Title: 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

Author: Elisa Gracia-Moreno Ricardo Lopez Vicente Ferreira

PII: S0021-9673(14)02006-8
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2014.12.074
Reference: CHROMA 356147

To appear in: *Journal of Chromatography A*

Received date: 3-10-2014
Revised date: 19-12-2014
Accepted date: 23-12-2014

Please cite this article as: E. Gracia-Moreno, R. Lopez, V. Ferreira, 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, *Journal of Chromatography A* (2014), http://dx.doi.org/10.1016/j.chroma.2014.12.074

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.

Elisa Gracia-Moreno*, Ricardo Lopez, Vicente Ferreira

Laboratorio de análisis del aroma y enología, Departamento de Química Analítica, Facultad de Ciencias, Aragon Institute of Engineering Research (I3A), Universidad de Zaragoza, E-50009 Zaragoza, Spain

telephone 34 876553330, fax 34 976761292

*Author to whom correspondence should be addressed: **elisagm@unizar.es**

Abstract

A method to analyse 2-methylpentanoic, 3-methylpentanoic and 4-methylpentanoic acids as well as cyclohexanecarboxylic acid has been developed and applied to wine and other alcoholic beverages. Selective isolation with solid phase extraction, derivatization with 2,3,4,5,6-pentafluorobenzyl bromide at room temperature for 30 minutes, and further analysis by gas chromatography-mass spectrometry in negative chemical ionization mode provides detection limits between 0.4 and 2.4 ng/L. Good linearity up to 3.6 μg/L, satisfactory reproducibility (RSD < 10%) and signal recovery of around 100% represents a robust method of analysis. Concentration data of these analytes in wine and other alcoholic beverages are reported for the first time. The levels found ranged from the method detection limits to 2630 ng/L, 2040 ng/L and 3810 ng/L for 2-, 3- and 4-methylpentanoic acids, respectively, and to 1780 ng/L for cyclohexanecarboxylic acid. There are significant differences depending on the type of wine or beverage. Distilled beverages, beer and aged wines have higher contents in methylpentanoic and cyclohexanecarboxylic acids.

Keywords: 2-, 3- and 4- methylpentanoic acids; cyclohexanecarboxylic acid; wine; SPE; GC-NCI-MS; selective isolation;

1. Introduction

Fatty acids are essential in living organisms as components of cellular membranes and as energy reservoirs in the form of triacylglycerols. They can be classified into long- and short-chain as well as into straight- and branched-chain fatty acids. In wine, short-chain fatty acids (SCFAs) are relevant because they are related to unpleasant aromas such as rancid, butter, cheese and sweat [1].

On the other hand, the esterification of fatty acids in the presence of ethanol produces their corresponding ethyl esters [2]. This has been amply studied because of the aromatic importance of ethyl esters in the overall aroma of wine [3-5]. Their fruity descriptors contribute to a positive balance in the aroma. A different behaviour during ageing has been found for esters of branched fatty acids and those of linear fatty acids. The first group increases in concentration during ageing, whereas the second one decreases [2]. Thus, short-chain branched fatty acids could act as reservoirs of fruity aromas to be developed during ageing.

In the last decade, Campo et al. identified four novel esters in wine as responsible for powerful strawberry aromas: 2-, 3-, and 4-methylpentanoate ethyl esters and cyclohexanecarboxylate ethyl ester [6, 7]. The same authors reported a connection between ageing of the samples and a higher content of the esters, and postulated that the origin of these ethyl esters could be the esterification of their corresponding acids [8]. These results suggest the plausibility of finding 2-, 3- and 4-methylpentanoic and cyclohexanecarboxylic acids in wine. To the best of our knowledge, none of the four analytes has yet been analysed in grape wine. However, the presence of 2- and 4-methylpentanoic acids, as well as 4-methylpentanoate and cyclohexanecarboxylate ethyl

esters, has already been described in Chinese liquors made from mixtures of cereals [9, 10]. 4-methylpentanoic acid has also been determined in rice wine [11] and 2-methylpentanoic acid has been identified in some commercially available yeast derivatives added to wine [12, 13]. Finding these acids in wine would be the first step towards eventually proving or refuting the hypothesis that the origin of the corresponding ethyl esters is esterification.

The ratio between acid and ethyl ester concentrations ranges from two up to ten for branched and linear acids [14]. Assuming a similar behaviour for the methylpentanoic and cyclohexanecarboxylic acids, the predictable concentrations of the acids studied in this paper could be expected to be higher than those obtained for their corresponding ethyl esters. Following this hypothesis, and taking into account the concentration of the ethyl esters obtained in [8, 15, 14], we could expect concentrations to be a few μg/L in the case of 4-methylpentanoic acid. For the rest of the acids, ng/L levels could be expected. In particular, low ng/L concentrations are expected for cyclohexanecarboxylic acid.

The sample preparation methods used to analyse methylpentanoic acids in other matrices have been based on the extraction of large quantities of brew or fish sauce with different sorbents (Tenax or Porapack Q) in classic columns [16, 17], solid-liquid extraction from tobacco leaves in an acidified medium [18] or HS-Tenax extraction in the case of dry fermented sausages [19]. In the case of Chinese liquors, liquid-liquid extraction with diethyl ether and further fractionation into acidic, basic and neutral fractions was used [9]. However, no quantitative data were provided with this method. The analysis and detection of the extracts in the aforementioned cases was carried out by gas chromatography (GC). The columns used for the isolation of the analytes were polar in most cases [16-18] with the exception of [19] in which an apolar column was

used. As for the detection, flame ionic detector (GC-FID) [16-18] and mass spectrometric detection in electronic impact mode and (GC-MS-EI) [16, 17, 19] were used. Fan et al. used both types of column and carried out the identification of compounds with an olfatometric detector (GC-O-FID) and GC-MS-EI [9].

Linear and branched short chain fatty acids have been analysed in wine by different methods such as liquid-liquid extraction with different solvents [20], solid phase extraction (SPE) [21] and solid phase micro-extraction (SPME) [22]. However, the expected low amount of the target acids in this study requires a method able to provide a good pre-concentration of the sample that can be provided by SPE. Furthermore, the use of the acid properties of the analytes can help with the pre-concentration and cleaning of the samples. Acid and basic properties of the analytes have been used in the past to improve the selectivity of the isolation: ionic or mixed-mode sorbents, selective elution or both [23, 24]. The bad chromatographic properties of the acids and their poor detectability in MS are addressed with a derivatization method.

The objectives of this paper are the development and validation of a method to analyse the three above-mentioned methylpentanoic acids and cyclohexanecarboxylic acid at the ng/L level, as well as to provide the first data relating to the four analytes in a variety of wines and other beverages.

2. Materials and methods

2.1. Reagents and standards

The standards of 2-methylpentanoic acid (2MePc), 3-methylpentanoic (3MePc) acid, 4-methylpentanoic acid (4MePc), cyclohexanecarboxylic acid and 2-ethylbutanoic (2EtBc) acid were supplied by Aldrich (Steinheim, Germany) with purity higher than 96% in all cases. 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) and tetrabutylamonium chloride (NBu_4Cl) (> 97%) were also obtained from Aldrich.

The solvents used were Unisolv quality hexane (Hx), Lichrosolv quality ethanol, Suprasolv quality methanol (MeOH) and dichloromethane (DCM), and diethyl ether, all supplied by Merck (Darmstadt, Germany). Toluene 99.5% was supplied by Panreac (Barcelona, Spain). Pure water was obtained from a milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

The sorbents used were: Oasis MAX (60 mg, 3 mL reservoir) supplied by Waters (Milford, U.S.A.), and LiChrolut EN resins both pre-packed (200 mg, 3 mL reservoirs) and in-house packed (50 mg in 1 mL reservoir) obtained from Merck. SPE was performed with the help of a Vac Elut 20 system supplied by Varian (Sunnyvale, CA, USA). Silica-gel 60 was obtained from Merck.

Standard solutions of the acids were prepared in hexane to avoid esterification. Those used to spike wine or synthetic wine were prepared in ethanol prior to spiking.

2.2. Wines and alcoholic beverages samples

Two commercial Spanish young red wines were used for the development of the method. Additionally, twenty-one samples were analysed, including red and white wines with diverse degrees of ageing, and other alcoholic beverages such as beer,

whisky and brandy. Detailed information about the samples can be found in the supplementary content (table 1).

2.3. SPE method development

2.3.1. Sorbent selection and breakthrough volumes

Mixed-mode anionic Oasis MAX sorbent (60 mg, 3 mL reservoir) was conditioned with 2 mL DCM, 2 mL MeOH and 4 mL hydroalcoholic solution (12% ethanol). Synthetic wine was spiked with 1.6 mg/L of the acids studied and its pH was adjusted to 7.0 prior to the loading of the cartridges. Vacuum suction was not applied in this particular experiment to avoid losses of the non-retained analytes due to their volatility. The percolated solutions (10 mL fractions up to 100 mL) were collected and the pH readjusted to 2.7. The solutions were then analysed with the method described in [21]. Lichrolut EN sorbent (200 mg, 3 mL reservoirs) conditioned with 4 mL DCM, 4 mL MeOH and 4 mL hydroalcoholic solution (12% ethanol) was used to analyse the samples. After loading the samples under vacuum suction, 1 mL of milli-Q water was used to clean the cartridges. The sorbent was dried under nitrogen and the analytes were eluted with 1.6 mL of DCM.

Generic hydrophobic LiChrolut EN sorbent (200 mg, 3 mL reservoirs) was also studied. Conditioning was done with 4 mL DCM, 4 mL MeOH and 4 mL hydroalcoholic solution (12% ethanol). A young red wine spiked in this case with the analytes in a concentration of 5 mg/L was loaded without vacuum suction. Different fractions (10 mL each) up to 100 mL of the percolated solution were recovered and analysed as described above. Ten mL of the spiked wine was analysed following the

same procedure as with the percolated fractions and was used as a reference to calculate the breakthrough volumes.

2.3.2. Removal of interferences and matrix compounds

Fifty mL of a young red wine from Rioja spiked with 5 mg/L of the analytes was loaded into a 200 mg LiChrolut EN cartridge. Five fractions (1 mL each) of a 40% MeOH solution in milli-Q water buffered at pH 3 with H_3PO_4/NaH_2PO_4, were used to clean the cartridge without vacuum suction. The percolated solutions were analysed as in [21].

2.3.3. Optimization of the elution strategy

Five LiChrolut EN cartridges conditioned as aforementioned were loaded with 50 ml each of a young red wine from Rioja spiked with 5 mg/L of the analytes. Five solutions of milli-Q water buffered at pH 7.0 with NaH_2PO_4/Na_2HPO_4, containing different percentages of MeOH (5, 15, 25, 35, and 40) were prepared and used to elute a different cartridge each (4 fractions of 5 mL). The 20 recovered eluates were each supplemented with 2 mL of a 0.625 M tartaric acid solution and the appropriate volume of MeOH in each case to reach a final concentration of 25% MeOH. All the eluates were then analysed following the method mentioned in [21].

2.3.4. Second SPE step

Two 200 mg LiChrolut EN cartridges were conditioned and loaded with 50 mL each of a young red wine from Rioja spiked with the analytes (2 mg/L). They were then rinsed with 3 mL of 40% MeOH/milli-Q water buffered at pH 3. The cartridges were eluted with 5 mL of 40% MeOH/milli-Q water buffered at pH 7. The eluted fractions were combined and then divided into two fractions of equal volume. 2 mL of a 0.625 M tartaric acid solution were added to each fraction. One fraction was diluted with milli-Q water up to 20 mL and the other to 10 mL. Each fraction was loaded into a cartridge (packed in house) containing 50 mg of LiChrolut EN (1 ml volume reservoir), previously conditioned with 1 mL DCM and 1 mL MeOH. The recovered eluates were analysed as described in [21]. The reproducibility of the whole extraction process was checked by analysing three different wines spiked at a level of 10 μg/L.

2.4. Derivatization

Initially, the derivatization was done as described in [25]. Two hundred μg of pure analyte was dissolved in 1 mL of DCM. To this was added 1 mL of an aqueous solution containing 0.1 M NBu_4Cl and 0.2 M NaOH. Then 20 μL of pure PFBBr was also added and the mixture was stirred during 30 minutes at room temperature. The organic phase was isolated and dried with Na_2SO_4, evaporated to dryness under a nitrogen stream and re-dissolved in diethyl ether.

Once the derivatives had been characterized, extracts from wine spiked at 1 mg/L obtained with the previously described SPE-method or 0.5 mL of synthetic solution containing the equivalent amount of the analytes were used to optimize the reaction. The following factors were checked: the solvents for the organic phase being synthetic solutions of the acids in hexane, hexane/ 25% diethyl ether (v/v) and DCM;

the temperature, 25 ºC and 60 ºC; the reaction time (up to 20 hours) and the pH (6 and 11). For these experiments 20 μL of pure PFBBr and 0.5 mL of aqueous solution 0.1 M in NBu_4Cl were used. The influence of the concentration of the reactant (20, 10 and 2 μL) and the NBu_4Cl phase-transfer catalyst (0.1 M, 0.05 M and 0.02 M in the aqueous solution) was checked once the solvent (DCM), temperature (25 ºC), time (30 minutes) and pH (6) had been established.

2.5. Gas Chromatography and Mass Spectrometry

The chromatographic analysis during the development of the SPE method was done with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass-spectrometric detection system supplied by Varian (Sunnyvale, CA, USA). The capillary column used was a DB-WAX ETR (J&W Scientific, Folsom, CA, USA) (60 m x 0.25 mm, 0.25 μm) preceded by a 3 m x 0.25 mm uncoated (deactivated, intermediate polarity) pre-column from Supelco (Bellefonte, USA). Helium was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature programme was 5 min at 40 ºC, then increasing by 8 ºC/min up to 170 ºC, with a second ramp at 4 ºC min^{-1} up to 190 ºC and a third ramp at 8 ºC min^{-1} up to 220 ºC. This temperature was maintained for 20 min. The MS-parameters were: MS transfer line 220 ºC and ionization chamber temperature 170 ºC. Electronic impact was used with a scan range of 40-360 m/z. The acquisition was done in automatic gain control (AGC) with a filament intensity current of 30 μA.

Two μL of the extract was injected in splitless mode for 2 min with a pulse pressure of 30 psi.

The optimization of the reaction was monitored with the help of an FID GC-8000 supplied by Carlo Erba (Milan, Italy), with hydrogen as the carrier gas (100 kPa), nitrogen as make-up gas (95 kPa) and hydrogen (35 kPa) and air (60 kPa) in the FID detector. The column used was a DB-WAX (30 m, 0.32 mm, 0.5 μm) with a deactivated pre-column (3 m, 0.25 mm). The oven temperature program was 40º C during 3 minutes followed by a 20º C ramp up to 220º C held during 20 min. Injection of 1 μL sample was done in splitless mode at 250 ºC.

The analysis of the extracts in the definitive method was done in a GC-MS Shimadzu QP-2010 Plus (Shimadzu Corp., Kyoto, Japan). The column was a CP-WAX 52 CB (25 m, 0.15 mm, 0.25 μm) supplied by Varian preceded by a 3 m x 0.25 mm uncoated (deactivated, intermediate polarity) pre-column obtained from Supelco (Bellefonte, USA). One μL of sample was injected at 250 ºC with 3 min of splitless time with helium at 45 cm/s as the carrier gas. The oven was programmed as follows: 40 ºC during 4 min, ramp of 80 ºC/min up to 80 ºC and held for 1 min, 4 ºC/min ramp up to 130 ºC, 30 ºC/min ramp up to 190 ºC and a final ramp of 100 ºC/min up to 230 ºC and held for 15 min. The spectrometer was operated in negative chemical ionization (NCI) mode with methane as ionization gas (2 bars of pressure). The temperature of the ion source was set at 220 ºC and the transfer line temperature was 250 ºC. A DB-5 column (20 m, 0.18 mm, 0.18 μm) was also fitted to this system to calculate the LRI of the analytes.

Some samples were analysed with different ionization modes to check which one provided the best results. GC-EI-MS in an ion-trap was compared with two other ionization modes in a Shimadzu quadrupole: GC-EI-MS (SIM) and GC-NCI-MS. The systems used are those above mentioned with the exception of GC-EI-MS (SIM). This experiment was done in the Shimadzu instrument but the column fitted to it was a DB-

WAX ETR (30 m, 0.25 mm, 0.25 μm). The chromatographic conditions were those already reported for the Shimadzu system. As for the ionization, two segments were done to acquire the internal standard and the methylpentanoic acids, and the cyclohexanecarboxylic acid respectively. The fragments used in the first segment were: 181, 268, 254, 240, 73 and 115 m/z; whereas in the second segment the fragments were: 181, 81 and 109 m/z.

2.6. Proposed method

Extraction of the analytes: 200 mg Lichrolut EN sorbent (pre-packed in 3 mL cartridges) is conditioned with 4 mL DCM, 4 mL MeOH and 4 mL hydroalcoholic solution (12%). Fifty mL of wine is spiked with 2EtBc acid (IS) to obtain a 10 μg/L concentration. Highly alcoholic beverages, such as whisky and brandy, are diluted prior to the analysis to 12% ethanol content. The sample is then loaded into the cartridges with the help of a vacuum manifold. The sorbents are washed with 3 mL of aqueous solution (40% MeOH) buffered at pH 3 with H_3PO_4/NaH_2PO_4. Elution of the analytes is done with 5 mL of aqueous solution (40% MeOH) buffered at pH 7.0 (NaH_2PO_4/Na_2HPO_4). The buffer is broken with the addition of 2 mL of 0.625 M tartaric acid solution to the collected eluate and is diluted to the required volume with milli-Q water in a 10 mL volumetric flask (final pH 3.0). The resulting solution is loaded into a 50 mg LiChrolut EN cartridge (1 mL volume) previously conditioned with 1 mL DCM and 1 mL MeOH. The sorbents are vacuum-dried and eluted with 0.5 mL DCM and recovered in 2 mL glass vials.

Derivatization reaction: 20 μL of pure PFBBr and 500 μL NBu_4Cl 0.1 M in aqueous buffered solution (pH 6.0) are added to the DCM extract. After stirring the solution for

30 minutes at room temperature, the reaction is stopped with concentrated HCl (37%). The organic phase is washed with 1 mL acidified milli-Q water (pH 1), dried with Na_2SO_4, and then purified through a 200 mg bed of silica-gel 60 (1 mL cartridge). For this, 1.5 mL of hexane is added and discarded. Elution of the analytes is done with 1 mL of hexane/ 40% toluene (v/v). One μL of the extract is finally injected in the GC-MS and analysed in NCI mode as described in the previous section.

2.7. Method validation

The linearity was studied by spiking the wines with known amounts of the standards up to 3.5 μg/L. The slopes were compared with an F-test (95% level of confidence) to detect matrix effects. The reproducibility and the signal recovery of the method were measured analysing 3 replicates of 2 wines spiked at around 1 μg/L: a young red (Montesierra, DO Somontano) and a very dry Fino (Tio Pepe, DO Manzanilla).

3. Results and discussion

3.1. SPE method development

3.1.1. Sorbent selection and breakthrough volumes

The most important parameter when designing an SPE based method is the breakthrough volume (V_B) of the analytes in the sorbent used, since this measures the capacity of an SPE system to isolate the analytes from a given liquid matrix. In this work V_B has been defined as the maximum volume of wine sample that can be loaded

into an SPE bed with losses of analyte in the percolated sample below 1% of the total amount of analyte loaded.

The first attempt to selectively isolate the analytes was based on the use of their acidic properties. The pKa of all the analytes studied is less than five. Consequently, pH 7.0 was chosen to have the analytes in their anionic form. Then, a mixed-mode anionic sorbent (Oasis MAX), combining anionic-exchange properties with hydrophobic retention, was assayed. However, the results were not good enough because the breakthrough volumes were less than 10 mL (data not shown). This option was then discarded because the small breakthrough volumes were not expected to provide a sufficiently high concentration factor to be able to detect the analytes, taking into account the low concentrations expected according to our preliminary experiments.

In a second attempt, a generic hydrophobic sorbent (LiChrolut EN) was selected. The loading was done at the natural pH of wine to have the analytes mainly in their neutral form. The breakthrough volumes were larger in this sorbent, with 50 mL in the case of the methylpentanoic acids and 80 mL for the cyclohexanecarboxylic acid.

Therefore, due to the unexpected poor performance of the anionic mixed sorbent, it was decided to choose the hydrophobic sorbent to carry out the SPE. The wine load volume in this sorbent was set at 50 mL to prevent losses of the least retained methylpentanoic acids.

3.1.2. Removal of interferences and matrix compounds

With the aim of having a cleaner extract, a previous washing step was introduced. The objective was to eliminate more polar acids (such as tartaric or lactic

311 acid) and the largest possible amounts of major wine alcohols as possible. Different
312 volumes of a 40% MeOH/ water solution were studied to remove as many interferences
313 as possible without loosing the analytes. The pH of the washing solution was set at 3.0
314 to avoid the possibility of losing the analytes in their ionic form. The results showed
315 that the amount of methylpentanoic acids removed with the first fraction of 5 mL was
316 less than 1% of the total, and even lower for the cyclohexanecarboxylic acid (fig.1).

317 An acid not present in wine, 2-ethylbutanoic acid (2EtBc), was selected as a
318 potential internal standard (IS). The fact that it has the same number of carbon atoms
319 suggested a similar behaviour to that of the analytes. However, the polarity is not the
320 same and, as a consequence, there were some differences. More than 2% of 2EtBc was
321 lost with just 5 mL (fig. 1) of the washing solution. As a result, a volume of 3 mL was
322 chosen to clean the sorbents after the loading of the wine. In this way only 1% of 2EtBc
323 was lost.

324 It was confirmed that this cleaning step removed completely some of the major
325 interfering compounds, and roughly 50% of the C_4 acids (2-methylpropanoic and
326 butanoic acids) and C_5 acids (2-methylbutanoic and 3-methylbutanoic acids)
327 endogenous in wine. Nevertheless, the quantity of other major compounds retained in
328 the sorbent was still considerable. In consequence, a 3 mL volume was chosen for the
329 washing step as a compromise between cleanliness and retention of the analytes and the
330 IS.

331

332 *3.1.3. Optimization of the elution strategy*

333 A selective step was designed to elute the analytes, minimizing the amount of
334 interferences. Different percentages of MeOH were tested to optimize the volume of

elution. The pH of the elution solutions was fixed at 7.0 to change the acids from their neutral to their ionic forms. Thus, the elution is eased because the interactions with the sorbent are hindered due to the electrical charge, while the interactions with the elution solution are favoured. It was decided not to use a more basic pH to avoid eluting polyphenols.

As can be seen in table 1, the most effective elution can be performed with 40% of MeOH in the solution. Just 5 mL were enough to elute the whole amount of the analytes retained. Lower percentages of MeOH would imply higher volumes of elution solution to completely elute the analytes.

3.1.4. Second SPE step

At this point in the development of the method, the analytes had already been selectively concentrated 10 times but this was still insufficient for a good quantification. In addition, the extract (a 40% MeOH aqueous solution) was not compatible with GC. For these reasons, a second extraction process was needed. A second SPE step with the same sorbent (LiChrolut EN) was selected, but this time using a 50 mg bed in a 1 mL cartridge. This reduction in the size of the bed was intended to allow a greater concentration of the analytes.

The aforementioned extract had the analytes in their anionic form. Tartaric acid (0.625 M) was used to reduce the pH from 7.0 to 3.0 and to convert the analytes into their neutral form, allowing their retention in the second SPE cartridge.

To check the influence of the MeOH percentage, two aliquots of the same extract diluted to 10 and 20% of MeOH respectively were compared. This experiment

was also used to check if a 50 mg sorbent bed was enough to retain the analytes present in the extract from the first cartridge. In consequence, those extracts were compared with a further extract that had been diluted to 20% MeOH and loaded into a 200 mg sorbent for the second SPE.

The samples containing 10% and 20% MeOH (prior to loading in the 50 mg bed of sorbent) showed no significant differences. In consequence, dilution to 20% MeOH was selected to save time during the loading of the second cartridge.

The loss of analytes was less than 1% in all cases (methylpentanoic acids, cyclohexanecarboxylic acid and IS). This implies that a 50 mg sorbent bed is adequate to retain the analytes in the second extraction. The elution of this second cartridge was done with 0.5 mL of DCM. Reproducibility (n=9) of the whole SPE method, tested with three wines spiked at a level of 10 μg/L and analysed three times each, was good with relative standard deviations below 7% for all analytes except for 4-methylpentanoic acid which, because of chromatographic interference, was 30%. The interference, identified as ethyl 4-hydroxybutanoate, presented isobaric coincidences with 4-methylpentanoic acid in all the relevant fragments. Improving the resolution by changing the temperature programming rate was not possible and the strategy of changing the column to avoid this co-elution was impractical because of the bad chromatographic properties of acids in apolar stationary phases. A washing step in the second cartridge allowed the interference to be reduced to 1%, but a large amount of the analytes was also eliminated (40-60%) and thus this option was discarded. Instead, derivatization was chosen to improve both the selectivity and the sensitivity of the method.

3.2. Derivatization

Methylation is an easy and frequently used reaction to derivatize fatty acids [26]. However, the addition of just one methyl group would not improve the detectability of analytes. Injection-port derivatization is another strategy [27]. This method uses tetraalkylammonium salts as ion-pair reagents to produce the corresponding carboxylate ion-pairs [$R\text{-}COO^-\ NBu_4^+$] that are transformed into their volatile butyl-esters in the injector at high temperature. The main drawback in this case might be the dirtiness accumulated in the injector and its influence on the chromatographic performance.

The reaction selected to transform the analytes was an alkylation in which the acids in their anionic form substituted the bromide of the reactant through a S_N2 mechanism, as shown in figure 2. Thus, the acids were transformed into their corresponding 2,3,4,5,6-pentafluorobenzyl (PFB) esters. One benefit of highly halogenated derivatives is the large fragment bonded to the carboxylate that can provide more selective ions. The use of a specific detector based on the stabilization of electrons enhances both selectivity and sensitivity. Two detection techniques can be used for this purpose: electron capture detection (ECD) [28] or mass-spectrometry with negative chemical ionization (MS-NCI) [29].

The characterization of the derivatives was done in an ion-trap analyzer in electronic ionization (EI) mode and in a quadrupole analyzer both in EI mode and in NCI mode. The spectra are shown in figures 1-3 in the supplementary material. The linear retention indices determined in a DB-5 and in a DB-WAX are presented in table 2.

To obtain the highest possible yield, the following derivatization parameters were optimized: organic phase solvents, temperature and time of the reaction, pH, and concentration of both the reactant and the phase-transfer catalyst.

Different solvents were assayed and it was found that DCM provided the best yield. In the case of hexane, the increase of temperature (60 ºC) doubled the yield with respect to room temperature. The use of high temperatures was not possible with DCM due to its low boiling point. However, the use of DCM at room temperature provided a reaction yield twenty times higher than the other two solvents tested (hexane/diethyl ether and hexane) in the same conditions. Thus, DCM at room temperature proved to be the best option regarding the reaction yield.

The study of the kinetic profiles showed an increase in the yield that doubled in 20 h as shown in figure 3. However, 30 minutes was selected as the reaction time as a compromise between adequate sensitivity and time efficiency.

The influence of the pH was minimal provided it was high enough to have the analytes in their anionic form. A pH of 6.0 was selected as there were no significant differences between pHs of 6 and 11.

The concentration of both the reactant (PFBBr) and the phase-transfer catalyst (NBu_4Cl) was a determining factor in the yield of the reaction. Moreover, there is an interaction between them as the phase-transfer catalyst favours the decomposition of the reactant: part of the PFBBr added to the reaction medium was transformed into PFBCl, as has already been reported [30]. For these reasons, and to minimize the amount of residues, different concentrations of both components were studied. However, using half the concentration of PFBBr or alternatively a fifth of the catalyser concentration resulted in a reduction of the yield between 10 and 20%. Thus, 20 μL of pure PFBBr and 500 μL of 0.1 M in NBu_4Cl buffered at pH 6 were selected as the optimum.

A silica-fractionation of the organic phase was used to eliminate the excess of PFBBr and its degradation products and to avoid damage to the chromatographic

system. The fractionation consisted of loading the organic phase into a 200 mg silica-gel bed (1 mL cartridge). An initial fraction of pure hexane allowed the elimination of most of the remaining by-products of the reaction. The PFB-esters were isolated with 1 mL of Hx/ 40% toluene (v/v).

Although the reaction yield is not very high, the optimized parameters allow the analytes to be derivatized in a reproducible and satisfactory fashion as will be shown in the validation of the whole method.

A comparison of the performance of different ionization modes was done. The detection limits calculated in each ionization mode are shown in table 3. Both variants of EI mode studied delivered worse results than NCI. Ion trap-EI allowed limits of detection between 47 and 237 ng/L, whereas EI mode, in general, gave worse results when performed in the quadrupole.

The best DL values were obtained with NCI mode, which provided a huge increase (more than a hundred-fold) in the sensitivity of the method as compared to the analysis of the same samples in EI mode. Values of DL in the low ng/L level for the four analytes allowed their detection in most of the samples. Two reasons are behind this improvement of the signal in NCI mode. First, NCI is very selective and overcomes the problems posed by interferences. Much less molecules are able to give signal in this ionization mode, thus reducing the noise and providing a high signal to noise ratio (figure 4). The second factor is the low number of fragments produced that contributes to the high sensitivity provided by this mode of ionization. The molecular ion is not present in the spectrum. The only fragment produced is that corresponding to the carboxylate anion, that is, the derivatized molecule breaks through the bond formed in the reaction. This can be explained taking into account the higher ability of the

carboxylate ion to stabilize a negative charge in relation to the ester. Thus, a low number of fragments contributes to the high sensitivity provided by this mode of ionization. This kind of fragmentation seems to be typical of PFB-esters, as PFB-esters from other branched and linear acids present in wine showed the same fragmentation pattern. Chromatograms were acquired in scan mode because, thanks to the high fragmentation selectivity, maximum sensitivity can be achieved without losing additional information about other compounds present in the sample.

3.3. Method validation

Detection limits 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 0.4 and 2.4 ng/L (figures of merit can be seen in table 4). These good values are due to the excellent signal to noise ratio provided by NCI and the cleanness of the samples. The detection limits allowed determination of all the compounds in all but one case (young red 4) in which cyclohexanecarboxylic acid was under the limit of detection. The method proved to be linear up to a concentration of more than 1 μg/L in wine for the four acids. Accuracy was estimated through a signal recovery experiment done in triplicate in a Fino and in a young red wine. The signal recovery was near 100% in most cases, although 4-methylpentanoic acid and cyclohexanecarboxylic acids had worse recoveries in the Fino wine. This would be explained by the higher matrix complexity of the Fino wine. Reproducibility was good (RSD equal to or better than 10%) in all cases, which is very good for a method with so many steps. As in the case of the signal recovery, the best reproducibility values were obtained for 2- and 3-methylpentanoic acids in both wines. There are big differences of

behaviour for 4-methylpentanoic acid and cyclohexanecarboxylic between the two wines. Matrix effects were studied through an F-test on the slopes of the calibration curves and significant differences were found at a 95% confidence level for all the compounds. However, the great variety of the samples accounts for most of the differences. The same statistical study done among similar wines, for example reds (both young and aged), revealed no significant differences. To solve this problem, a standard addition had to be done for each type of wine: white, red, distilled beverages and so on.

3.4. Occurrence in different wines and alcoholic beverages

The concentrations of the analytes are presented in table 5. The variety of wines and alcoholic beverages explains the great variability in the results.

In general, 2- and 3-methylpentanoic acid concentrations are similar in most of the wines. Comparing the concentration of the four acids by sample, the highest values correspond to 4-methylpentanoic acid in all the samples analysed but one (natural sweet wine 1). The ratio between this acid and the other methylpentanoic acids is around 10:1 in many cases, although it can reach even around 50:1 (Fino and Manzanilla wines for the 2-methylpentanoic acid).

The quantification of 4-methylpentanoic acid in a Chinese rice wine [11] showed a concentration of 294 μg/L, which is a hundred-fold higher than any of the values found in this paper. This great difference could be attributed to the rice composition itself, although other factors such as the raw materials used and the manufacturing process cannot be excluded without further studies.

Cyclohexanecarboxylic acid has the lowest concentrations of the four acids except in the case of natural sweet wine 1, which has 1780 μg/L. This is very surprising because this concentration level is much higher than in any other sample, including the other natural sweet wine. The only difference between the two natural sweet wines (not fermented) lies in the grapes used. In “natural sweet wine 1” the grapes were unripe. This is very interesting because it points to a grape origin of the cyclohexanecarboxylic acid. Furthermore, the comparison with “natural sweet wine 2” suggests that the cyclohexanecarboxylic acid degrades through ripening given that this sample was produced with ripe grapes and had the lower concentration of the two. Furthermore, if the hypothesis that these acids are precursors of their corresponding ethyl esters is true, it opens up the possibility of technologically controlling their content in wine through grape ripeness.

Some interesting parallels can be found between the concentrations of the four acids analysed in this work and their corresponding ethyl esters that were analysed for the first time in [8]. First, only ethyl 4-methylpentanoate was found in young wines in [8], always below 300 ng/L. The four acids have been quantified in all the young wines analysed here, 4-methylpentanoic acid in a range between 600 and 2000 ng/L while none of the other three acids is above 150 ng/L in any sample. This is in accordance with the esterification hypothesis exposed by Campo et al. [8]. The low levels of 2- and 3-methylpentanoic acids and cyclohexanecarboxylic acid would provide low levels of their esters while 4-methylpentanoic acid would give rise to detectable amounts of its ester even with a low esterification rate. Second, aged wines have larger amounts of the acids than young wines, both white and red. As reported for the ethyl esters [8], there is a great variability in the levels of the acids among white wines with special ageing (Fino, Manzanilla, Oloroso and Pedro Ximenez). This could be attributed to the

526 different fermentation processes used to produce each wine and would be in accordance
527 with the theory that the esters are produced because of the metabolism of yeasts [8].

528 As for the other alcoholic beverages, the whisky sample has the highest
529 concentrations of the three methylpentanoic acids, brandy is rich in 2- and 4-
530 methylpentanoic acids and beer has high concentrations of the three methylpentanoic
531 acids. The presence of 3-methylpentanoic acid has already been described in beer [31].
532 However, its identification was tentative, based only in its EI mass spectra. The
533 retention index provided by the authors in a BP-20 column (1987) [31] differs greatly
534 from that calculated in this paper (1774) and reported by other authors [12] in WAX
535 type columns.

536

537 **4. Conclusions**

538 A robust and very selective method has been developed to analyse 2-, 3- and 4-
539 methylpentanoic and cyclohexanecarboxylic acids in wine, a complex matrix. The
540 removal of interferences throughout the method as well as the use of the selective
541 ionization mode has provided detection limits in the range of a few ng/l, low enough to
542 quantify these acids in different beverages.

543 The first concentration data for 2-, 3- and 4-methylpentanoic and
544 cyclohexanecarboxylic acids in wine and other alcoholic beverages are reported,
545 showing interesting differences depending on the kind of wine and the ageing process.

546 The availability of the method presented enables further research to be carried
547 out into the origin of the analytes and their capacity as precursors of the

methylpentanoic and cyclohexanecarboxylic esters. This research is currently in development.

5. Acknowledgements

The authors acknowledge the Spanish Government for the funding of this work through 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 non-commercial sweet wines. The authors appreciate very much the work done by Julian Chancelor providing useful comments about English usage.

6. References

[1] P.X. Etievant, Wine, in: M.H. Dekker (Ed.), Volatile Compounds of Food and Beverages, New York, USA, 1991, pp. 498-499.

[2] M.C. Diaz-Maroto, R. Schneider, R. Baumes, Formation pathways of ethyl esters of branched short-chain fatty acids during wine aging, J. Agric. Food Chem. 53 (2005) 3503-3509.

[3] M. Aznar, R. Lopez, J.F. Cacho, V. Ferreira, Identification and quantification of impact odorants of aged red wines from Rioja. GC-olfactometry, quantitative GC-MS, and odor evaluation of HPLC fractions, J. Agric. Food Chem. 49 (2001) 2924-2929.

[4] V. Ferreira, P. Fernandez, C. Pena, A. Escudero, J.F. Cacho, Investigation on the Role Played by Fermentation Esters in the Aroma of Young Spanish Wines by Multivariate-Analysis, J. Sci. Food Agric. 67 (1995) 381-392.

[5] H. Guth, Identification of character impact odorants of different white wine varieties, J. Agric. Food Chem. 45 (1997) 3022-3026.

[6] E. Campo, V. Ferreira, R. Lopez, A. Escudero, J. Cacho, Identification of three novel compounds in wine by means of a laboratory-constructed multidimensional gas chromatographic system, J. Chromatogr. A 1122 (2006) 202-208.

[7] E. Campo, J. Cacho, V. Ferreira, 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 (2006) 223-230.

[8] E. Campo, J. Cacho, V. Ferreira, Solid phase extraction, multidimensional gas chromatography mass spectrometry determination of four novel aroma powerful ethyl esters - Assessment of their occurrence and importance in wine and other alcoholic beverages, J. Chromatogr. A 1140 (2007) 180-188.

583 [9] W.L. Fan, M.C. Qian, Characterization of aroma compounds of Chinese "Wuliangye"
584 and "Jiannanchun" liquors by aroma extract dilution analysis, J. Agric. Food Chem. 54 (2006)
585 2695-2704.
586 [10] W.L. Fan, M.C. Qian, Headspace solid phase microextraction and gas chromatography-
587 olfactometry dilution analysis of young and aged Chinese "Yanghe Daqu" liquors, J. Agric. Food
588 Chem. 53 (2005) 7931-7938.
589 [11] S. Chen, Y. Xu, M.C. Qian, Aroma Characterization of Chinese Rice Wine by Gas
590 Chromatography-Olfactometry, Chemical Quantitative Analysis, and Aroma Reconstitution, J.
591 Agric. Food Chem. 61 (2013) 11295-11302.
592 [12] P. Comuzzo, L. Tat, A. Tonizzo, F. Battistutta, Yeast derivatives (extracts and
593 autolysates) in winemaking: Release of volatile compounds and effects on wine aroma
594 volatility, Food Chem. 99 (2006) 217-230.
595 [13] P. Comuzzo, L. Tat, A. Liessi, L. Brotto, F. Battistutta, R. Zironi, Effect of Different Lysis
596 Treatments on the Characteristics of Yeast Derivatives for Winemaking, J. Agric. Food Chem. 60
597 (2012) 3211-3222.
598 [14] F. San Juan, J. Cacho, V. Ferreira, A. Escudero, Aroma Chemical Composition of Red
599 Wines from Different Price Categories and Its Relationship to Quality, J. Agric. Food Chem. 60
600 (2012) 5045-5056.
601 [15] E. Campo, J. Cacho, V. Ferreira, The chemical characterization of the aroma of dessert
602 and sparkling white wines (Pedro Ximenez, Fino, Sauternes, and Cava) by gas chromatography-
603 olfactometry and chemical quantitative analysis, J. Agric. Food Chem. 56 (2008) 2477-2484.
604 [16] Y. Sekiwa, K. Kubota, A. Kobayashi, Characteristic flavor components in the brew of
605 cooked clam (Meretrix lusoria) and the effect of storage on flavor formation, J. Agric. Food
606 Chem. 45 (1997) 826-830.
607 [17] R.R. Peralta, M. Shimoda, Y. Osajima, Further identification of volatile compounds in
608 fish sauce, J. Agric. Food Chem. 44 (1996) 3606-3610.
609 [18] X. Zhu, Q. Su, J. Cai, J. Yang, Optimization of microwave-assisted solvent extraction for
610 volatile organic acids in tobacco and its comparison with conventional extraction methods,
611 Anal. Chim. Acta 579 (2006) 88-94.
612 [19] J.R. Bruna, M. Fernandez, E.M. Hierro, J.A. Ordonez, L. de la Hoz, Combined use of
613 Pronase E and a fungal extract (Penicillium aurantiogriseum) to potentiate the sensory
614 characteristics of dry fermented sausages, Meat Sci. 54 (2000) 135-145.
615 [20] H. Guth, Quantitation and sensory studies of character impact odorants of different
616 white wine varieties, J. Agric. Food Chem. 45 (1997) 3027-3032.
617 [21] R. Lopez, M. Aznar, J. Cacho, V. Ferreira, Determination of minor and trace volatile
618 compounds in wine by solid-phase extraction and gas chromatography with mass
619 spectrometric detection, J. Chromatogr. A 966 (2002) 167-177.
620 [22] A.L. Robinson, P.K. Boss, H. Heymann, P.S. Solomon, R.D. Trengove, Development of a
621 sensitive non-targeted method for characterizing the wine volatile profile using headspace
622 solid-phase microextraction comprehensive two-dimensional gas chromatography time-of-
623 flight mass spectrometry, J. Chromatogr. A 1218 (2011) 504-517.
624 [23] R. Lopez, E. Gracia-Moreno, J. Cacho, V. Ferreira, Development of a mixed-mode solid
625 phase extraction method and further gas chromatography mass spectrometry for the analysis
626 of 3-alkyl-2-methoxypyrazines in wine, J. Chromatogr. A 1218 (2011) 842-848.
627 [24] L. Cullere, M. Bueno, J. Cacho, V. Ferreira, Selectivity and efficiency of different
628 reversed-phase and mixed-mode sorbents to preconcentrate and isolate aroma molecules, J.
629 Chromatogr. A 1217 (2010) 1557-1566.
630 [25] D.G. Watson, Chemical derivatization in gas chromatography, in: P.J. Bauch (Ed.), Gas
631 chromatography. A practical approach, Oxford University Press, New York, 1993, p. 426.
632 [26] M. Gallart, S. Francioli, A. ViuMarco, E. LopezTamames, S. Buxaderas, Determination of
633 free fatty acids and their ethyl esters in musts and wines, J. Chromatogr. A 776 (1997) 283-291.

634 [27] C.-L. Hsu, W.-H. Ding, Determination of low-molecular-weight dicarboxylic acids in
635 atmospheric aerosols by injection-port derivatization and gas chromatography-mass
636 spectrometry, Talanta 80 (2009) 1025-1028.
637 [28] O. Gyllenhaal, H. Brotell, P. Hartvig, Determination of Free Fatty-Acids as
638 Pentafluorobenzyl Esters by Electron-Capture Gas-Chromatography, J. Chromatog. 129 (1976)
639 295-302.
640 [29] L. Mateo-Vivaracho, J. Cacho, V. Ferreira, Improved solid-phase extraction procedure
641 for the isolation and in-sorbent pentafluorobenzyl alkylation of polyfunctional mercaptans
642 Optimized procedure and analytical applications, J. Chromatogr. A 1185 (2008) 9-18.
643 [30] O. Gyllenhaal, Degradation of Pentafluorobenzyl Bromide in Extractive Alkylation
644 Procedure, J. Chromatog. 153 (1978) 517-520.
645 [31] F. Rodrigues, M. Caldeira, J.S. Camara, Development of a dynamic headspace solid-
646 phase microextraction procedure coupled to GC-qMSD for evaluation the chemical profile in
647 alcoholic beverages, Anal. Chim. Acta 609 (2008) 82-104.

648

649

650

651

652

653

654

655

656

657

658

659

660

661

FIGURES CAPTIONS

Fig. 1. Effect of an acid washing solution (pH 3.0 water/ 40% methanol) in the retained acids. Cumulative analyte loss versus volume (mL) of washing solution.

Fig. 2. Sketch of the derivatization reaction used. The carboxylate ion produced in the first step attacks the reactive (S_N2 mechanism) to produce the corresponding ester.

Fig. 3. Evolution of the yield (%) of the production of PFB-ester with time (hours) when 20 μL of reactive (PFBBr) and 0.1 M of transfer phase catalyser (NBu_4Cl) in a pH 6.0 buffered solution are used.

Fig. 4. SPE//GC-MS-NCI chromatogram (CP-WAX column) of a Pedro Ximenez wine: 163 ng/L 2-methylpentanoic acid (m/z 115), 110 ng/L 3-methylpentanoic acid (m/z 115), 759 ng/L 4-methylpentanoic acid (m/z 115) and 116 ng/L cyclohexanecarboxylic acid (m/z 127) and IS (m/z 115). The peaks signalled by the arrows correspond to the derivatized PFB-esters.

HIGHLIGHTS

- Development of a method of analysis for low concentration branched acids in wine
- The analytes are 2-, 3-, 4-methylpentanoic and cyclohexanecarboxylic acids
- SPE//PFBBr derivatization//GC-MS-NCI analysis for high selectivity and sensitivity
- First quantitative data on these analytes reported in wine, beer, whisky and brandy

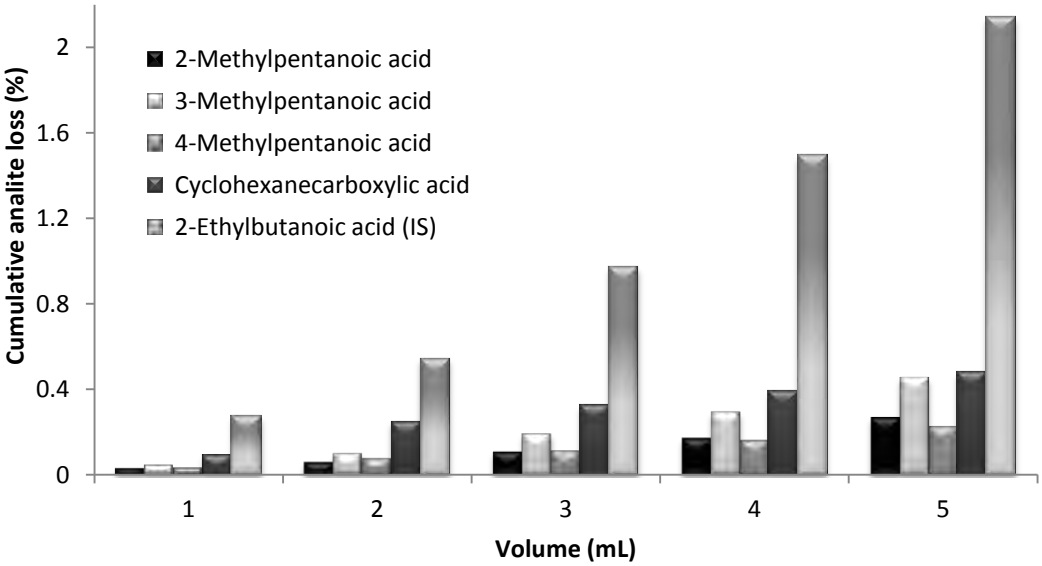

Fig. 1. Effect of an acid washing solution (pH 3.0 water/ 40% methanol) in the retained acids. Cumulative analyte loss versus volume (mL) of washing solution.

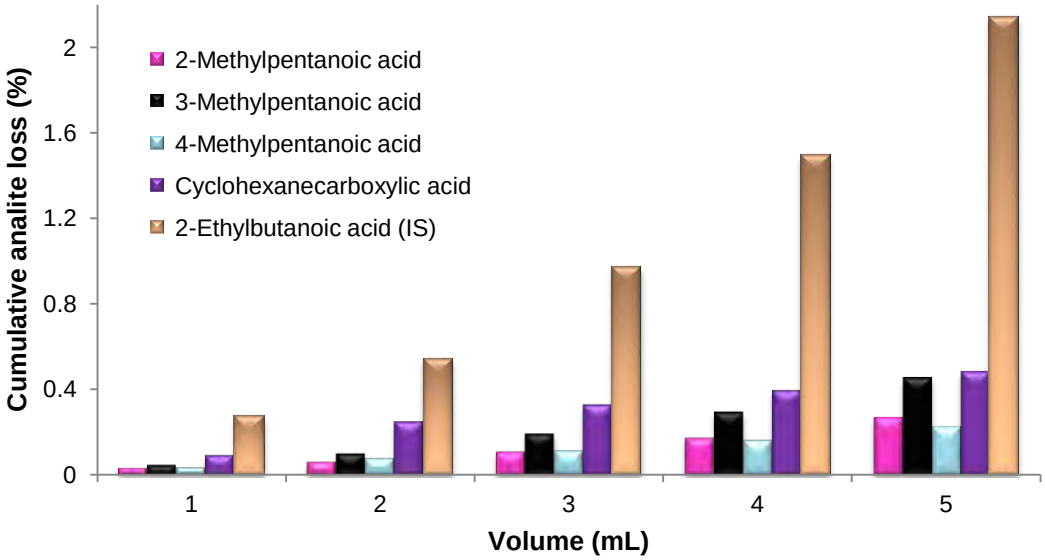

Fig. 1. Effect of an acid washing solution (pH 3.0 water/ 40% methanol) in the retained acids. Cumulative analyte loss versus volume (mL) of washing solution.

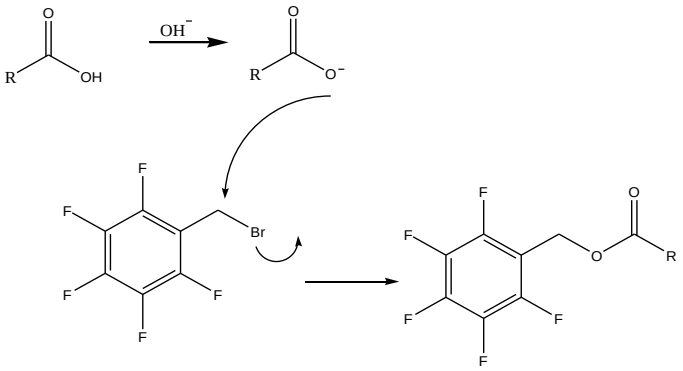

Fig. 2. Sketch of the derivatization reaction used. The carboxylate ion produced in the first step attacks the reactive (S_N2 mechanism) to produce the corresponding ester.

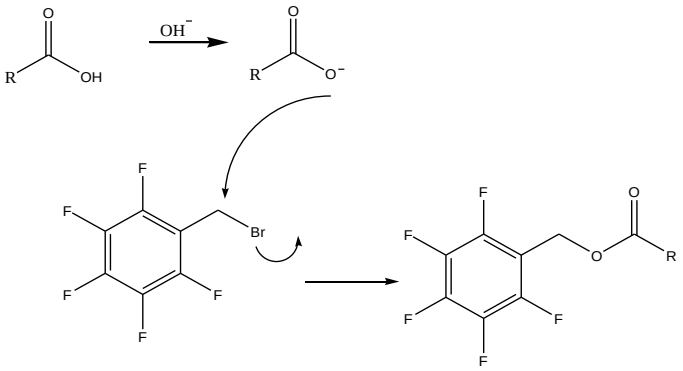

Fig. 2. Sketch of the derivatization reaction used. The carboxylate ion produced in the first step attacks the reactive (S_N2 mechanism) to produce the corresponding ester.

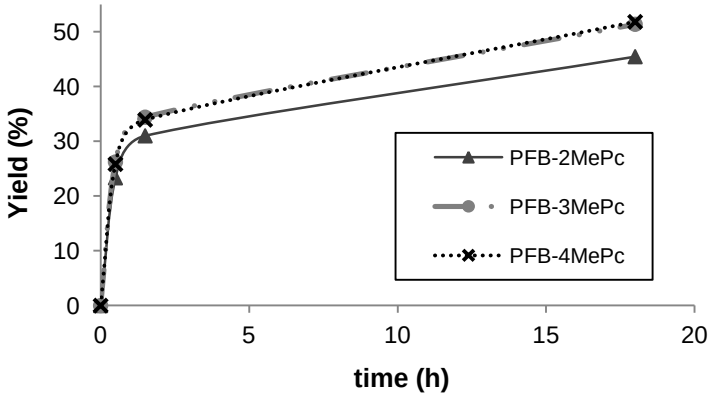

Fig. 3. Evolution of the yield (%) of the production of PFB-ester with time (hours) when 20 μL of reactive (PFBBr) and 0.1 M of transfer phase catalyser (NBu_4Cl) in a pH 6.0 buffered solution are used.

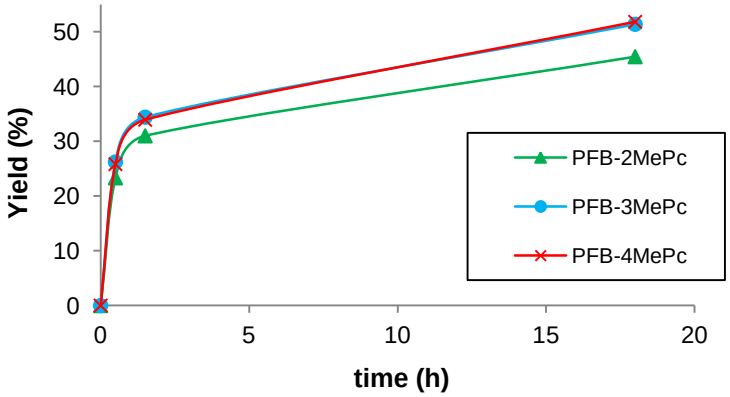

Fig. 3. Evolution of the yield (%) of the production of PFB-ester with time (hours) when 20 μL of reactive (PFBBr) and 0.1 M of transfer phase catalyser (NBu_4Cl) in a pH 6.0 buffered solution are used.

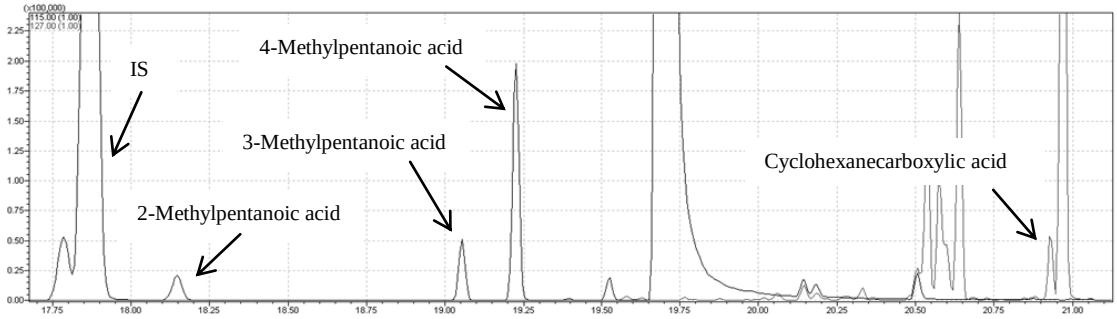

Fig. 4. SPE//GC-MS-NCI chromatogram (CP-WAX column) of a Pedro Ximenez wine: 163 ng/L 2-methylpentanoic acid (m/z 115), 110 ng/L 3-methylpentanoic acid (m/z 115), 759 ng/L 4-methylpentanoic acid (m/z 115) and 116 ng/L cyclohexanecarboxylic acid (m/z 127) and IS (m/z 115). The peaks signalled by the arrows correspond to the derivatized PFB-esters.

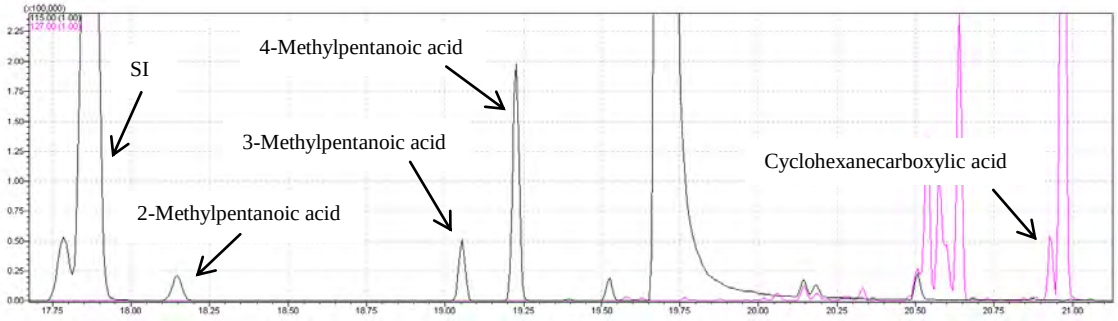

Fig. 4. SPE//GC-MS-NCI chromatogram (CP-WAX column) of a Pedro Ximenez wine: 163 ng/L 2-methylpentanoic acid (m/z 115), 110 ng/L 3-methylpentanoic acid (m/z 115), 759 ng/L 4-methylpentanoic acid (m/z 115) and 116 ng/L cyclohexanecarboxylic acid (m/z 127). The peaks signalled by the arrows correspond to the derivatized PFB-esters.

Table 1
Volume of elution solution needed to completely elute the analytes (mL)

Compound	% MeOH				
	5%	15%	25%	35%	40%
2-Ethylbutanoic acid[a]	>20	15	10	5	5
2-Methylpentanoic acid	>20	15	10	5	5
3-Methylpentanoic acid	>20	15	10	5	5
4-Methylpentanoic acid	>20	15	15	5	5
Cyclohexanecarboxylic acid	>20	>20	20	10	5

[a] Internal standard.

Table 2
Linear retention indices (LRI) for the analytes and IS studied and their corresponding PFB esters in DB-WAX and DB-5 columns

Compound	CAS number	Mw		LRI (DB-WAX)		LRI (DB-5)	
		Acid	PFB-ester	Acid	PFB-ester	Acid [a]	PFB-ester
2-Ethylbutanoic acid[b]	88-09-5	116	296	1768	1666	------	1371
2-Methylpentanoic acid	97-61-0	116	296	1774	1674	------	1377
3-Methylpentanoic acid	105-43-1	116	296	1800	1731	------	1408
4-Methylpentanoic acid	646-07-1	116	296	1811	1745	------	1416
Cyclohexanecarboxylic acid	98-89-5	128	308	2054	2009	------	1603

[a] LRI for acids not calculated in DB-5 due to the bad chromatographic properties of acids in this column
[b] Internal standard.

Table 3. Comparison of the limits of detection (ng/L) in different ionization modes: the numbers between brackets are the m/z values of the fragments used.

Analyte	**Ion trap[a]**	**Quadrupole[b]**	
	EI (SCAN)	**EI (SIM)**	**NCI (SCAN)**
Column	*DB-WAX ETR (60 m, 0.25 mm, 0.25 μm)*	*DB-WAX ETR (30 m, 0.25 mm, 0.25 μm)*	*CP-WAX (25 m, 0.15 mm, 0.25 μm)*
2MePc	47 (206)	81 (254)	2.4 (115)
3MePc	111 (115)	156 (240/115)[c]	0.4 (115)
4MePc	-----[d]	-----[d]	1.2 (115)
Cyclohxc	237 (81)	92 (81)	0.6 (127)

[a] 2μL cold splitless
[b] 1μL hot splitless
[c] The LD was the same with both fragments.
[d] Co-elution with the sub-product of the reaction PFBOH that hindered the analysis.

Table 4
Method figures of merit

Compound	$R^{2\,a}$	DL[b] (ng/L)	Linear range (ng/L)	Recovery (%) ± RSD (%)	
				Fino	Red young 4
2-Methylpentanoic acid	0.9990	2.4	8-3300	98 ± 6	99 ± 1
3-Methylpentanoic acid	0.9985	0.4	1.3-1500	93 ± 8	101 ± 1
4-Methylpentanoic acid	0.9884	1.2	4-3600	80 ± 10	104 ± 5
Cyclohexanecarboxylic acid	0.9974	0.6	2-1900	121 ± 9	107 ± 4

[a] Average R^2 (n= 21)

[b] Detection limits for the overall method

Table 5
Wines and other alcoholic beverages analysed: type, year, ethanol content and concentration (ng/L) of 2-, 3-, 4-methylpentanoic and cyclohexanecarboxylic acids.

Sample type	Year	% Ethanol	2-MePc	3-MePc	4-MePc	Cyclohx
Young white 1	2011	13.5	87	75	938	19
Young white 2	2012	13.0	127	54	625	61
Young white 3	2012	13.0	128	150	1140	92
Rosé 1	2012	13.5	73	85	802	125
Rosé 2	2012	13.0	62	77	632	120
Young red 1	2011	13.5	74	90	1370	62
Young red 2	2011	14.0	154	103	1540	40
Young red 3	2011	13.5	121	135	1930	14
Young red 4	2012	14.5	116	84	781	<DL
Barrel aged red 1	2006	14.0	91	102	1880	190
Barrel aged red 2	2007	13.5	140	143	1220	64
Barrel aged red 3	2010	13.0	335	217	2050	109
Natural sweet wine 1	2012	15.2	53	67	143	1780
Natural sweet wine 2	2012	15.5	120	52	431	15
Fino Sherry	3[a]	15.0	73	1390	3430	18
Oloroso Sherry	4[a]	18.0	106	170	647	84
Manzanilla Sherry	3[a]	15.0	75	535	3730	35
Pedro Ximenez Sherry	2[a]	15.0	163	110	759	116
Beer	-------	5.2	421	743	3520	56
Imperial Brandy	5[a]	38.0	735	148	1950	91
Pure Malt Scotch Whisky	10[a]	40.0	2630	2040	3810	177

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

DL: Detection limit