidified milli-Q water, vacuum-drying and eluting with 1 mL DCM. The other samples were prepared with 10 mL aliquots and changing just one variable at a time. Sorbent bed length: One aliquot was loaded into a 1-mL cartridge containing 200 mg LiChrolut EN (packed in house). Effect of ethanol content: An aliquot was diluted with milli-Q water (1:1, *v*/*v*) prior to loading. Acid washing to clean the extract: An aliquot was processed as the reference but without the washing step. Elution optimization: Two aliquots of the spiked wine were eluted with DCM (reference sample) and DCM/2 % TEA respectively with fractions of 0.5 mL volume.

Table 1

Optimization of the SPE process for hydroxy acid extraction

Experiment	Ref.	Longer bed length	Diluted wine	Without washing	TEA (2 %) elution
Sorbent bed (mg)	200	200	200	200	200
Cartridge volume (mL)	3	1	3	3	3
Wine (mL)	10	10	10:10	10	10
Acid washing step (mL)	3	3	3	–	3
Elution (1 mL)	DCM	DCM	DCM	DCM	TEA (2 %)/ DCM[a]

[a] Four fractions (0.5 mL each) were used

The recovery of analyte in the SPE process was evaluated after its

optimization comparing two extracts. Two aliquots of 10 mL each of a red wine were used. One of the aliquots was spiked with 75 μL of a solution containing 450 mg L^{-1} of the analytes and 25 μL of a solution containing 1200 mg L^{-1} of the internal standards (2OH2MPr acid and 2OH33diMB acid), both in ethanol, prior to the analysis with the final method, while the other aliquot was processed without being spiked. The extract of the unspiked sample was spiked with the same amount of the analytes and internal standards. To avoid any difference produced by the ethanol added to the spiked extract, the same amount of ethanol (100 μL) was added to the other extract. The same conditions were used to derivatize and analyse the two extracts.

Derivatization reaction

The derivatization procedure used in this study is based on a procedure optimized for branched acids in a previous work [35]. That is, to say, 20 μL of pure 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) and 500 μL of a solution of tetrabutylamonium chloride 0.1 M at pH 6 were added to 500 μL of DCM containing the analytes. After 30 min of stirring at room temperature, the reaction is stopped with HCl (37 %). The extract is purified through a 200-mg silica gel bed and, after a washing step with 1.5 mL of hexane, is eluted with 1 mL of 20 % of diethyl ether in hexane. Firstly, the aforementioned derivatization and further purification were applied to dichloromethane solutions (50 mg/L) of the standards to characterize the derivatized compounds. They were injected in a GC-MS with a DB-WAX column, and their linear retention indexes were determined (Table 2). Their spectra, both in EI and in NCI mode, are shown in the ESM of this paper.

Table 2

Linear retention indices for the hydroxy acids studied and their corresponding PFB esters in a DB-WAX column

Compound	CAS number	pKa	log P	Mw		LRI (DB-WAX)	
				Acid	PFB-ester	Acid	PFB-ester
2OH2MPr[a]	594-61-6	4.01	−0.44	104	284	2047	1878

2OH2MB	3739-30-8	4.05	0.07	118	298	2093	1920
3OH3MB	625-08-1	4.38	−0.35	118	298	2090	2027
2OH33diMB[b]	4026-20-4	3.91	0.42	132	312	2278	2033
2OH3MB	4026-18-0	3.87	0.01	118	298	2259	2034
2OH4MP	498-36-2	3.86	0.52	132	312	2416	2082
3OHBc	300-85-6	4.36	−0.76	104	284	2226	2087
[a] Internal standard (IS-1)							
[b] Internal standard (IS-2)							

The purification after the reaction had to be optimized. For this purpose, a DCM solution of the standards was derivatized as already explained. Then, the purification through the silica was done with several fractions of hexane (1 mL each) containing increasing amounts of ether (20, 40 and 60 %).

Chromatographic conditions

A CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass-spectrometric detection system from Varian (Sunnyvale, CA, USA) was used during the development of the method. The capillary column used was a DB-WAX ETR (J&W Scientific, Folsom, CA, USA) (60 m, 0.25 mm, 0.25 μm) preceded by a 3 m × 0.25 mm uncoated (deactivated, intermediate polarity) pre-column from Supelco (Bellefonte, USA). Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature programme was 4 min at 40 °C, increased by 10 °C min^{-1} up to 150 °C, with a second ramp at 4 °C min^{-1} up to 190 °C and a third at 2 °C min^{-1} up to 220 °C and finally held at this temperature for 20 min. The MS-parameters were: MS transfer line 220 °C and ionization chamber temperature 170 °C. Two microliters of the extract were injected in splitless mode for 2 min with a pulse pressure of 30 psi.

The analysis of the extracts in the final method was done in an Agilent 7890A GC coupled to a 5975 MS in NCI mode. In this case, the column was a CP-WAX 52 CB (25 m, 0.15 mm, 0.25 μm) from Varian preceded by a 3 m × 0.25 mm uncoated (deactivated, intermediate polarity) pre-column from

Supelco (Bellefonte, USA). Two microliters of sample were automatically injected at 250 °C for 4.5 min of splitless time with helium at 45 cm s^{-1} as the carrier gas and a pulse pressure of 59 psi during the splitless time. The oven was programed as follows: 40 °C during 5 min, ramp of 10 °C min^{-1} up to 115 °C and a final ramp of 4 °C min^{-1} up to 230 °C. This temperature was held for 20 min. The spectrometer operated in negative chemical ionization (NCI) with methane as ionization gas (3 bars of pressure). The temperature of the ion source and quadrupole were set at 150 °C and transferline temperature was 150 °C. The acquisition of mass spectra was done after 10 min solvent delay, scanning from 40 to 300 amu.

Final method

For the extraction of the analytes, conditioning of 200 mg of LiChrolut sorbents (1 mL cartridge, in house packed) is done with 4 mL of DCM, 4 mL of MeOH and 4 mL of hydroalcoholic solution (12 % ethanol). Highly alcoholic beverages, such as whisky and brandy, are diluted prior to the analysis to 12 % content of ethanol. A volume of 10 ml of wine spiked with 2OH3MPr (IS-1) and 2OH33diMB (IS-2) to obtain a concentration of 3.4 and 2.7 mg L^{-1}, respectively, is loaded into the sorbents at the natural pH of wine with the help of a vacuum device. Then, the sorbents are vacuum-dried and eluted with 1 mL of DCM with 2 % of TEA. The extracts are recovered in 2 mL glass vials.

The derivatization procedure used is the same as that described in [35], whereas the purification requires the use of more polar solvents. The addition of 20 µL pure PFBBr and 500 µL NBu_4Cl 0.1 M in buffered solution (pH 6.0) to each of the extracts is followed by 30 min of stirring at room temperature. The reaction ends by adding HCl (37 %). After discarding the aqueous phase, the organic phase is washed with acidified milli-Q water and dried with Na_2SO_4. The purification is done in cartridges (1 mL) containing 200 mg bed of silica-gel 60. After a washing step with 1.5 mL of hexane, the analytes are eluted with 1 mL of hexane/ 60 % diethyl ether (*v*/*v*).

Analysis: 2 µL of the extract is injected in splitless mode in a GC-MS and analysed in NCI mode as described in the previous section.

Method linearity and reproducibility

The external calibration method with internal standard was used to assess the linearity and to calculate the concentrations. The linearity was studied spiking several wines with known amounts of the standards solutions (prepared in pure ethanol) up to 10 mg L^{-1} (six points per linearity curve). The relative area obtained for the unspiked wine was subtracted to each of the points in the curve. The concentrations were calculated by the direct interpolation in the calibration curve of the peak area of each compound divided by the corresponding internal standard peak area. Calibration curves linearity was confirmed by evaluating residual distribution, and no weighing factors were necessary. The slopes of the calibration curves obtained from different types of wines and alcoholic beverages were compared with an F-test (95 % level of confidence) to detect matrix effects. The reproducibility and recovery of signal were measured analysing three replicates of two wines spiked with 3 mg L^{-1} of each of the analytes: a white (Albada, DO Calatayud) and a rosé (Homenaje, DO Navarra).

Results and discussion

Method optimization

The sorbent (200 mg LiChrolut EN) and the volume of wine (10 mL) were set based on previous experience [34, 37]. No significant improvement was found by lowering the pH, and therefore, the pH of the wine was not changed in further studies. By contrast, and as expected, the length of the SPE bed had a marked influence, as shown in Fig. S3 of the ESM. This occurs because the chromatographic retention factors for these polar compounds are very small, and hence, breakthrough volumes are easily reached so that the bed length becomes critical. A longer sorbent bed with the same amount of sorbent provided much higher retentions (50–110 % increment) for the four hydroxy acids. In consequence, it was decided to pack 200 mg of the sorbents in 1 mL cartridges.

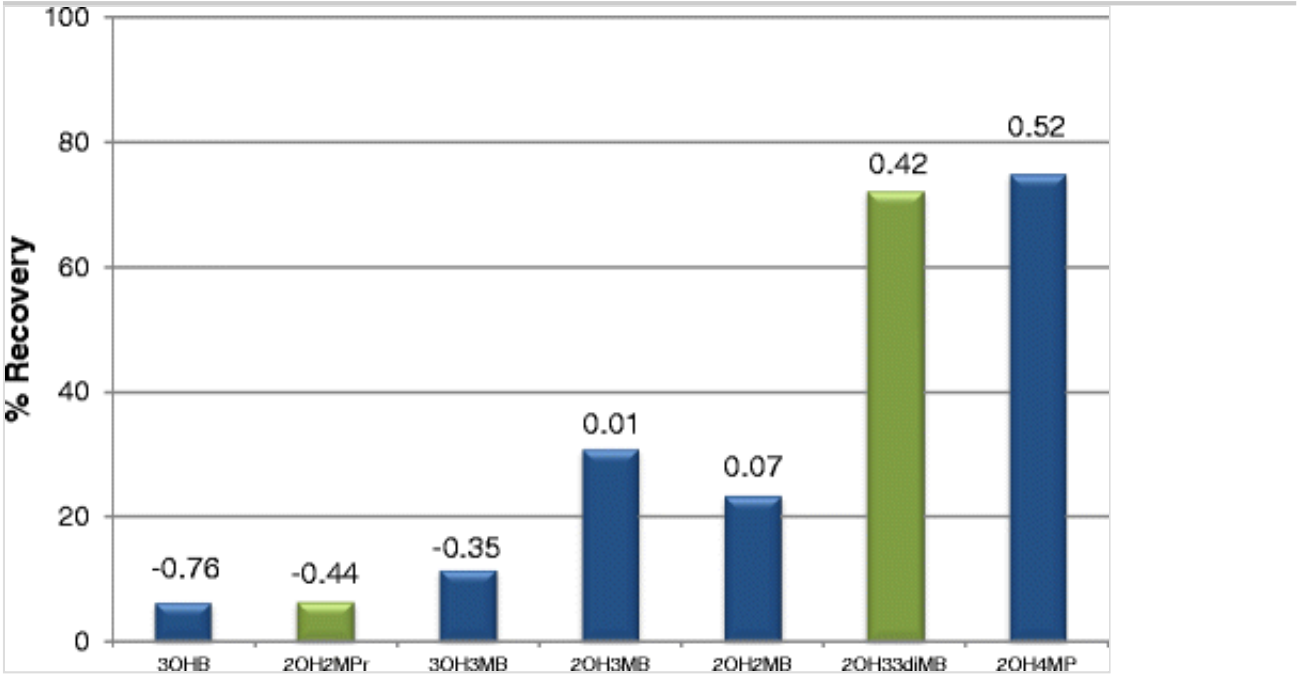
Sample dilution with water (1:1) improved retention of 2OH2MB, suggesting that this compound has the lowest breakthrough volume. Similarly, washing the cartridge with acid water reduced recoveries of all analytes, suggesting that even in acid water, breakthrough volumes are very small which does not

leave room for a cleanup. A 2-mL volume of DCM was required for the elution, but this volume could be reduced to 1 mL by adding 2 % triethylamine (TEA). Summarizing, the optimum conditions for the extraction process included the use of 200 mg of sorbent in a 1-mL volume cartridge without dilution of the wine or washing step and elution with 1 mL of DCM/ 2 % TEA.

Even under such conditions, recoveries were very low for some of the analytes, as shown in Fig. 1 and as expected, were inversely related to the polarity of the hydroxy acids. We tried to correct the potential bias and imprecision caused by these low recoveries by using two internal standards (IS) similar in polarity and retention to each one of the analytes displaying extreme behaviour, as shown in Fig. 1 . 2-hydroxy-2-methyl propanoic (2OH2MPr) is very close in properties and retention to the most polar 3OHB, while 2-hydroxy-3,3-dimethylbutanoic (2OH33diMB) is very similar to the least polar 2OH4MP.

Fig. 1

Recovery (%) of the hydroxy acids through the SPE process. The two IS are presented in a different colour. The *numbers above the bars* represent the polarity (logP values)



The polar nature of the analytes and their small size make convenient the use

of a derivatization process to improve their chromatographic and detectability properties. Derivatization with PFBBr is suitable for anionic compounds, which can undergo nucleofilic substitution [35, 38]. In particular, this reaction has been recently optimized for a group of branched chain fatty acids [35]. The hydroxy acids studied in the present work are very similar to those branched acids; in consequence, a similar behaviour towards PFBBr can be expected. Thus, the derivatization reaction conditions developed for branched acids in [35] were tested and found to be appropriate for hydroxy acids. However, the silica cleanup step had to be modified by using diethyl ether instead of toluene to account for the higher polarity of these analytes because toluene' strength was not enough to remove the hydroxy acids from the silica-gel. The less polar PFB-hydroxy esters start eluting with just 20 % of diethyl ether in hexane, whereas the most polar one (PFB-3OHBc) needs 40 % diethyl ether to start eluting and 60 % to complete its full elution. Therefore, 60 % of diethyl ether is used in the silica cleanup.

Derivatized analytes can be quantified in electron impact (EI) mode when their fragmentation is selective enough. The use of highly halogenated reagents together with negative chemical ionization (NCI) allows increasing the sensitivity of the method. The PFB esters obtained in this case allowed the use of NCI due to their high affinity for negative electrons and their capacity to stabilize a negative charge. EI and NCI mass spectra of the PFB-esters obtained are given in the ESM. Linear retention indices determined in a DB-WAX column are presented in Table 2. In EI mode, the fragment with highest abundance (*m*/*z* 181) corresponded to the derivatizing reagent. This fragment presented many interfering compounds, which could not be resolved by adjusting the chromatographic conditions. Hence, more specific and selective but less sensitive fragments were used to quantify with this ion source (Table 3). Unfortunately, 3OH3MB was present at such low levels in the extracts that the sensitivity provided by EI did not suffice since its mass spectrum lacks characteristic ions. 3OHB concentrations were higher, but again, the poor fragmentation provided by EI mode did not allow its detection. By contrast, in the case of NCI, fragmentation is limited to the cleavage of the ester bond and the main ion corresponds to the deprotonated original hydroxy acid (see ESM) because of its ability to stabilize the negative charge [35]. A typical chromatogram (Oloroso wine sample) obtained by both ionization modes can be seen in Figs. 2 (EI mode) and 3 (NCI mode). Both figures

show the ionic chromatograms of the PFB esters with the fragments used to quantify each of the analytes and the internal standards.

Table 3

Fragments, IS and ionization mode used to quantify the analytes and figures of merit of the analysis

Compound	*m/z*		IS	LOD (µg/L)	LOQ (µg/L)	Linear range (µg/L)	Recovery (%)[c] ± RSD (%)	
	EI	NCI					White	Rosé
PFB-2OH2MPr[a]	59	103	–	–	–	–	–	–
PFB-2OH2MB[d]	73	117	IS-1	29	97	97-9500	103 ± 12	91 ± 10
PFB-3OH3MB[e]	283	117	IS-1	0.5	1.7	1.7-3900	104 ± 3	89 ± 8
PFB-2OH33diMB[b]	236	131	–	–	–	–	–	–
PFB-2OH3MB[d]	73	117	IS-1	8.6	29	29-4700	103 ± 6	85 ± 17
PFB-2OH4MP[d]	69	131	IS-2	7.2	24	24-3600	106 ± 5	101 ± 3
PFB-3OHBc[e]	87	103	IS-1	3.6	12	12-12500	101 ± 4	102 ± 5

[a]Internal standard (IS-1)

[b]Internal standard (IS-2)

[c]The number of replicates for the recovery of signal study and the reproducibility was 3

[d]Figures of merit correspond to the EI detection method

[e] Figures of merit correspond to the NCI detection method

Fig. 2

SPE//GC-MS-EI chromatogram (DB-WAX ETR) of an Oloroso wine: 7090 mg L^{-1} 2OH2MB (*m/z* 73, *red*), 2410 mg L^{-1} 2OH3MB (*m/z* 73, *red*), 1180 mg L^{-1} 2OH4MP (*m/z* 69, *dark blue*). The peaks signaled by the *arrows*

correspond to the derivatized PFB-esters of the analytes and internal standards

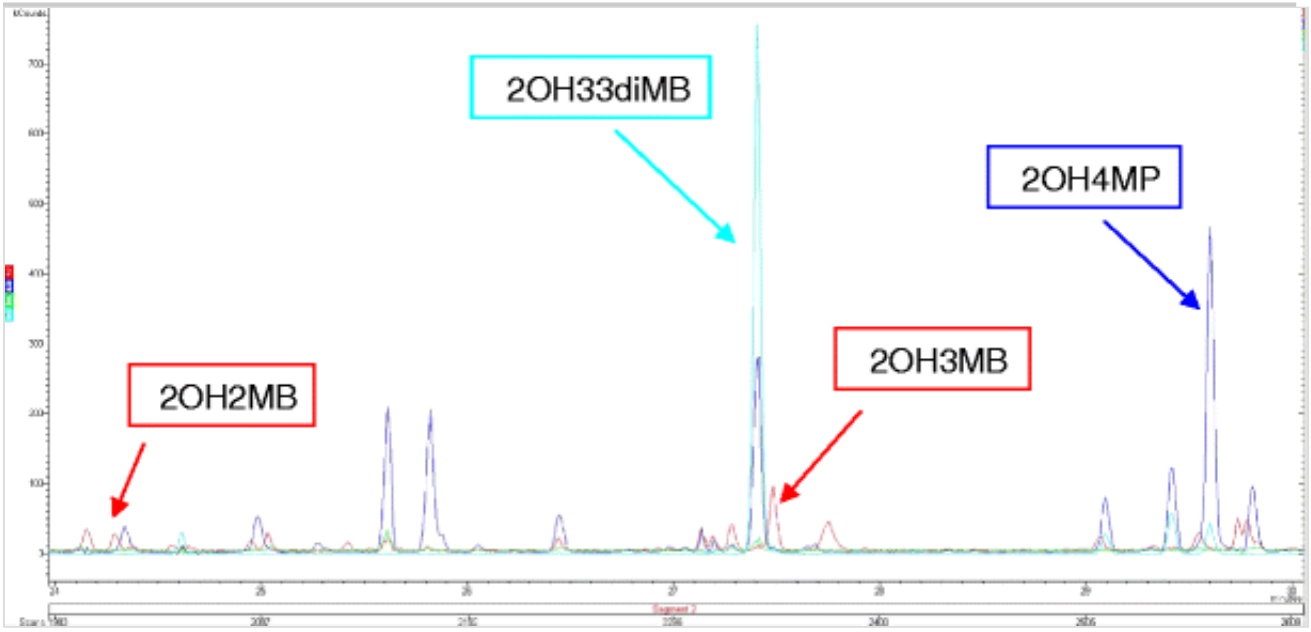
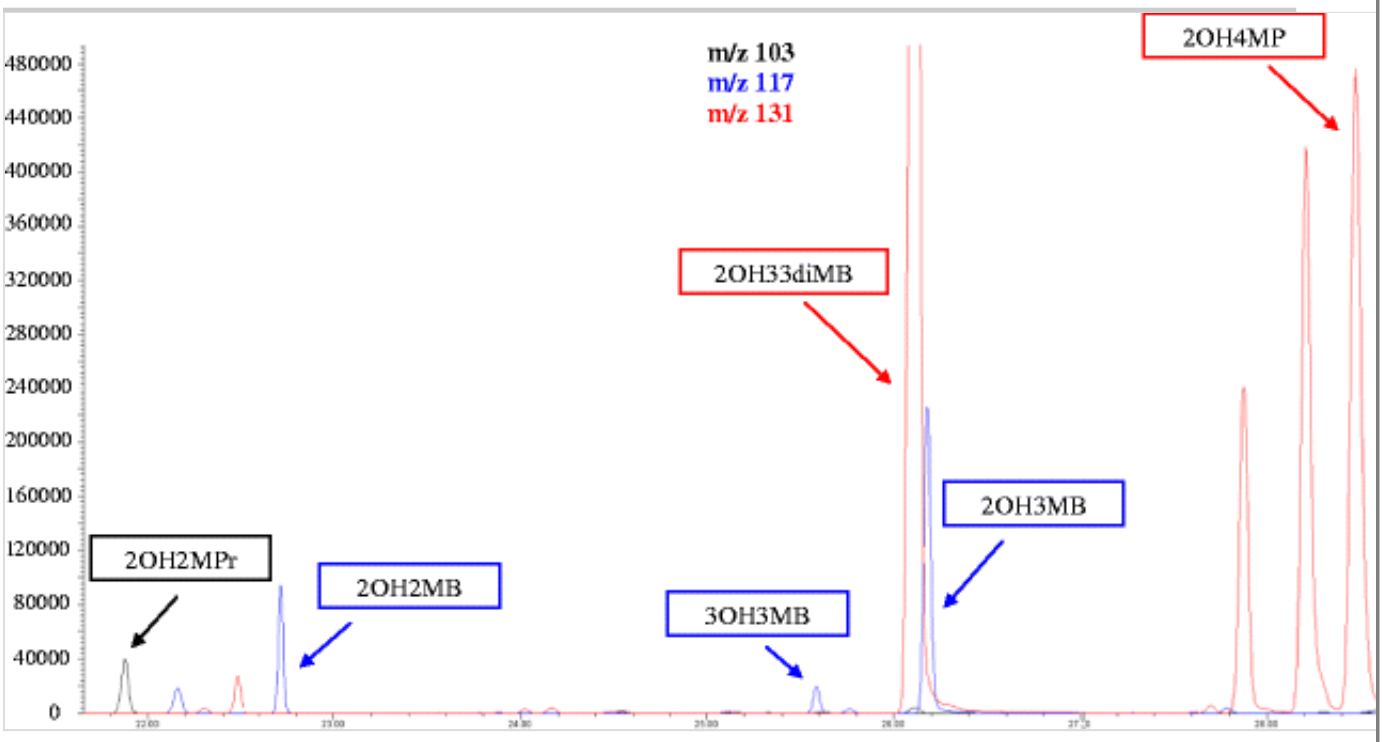


Fig. 3

SPE//GC-MS-NCI chromatogram (CP-WAX 52 CB) of an Oloroso wine: 7090 mg L^{-1} 2OH2MB (*m/z* 117, blue), 519 mg L^{-1} 3OH3MB (*m/z* 117, *blue*), 2410 mg L^{-1} 2OH3MB (*m/z* 117, *blue*), 1180 mg L^{-1} 2OH4MP (*m/z* 131, *red*), 863 mg L^{-1} 3OHB (*m/z* 103, *black*). The peaks signaled by the *arrows* correspond to the derivatized PFB-esters of the analytes and internal standards



For each of the studied analytes, the internal standard providing the most accurate results was chosen for calibration. In all cases, very good signal recoveries with reasonable RSD were obtained, as shown in Table 3, in spite of the low extraction recoveries of analyte mass obtained in the SPE process, as was mentioned previously. This is remarkable because it shows that the use of a good internal standard can counterweight low recoveries of analyte during extraction.

Limits of detection (LODs) were estimated by the analysis of real samples and the figures obtained correspond to the concentration at which the signal-to-noise ratio becomes 3. The detection limits ranged between 0.5 and 29 μg L^{-1}, as seen in Table 3. In a similar fashion, limits of quantitation (LOQs) were calculated as the concentration at which the signal-to-noise ratio becomes 10 (Table 3). The method proved to be linear for all the analytes from the LOQs up to a few mg L^{-1}. Reproducibility was satisfactory (RSD equal to or better than 10 %) in nearly all cases, particularly taking into account that the method includes several steps. In the case of 2OH3MB, a high standard deviation was observed in the rosé wine. Matrix effects were studied through a comparison of the slopes obtained in the calibration for each analyte. Only 3OH3MB was free of such an effect at 95 % confidence. For the other four hydroxy acids investigated, it is necessary to do a calibration curve for each type of beverage analysed: white, red, distilled beverages and so on. Unfortunately, the presence of matrix effects is quite common in complex matrices such as wine and in particular when derivatization is used in the process [11, 39].

Occurrence in different wines and alcoholic beverages

The concentrations of the analytes are presented in Table 4. The great differences among the ranges of the analyte concentrations give an idea of their different biological origins. Three hydroxy acids have been detected in most of the samples analysed (2OH2MB, 2OH4MP and 3OHBc), whereas 3OH3MB was detected only in a few of them. 2OH2MB and 2OH3MB have higher ranges of concentration due to the large values found for these two compounds in some Sherry wines, up to 7 or 8 mg L^{-1}. 2OH4MP and 3OHBc have concentrations lower than 4 mg L^{-1} and 3OH3MB lower than 600 μg L^{-1}. The concentrations of 2OH4MP reported in wine by Drawert et al. were

<10 mg L^{-1} [24], whereas in a by-product of a Chinese liquor the concentrations were larger, up to 60 mg L^{-1} [40].

Table 4

Concentrations of the hydroxy acids (μg/L) studied

Sample type	Year	% EtOH	2OH2MB	3OH3MB	2OH3MB	2OH4MP	3OH
Young white 1	2012	14.0	1940	< DL	< DL	320	775
Young white 2	2012	13.5	198	< DL	214	876	726
Rosé 1	2012	13.5	408	< DL	< DL	525	1120
Rosé 2	2012	13.0	< DL	< DL	< DL	265	931
Rosé 3	2012	13.5	< DL	< DL	< DL	270	937
Young red 1	2011	13.5	1070	< DL	191	1370	2100
Young red 2	2012	13.5	1160	< DL	1580	1750	1220
Young red 3	2012	13.0	< DL	< DL	899	985	2120
Barrel aged red 1	2008	14.1	211	13	397	1060	2500
Barrel aged red 2	2007	13.5	3020	12	1411	1110	1450
Barrel aged red 3	2008	13.5	1250	< DL	< DL	1050	2220
Barrel aged red 4	2010	14.5	1150	< DL	< DL	911	2360
Grape must alcoholic bv 1	2012	15.2	1740	7.9	< DL	< DL	< DI
Grape must alcoholic bv 2	2012	15.5	754	294	< DL	< DL	428
Oloroso							

Sherry	4[a]	18.0	7090	519	2410	1180	863
Manzanilla Sherry	3[a]	15.0	7820	< DL	8510	3470	2110
Pedro Ximenez Sherry	2[a]	15.0	611	< DL	525	803	782
Beer	–	5.2	83	129	205	266	194
Imperial Brandy	5[a]	38.0	< DL	< DL	< DL	< DL	300
Pure Malt Scotch Whisky	10[a]	40.0	< DL	< DL	< DL	< DL	327

DL detection limit

[a] Sample with no attributable vintage date on the bottle. Instead, the aging period (yea is indicated

The low presence of hydroxy acids in rosé wines is notable. Neither 3OH3MB nor 2OH3MB was detected in this type of wines, whereas 2OH2MB was present in only one of the rosé samples analysed. In general, red wines had larger amounts of the five hydroxy acids studied than rosé and white wines. This could be the result of the different types of vinification process.

2OH3MB and 2OH4MP were not detected in either of the two- grape must-derived alcoholic beverages analysed, strongly suggesting an exclusive fermentative origin for both compounds. The ability of some bacteria genus to transform leucine into 2OH4MP and valine into 2OH3MB has already been reported in the literature [41–43]. Besides, the concentrations of 2OH4MP and its ethyl ester in Chinese liquor showed significant differences, depending on the yeast used [40]. By contrast, the relatively high levels of 2OH2MB and 3OH3MB in this kind of unfermented products suggest that these compounds are originated in grapes.

With the exception of 3OHBc, the highest concentrations of the hydroxy acids studied were found in the Sherry wines. The differences in concentration with respect to other types of wine are particularly significant in the case of 2OH2MB and 2OH3MB. In Sherry wines, the concentrations are between 2

and 40 times higher than in the other types of wine. This might be related with the special vinification and ageing processes undergone by Sherry wines. Further details of the comparison of samples and their oenological meaning can be found in [44].

As for the other alcoholic beverages, beer had low amounts of the hydroxy acids. Nevertheless, the 3OH3MB concentration in beer was among the highest values found in the whole set of samples. This is curious taking into account the scarce presence of this compound in the rest of the samples and could be due to the use of barley instead of grapes. To the best of the authors' knowledge, to date, this acid has only been quantified in honey samples [27]. In the distilled beverages, only 3OHBc was detected and the concentrations were low. This could be explained, taking into account the high polarity of these compounds that would hinder their volatilization during the distillation process.

In general, the concentrations of 2OH4MP are lower in white than in red wines, as was already reported for its ethyl ester in the same kind of wines [13]. Although average values of 2OH4MP are higher in young than in aged red wines, the differences are not significant due to the huge range at which these compounds seem to be naturally present in wines.

Conclusions

A specific method has been developed to analyse five hydroxy acids present in wine and other beverages: 2OH2MB, 3OH3MB, 2OH3MB, 2OH4MP and 3OHBc.

The first specific analysis of these hydroxy acids in a wide variety of wines and other alcoholic beverages has been done, showing interesting differences in concentration depending on the sample type.

The availability of the method presented enables further research to be carried out into the origin of the analytes and their capacity as precursors of the corresponding hydroxy esters.

Acknowledgments

The Spanish Government funded this work: project CICYT AGL2010-22355-C02-01. E. Gracia-Moreno is grateful to the Spanish Government for support from the “Formación de Profesorado Universitario” (FPU) program (grant number: AP2008-03811). The authors acknowledge E. Franco Aladrén for providing the grape must derived alcoholic beverages produced in “Unidad de Enología del Gobierno de Aragón” and used in this experiment.

Conflict of interest The authors declare that they have no conflict of interest.

Electronic supplementary material

Below is the link to the electronic supplementary material.

ESM 1

(PDF 1761 kb)

References

1. Etievant PX (1991) In: Dekker MH (ed) Volatile compounds of food and beverages, New York, USA

2. Mirandalopez R, Libbey LM et al (1992) Identification of additional odor-active compounds in Pinot-Noir wines. Am J Enol Vitic 43(1):90–92

3. Peinado RA, Moreno J et al (2004) Changes in volatile compounds and aromatic series in sherry wine with high gluconic acid levels subjected to aging by submerged flor yeast cultures. Biotechnol Lett 26(9):757–762

4. Chaves M, Zea L et al (2007) Changes in color and odorant compounds during oxidative aging of Pedro Ximenez sweet wines. J Agric Food Chem 55(9):3592–3598

5. Pineau B, Barbe J-C et al (2009) Examples of perceptive interactions involved in specific "Red-" and "Black-berry" aromas in red wines. J Agric Food Chem 57(9):3702–3708

6. Makhotkina O, Kilmartin PA (2012) Hydrolysis and formation of volatile esters in New Zealand Sauvignon blanc wine. Food Chem 135(2):486–493

7. Ramey DD, Ough CS (1980) Volatile ester hydrolysis or formation during storage of model solutions and wines. J Agric Food Chem 28(5):928–934

8. Diaz-Maroto MC, Schneider R et al (2005) Formation pathways of ethyl esters of branched short-chain fatty acids during wine aging. J Agric Food Chem 53(9):3503–3509

9. Campo E, Ferreira V et al (2006) Identification of three novel compounds in wine by means of a laboratory-constructed multidimensional gas chromatographic system. J Chromatogr A 1122(1-2):202–208

10. Campo E, Cacho J et al (2006) Multidimensional chromatographic approach applied to the identification of novel aroma compounds in wine - Identification of ethyl cyclohexanoate, ethyl 2-hydroxy-3-methylbutyrate and ethyl 2-hydroxy-4-methylpentanoate. J Chromatogr A 1137(2):223–230

11. Gracia-Moreno E, Lopez R et al (2015) Determination of 2-, 3-, 4-methylpentanoic and cyclohexanecarboxylic acids in wine: development of a selective method based on solid phase extraction and gas chromatography-negative chemical ionization mass spectrometry and its application to different wines and alcoholic beverages. J Chromatogr A 1381:210–218

12. Falcao LD, Lytra G et al (2012) Identification of ethyl 2-hydroxy-4-methylpentanoate in red wines, a compound involved in blackberry aroma. Food Chem 132(1):230–236

13. Lytra G, Tempere S et al (2012) Distribution and organoleptic impact of ethyl 2-hydroxy-4-methylpentanoate enantiomers in wine. J Agric Food Chem 60(6):1503–1509

14. Lytra G, Tempere S et al (2012) Impact of perceptive interactions on red wine fruity aroma. J Agric Food Chem 60(50):12260–12269

15. Lytra G, Tempere S et al (2013) Study of sensory interactions among red wine fruity esters in a model solution. J Agric Food Chem 61(36):8504–8513

16. Schreier P, Drawert F et al (1976) GLC-mass-spectrometrical investigation of volatile components of wines 6. Aroma compounds of Tokaj-Aszu wines A) neutral compounds. Z Lebensm Unters Forsch 161(3):249–258

17. Umano K, Hagi Y et al (1992) Volatile constituents of green and ripened pineapple (Ananas comosus [L.] Merr.). J Agric Food Chem 40(4):599–603

18. Sampaio KL, Garruti DS et al (2011) Aroma volatiles recovered in the water phase of cashew apple (Anacardium occidentale L.) juice during concentration. J Sci Food Agric 91(10):1801–1809

19. Aurore G, Ginies C et al (2011) Comparative study of free and glycoconjugated volatile compounds of three banana cultivars from French West Indies: Cavendish, Frayssinette and Plantain. Food Chem 129(1):28–34

20. Tiitinen K, Hakala M et al (2006) Headspace volatiles from frozen berries of sea buckthorn (Hippophae rhamnoides L.) varieties. Eur Food Res Technol 223(4):455–460

21. Umano K, Hagi Y et al (2002) Volatile chemicals identified in extracts from newly hybrid citrus, dekopon (Shiranuhi mandarin Suppl. J.). J Agric Food Chem 50(19):5355–5359

22. Kepner RE, Webb AD et al (1968) Sherry aroma.7. Some volatile components of flor Sherry of Spanish origin. Acidic compounds. Am J Enol Vitic 19(2):116–120

23. Drawert F, Schreier P et al (1974) Glc-mass-spectrometrical investigation of volatile components of wines 3. Acidic compounds of wine aroma. Z Lebensm Unters Forsch 155(6):342–347

24. Drawert F, Schreier P et al (1976) GLC-mass-spectrometrical investigation of the volatile components of wines vii. Aroma compounds of tokaj aszu wines b) organic acids (author's transl). Z Lebensm Unters Forsch 162(1):11–20

25. Meng J, Fang Y et al (2011) Changes in aromatic compounds of cabernet sauvignon wines during ageing in stainless steel tanks. Afr J Biotechnol 10(55):11640–11647

26. Gonzalez-Rodriguez J, Perez-Juan P et al (2003) Superheated liquids for extraction of solid residues from winemaking processes. Anal Bioanal Chem 377(7-8):1190–1195

27. Tan ST, Wilkins AL et al (1990) Extractive from New-Zealand honeys.3. Unifloral thyme and willow honey constituents. J Agric Food Chem 38(9):1833–1838

28. Abd El-Hady FK, Shaker KH (2013) Honey protects human low density lipoprotein (LDL) from peroxidation (in -vitro study). Int J Pharm Sci Rev Res 23(2):191–197

29. van der Wal B, Kettenes DK et al (1971) New volatile components of roasted cocoa. J Agric Food Chem 19(2):276–280

30. Wang L-J, Mu H-L et al (2010) Volatile components in three commercial Douchies, a Chinese traditional salt-fermented soybean food. Int J Food Prop 13(5):1117–1133

31. Temina M, Rezankova H et al (2007) Diversity of the fatty acids of the Nostoc species and their statistical analysis. Microbiol Res 162(4):308–321

32. Batovska DI, Todorova IT et al (2008) Preliminary study on

biomarkers for the fungal resistance in Vitis vinifera leaves. J Plant Physiol 165(8):791–795

33. Batovska DI, Todorova IT et al (2009) Biomarkers for the prediction of the resistance and susceptibility of grapevine leaves to downy mildew. J Plant Physiol 166(7):781–785

34. Lopez R, Aznar M et al (2002) Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. J Chromatogr A 966(1-2):167–177

35. Gracia-Moreno E, Lopez R et al (2015) Determination of 2-, 3-, 4-methylpentanoic and cyclohexanecarboxylic acids in wine: development of a selective method based on solid phase extraction and gas chromatography-negative chemical ionization mass spectrometry and its application to different wines and alcoholic beverages. J Chromatogr A(0)

36. Lu X, Cai JL et al (2003) Analysis of cigarette smoke condensates by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry 1 acidic fraction. Anal Chem 75(17):4441–4451

37. Cullere L, Bueno M et al (2010) Selectivity and efficiency of different reversed-phase and mixed-mode sorbents to preconcentrate and isolate aroma molecules. J Chromatogr A 1217(10):1557–1566

38. Mateo-Vivaracho L, Cacho J et al (2007) Quantitative determination of wine polyfunctional mercaptans at nanogram per liter level by gas chromatography-negative ion mass spectrometric analysis of their pentafluorobenzyl derivatives. J Chromatogr A 1146(2):242–250

39. Zapata J, Mateo-Vivaracho L et al (2010) Comparison of extraction techniques and mass spectrometric ionization modes in the analysis of wine volatile carbonyls. Anal Chim Acta 660(1-2):197–205

40. Xia Q, Wu C et al (2014) Selection and application of potential whole-cell enzymes in the esterification of Huangshui, a by-product formed

during Chinese liquor-making. J Inst Brew 120(1):45–51

41. Bader J, Rauschenbach P et al (1982) On a hitherto unknown fermentation path of several amimo-acids by proteolytic clostridia. FEBS Lett 140(1):67–72

42. Khelifa N, Butel MJ et al (1998) Synthesis of 2-hydroxy acid from 2-amino acid by Clostridium butyricum. Bioorg Med Chem Lett 8(23):3429–3434

43. Butel MJ, Rimbault A et al (1995) Formation of 2-hydroxy-4-methylpentanoic acid from L-leucine by clostridium-butyricum. FEMS Microbiol Lett 132(1-2):171–176

44. Gracia-Moreno E (due 2015) Nuevos métodos analíticos para la determinación selectiva de pirazinas, ácidos y otros compuestos de interés aromático presentes en cantidades traza. (Title in English: New analytical methods for the selective determination of pyrazines, acids and other relevant aroma compounds present at traze levels). Universidad de Zaragoza (Spain), Language: English, In realization