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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

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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

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1 # Determination of 2-, 3-, 4-methylpentanoic and cyclohexanecarboxylic
2 acids in wine: development of a selective method based on solid phase
3 extraction and gas chromatography-negative chemical ionization mass
4 spectrometry and its application to different wines and alcoholic
5 beverages.

6

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8

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14

15 **Abstract**

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

30

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

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33

34 ## 1. Introduction

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

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

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

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57 esters, has already been described in Chinese liquors made from mixtures of cereals [9,
58 10]. 4-methylpentanoic acid has also been determined in rice wine [11] and 2-
59 methylpentanoic acid has been identified in some commercially available yeast
60 derivatives added to wine [12, 13]. Finding these acids in wine would be the first step
61 towards eventually proving or refuting the hypothesis that the origin of the
62 corresponding ethyl esters is esterification.

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

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

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

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

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

101

102 **2. Materials and methods**

103 *2.1. Reagents and standards*

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104 The standards of 2-methylpentanoic acid (2MePc), 3-methylpentanoic (3MePc)
105 acid, 4-methylpentanoic acid (4MePc), cyclohexanecarboxylic acid and 2-ethylbutanoic
106 (2EtBc) acid were supplied by Aldrich (Steinheim, Germany) with purity higher than
107 96% in all cases. 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) and tetrabutylamonium
108 chloride (NBu_4Cl) (> 97%) were also obtained from Aldrich.

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

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

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

121

122 *2.2. Wines and alcoholic beverages samples*

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

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

128

129 *2.3. SPE method development*

130 *2.3.1. Sorbent selection and breakthrough volumes*

131 Mixed-mode anionic Oasis MAX sorbent (60 mg, 3 mL reservoir) was
132 conditioned with 2 mL DCM, 2 mL MeOH and 4 mL hydroalcoholic solution (12%
133 ethanol). Synthetic wine was spiked with 1.6 mg/L of the acids studied and its pH was
134 adjusted to 7.0 prior to the loading of the cartridges. Vacuum suction was not applied in
135 this particular experiment to avoid losses of the non-retained analytes due to their
136 volatility. The percolated solutions (10 mL fractions up to 100 mL) were collected and
137 the pH readjusted to 2.7. The solutions were then analysed with the method described in
138 [21]. Lichrolut EN sorbent (200 mg, 3 mL reservoirs) conditioned with 4 mL DCM, 4
139 mL MeOH and 4 mL hydroalcoholic solution (12% ethanol) was used to analyse the
140 samples. After loading the samples under vacuum suction, 1 mL of milli-Q water was
141 used to clean the cartridges. The sorbent was dried under nitrogen and the analytes were
142 eluted with 1.6 mL of DCM.

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

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

151

152 *2.3.2. Removal of interferences and matrix compounds*

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

158

159 *2.3.3. Optimization of the elution strategy*

160 Five LiChrolut EN cartridges conditioned as aforementioned were loaded with
161 50 ml each of a young red wine from Rioja spiked with 5 mg/L of the analytes. Five
162 solutions of milli-Q water buffered at pH 7.0 with NaH_2PO_4/Na_2HPO_4, containing
163 different percentages of MeOH (5, 15, 25, 35, and 40) were prepared and used to elute a
164 different cartridge each (4 fractions of 5 mL). The 20 recovered eluates were each
165 supplemented with 2 mL of a 0.625 M tartaric acid solution and the appropriate volume
166 of MeOH in each case to reach a final concentration of 25% MeOH. All the eluates
167 were then analysed following the method mentioned in [21].

168

169 *2.3.4. Second SPE step*

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

181

182 *2.4. Derivatization*

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

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

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

200

201 *2.5. Gas Chromatography and Mass Spectrometry*

202 The chromatographic analysis during the development of the SPE method was
203 done with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass-
204 spectrometric detection system supplied by Varian (Sunnyvale, CA, USA). The
205 capillary column used was a DB-WAX ETR (J&W Scientific, Folsom, CA, USA) (60
206 m x 0.25 mm, 0.25 μm) preceded by a 3 m x 0.25 mm uncoated (deactivated,
207 intermediate polarity) pre-column from Supelco (Bellefonte, USA). Helium was used as
208 a carrier gas at a flow rate of 1 mL/min. The oven temperature programme was 5 min at
209 40 ºC, then increasing by 8 ºC/min up to 170 ºC, with a second ramp at 4 ºC min^{-1} up to
210 190 ºC and a third ramp at 8 ºC min^{-1} up to 220 ºC. This temperature was maintained for
211 20 min. The MS-parameters were: MS transfer line 220 ºC and ionization chamber
212 temperature 170 ºC. Electronic impact was used with a scan range of 40-360 m/z. The
213 acquisition was done in automatic gain control (AGC) with a filament intensity current
214 of 30 μA.

215 Two μL of the extract was injected in splitless mode for 2 min with a pulse pressure of
216 30 psi.

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217 The optimization of the reaction was monitored with the help of an FID GC-
218 8000 supplied by Carlo Erba (Milan, Italy), with hydrogen as the carrier gas (100 kPa),
219 nitrogen as make-up gas (95 kPa) and hydrogen (35 kPa) and air (60 kPa) in the FID
220 detector. The column used was a DB-WAX (30 m, 0.32 mm, 0.5 μm) with a deactivated
221 pre-column (3 m, 0.25 mm). The oven temperature program was 40º C during 3 minutes
222 followed by a 20º C ramp up to 220º C held during 20 min. Injection of 1 μL sample
223 was done in splitless mode at 250 ºC.

224 The analysis of the extracts in the definitive method was done in a GC-MS
225 Shimadzu QP-2010 Plus (Shimadzu Corp., Kyoto, Japan). The column was a CP-WAX
226 52 CB (25 m, 0.15 mm, 0.25 μm) supplied by Varian preceded by a 3 m x 0.25 mm
227 uncoated (deactivated, intermediate polarity) pre-column obtained from Supelco
228 (Bellefonte, USA). One μL of sample was injected at 250 ºC with 3 min of splitless
229 time with helium at 45 cm/s as the carrier gas. The oven was programmed as follows:
230 40 ºC during 4 min, ramp of 80 ºC/min up to 80 ºC and held for 1 min, 4 ºC/min ramp
231 up to 130 ºC, 30 ºC/min ramp up to 190 ºC and a final ramp of 100 ºC/min up to 230 ºC
232 and held for 15 min. The spectrometer was operated in negative chemical ionization
233 (NCI) mode with methane as ionization gas (2 bars of pressure). The temperature of the
234 ion source was set at 220 ºC and the transfer line temperature was 250 ºC. A DB-5
235 column (20 m, 0.18 mm, 0.18 μm) was also fitted to this system to calculate the LRI of
236 the analytes.

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

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242 WAX ETR (30 m, 0.25 mm, 0.25 μm). The chromatographic conditions were those
243 already reported for the Shimadzu system. As for the ionization, two segments were
244 done to acquire the internal standard and the methylpentanoic acids, and the
245 cyclohexanecarboxylic acid respectively. The fragments used in the first segment were:
246 181, 268, 254, 240, 73 and 115 m/z; whereas in the second segment the fragments were:
247 181, 81 and 109 m/z.

248

249 *2.6. Proposed method*

250 Extraction of the analytes: 200 mg Lichrolut EN sorbent (pre-packed in 3 mL
251 cartridges) is conditioned with 4 mL DCM, 4 mL MeOH and 4 mL hydroalcoholic
252 solution (12%). Fifty mL of wine is spiked with 2EtBc acid (IS) to obtain a 10 μg/L
253 concentration. Highly alcoholic beverages, such as whisky and brandy, are diluted prior
254 to the analysis to 12% ethanol content. The sample is then loaded into the cartridges
255 with the help of a vacuum manifold. The sorbents are washed with 3 mL of aqueous
256 solution (40% MeOH) buffered at pH 3 with H_3PO_4/NaH_2PO_4. Elution of the analytes is
257 done with 5 mL of aqueous solution (40% MeOH) buffered at pH 7.0
258 (NaH_2PO_4/Na_2HPO_4). The buffer is broken with the addition of 2 mL of 0.625 M
259 tartaric acid solution to the collected eluate and is diluted to the required volume with
260 milli-Q water in a 10 mL volumetric flask (final pH 3.0). The resulting solution is
261 loaded into a 50 mg LiChrolut EN cartridge (1 mL volume) previously conditioned with
262 1 mL DCM and 1 mL MeOH. The sorbents are vacuum-dried and eluted with 0.5 mL
263 DCM and recovered in 2 mL glass vials.

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

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266 30 minutes at room temperature, the reaction is stopped with concentrated HCl (37%).
267 The organic phase is washed with 1 mL acidified milli-Q water (pH 1), dried with
268 Na_2SO_4, and then purified through a 200 mg bed of silica-gel 60 (1 mL cartridge). For
269 this, 1.5 mL of hexane is added and discarded. Elution of the analytes is done with 1 mL
270 of hexane/ 40% toluene (v/v). One μL of the extract is finally injected in the GC-MS
271 and analysed in NCI mode as described in the previous section.

272

273 *2.7. Method validation*

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

280

281 **3. Results and discussion**

282 *3.1. SPE method development*

283 *3.1.1. Sorbent selection and breakthrough volumes*

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

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

290 The first attempt to selectively isolate the analytes was based on the use of their
291 acidic properties. The pKa of all the analytes studied is less than five. Consequently, pH
292 7.0 was chosen to have the analytes in their anionic form. Then, a mixed-mode anionic
293 sorbent (Oasis MAX), combining anionic-exchange properties with hydrophobic
294 retention, was assayed. However, the results were not good enough because the
295 breakthrough volumes were less than 10 mL (data not shown). This option was then
296 discarded because the small breakthrough volumes were not expected to provide a
297 sufficiently high concentration factor to be able to detect the analytes, taking into
298 account the low concentrations expected according to our preliminary experiments.

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

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

307

308 *3.1.2. Removal of interferences and matrix compounds*

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

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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

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

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

344

345 *3.1.4. Second SPE step*

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

353 The aforementioned extract had the analytes in their anionic form. Tartaric acid
354 (0.625 M) was used to reduce the pH from 7.0 to 3.0 and to convert the analytes into
355 their neutral form, allowing their retention in the second SPE cartridge.

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

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

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

365 The loss of analytes was less than 1% in all cases (methylpentanoic acids,
366 cyclohexanecarboxylic acid and IS). This implies that a 50 mg sorbent bed is adequate
367 to retain the analytes in the second extraction. The elution of this second cartridge was
368 done with 0.5 mL of DCM. Reproducibility (n=9) of the whole SPE method, tested with
369 three wines spiked at a level of 10 µg/L and analysed three times each, was good with
370 relative standard deviations below 7% for all analytes except for 4-methylpentanoic acid
371 which, because of chromatographic interference, was 30%. The interference, identified
372 as ethyl 4-hydroxybutanoate, presented isobaric coincidences with 4-methylpentanoic
373 acid in all the relevant fragments. Improving the resolution by changing the temperature
374 programming rate was not possible and the strategy of changing the column to avoid
375 this co-elution was impractical because of the bad chromatographic properties of acids
376 in apolar stationary phases. A washing step in the second cartridge allowed the
377 interference to be reduced to 1%, but a large amount of the analytes was also eliminated
378 (40-60%) and thus this option was discarded. Instead, derivatization was chosen to
379 improve both the selectivity and the sensitivity of the method.

380

381 *3.2. Derivatization*

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

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

398 The characterization of the derivatives was done in an ion-trap analyzer in
399 electronic ionization (EI) mode and in a quadrupole analyzer both in EI mode and in
400 NCI mode. The spectra are shown in figures 1-3 in the supplementary material. The
401 linear retention indices determined in a DB-5 and in a DB-WAX are presented in table
402 2.

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

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

413 The study of the kinetic profiles showed an increase in the yield that doubled in
414 20 h as shown in figure 3. However, 30 minutes was selected as the reaction time as a
415 compromise between adequate sensitivity and time efficiency.

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

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

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

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

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

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

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

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

461

462 *3.3. Method validation*

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

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

486

487 *3.4. Occurrence in different wines and alcoholic beverages*

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

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

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

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

513 Some interesting parallels can be found between the concentrations of the four
514 acids analysed in this work and their corresponding ethyl esters that were analysed for
515 the first time in [8]. First, only ethyl 4-methylpentanoate was found in young wines in
516 [8], always below 300 ng/L. The four acids have been quantified in all the young wines
517 analysed here, 4-methylpentanoic acid in a range between 600 and 2000 ng/L while
518 none of the other three acids is above 150 ng/L in any sample. This is in accordance
519 with the esterification hypothesis exposed by Campo et al. [8]. The low levels of 2- and
520 3-methylpentanoic acids and cyclohexanecarboxylic acid would provide low levels of
521 their esters while 4-methylpentanoic acid would give rise to detectable amounts of its
522 ester even with a low esterification rate. Second, aged wines have larger amounts of the
523 acids than young wines, both white and red. As reported for the ethyl esters [8], there is
524 a great variability in the levels of the acids among white wines with special ageing
525 (Fino, Manzanilla, Oloroso and Pedro Ximenez). This could be attributed to the

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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

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

550

551 ## 5. Acknowledgements

552 The authors acknowledge the Spanish Government for the funding of this work through
553 project CICYT AGL2010-22355-C02-01. E. Gracia-Moreno is grateful to the Spanish
554 Government for support from the *"Formación de Profesorado Universitario"* (FPU)-
555 program (grant number: AP2008-03811). The authors acknowledge E. Franco Aladrén
556 for providing the non-commercial sweet wines. The authors appreciate very much the
557 work done by Julian Chancelor providing useful comments about English usage.

558

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667 FIGURES CAPTIONS

668

669 Fig. 1. Effect of an acid washing solution (pH 3.0 water/ 40% methanol) in the retained acids.
670 Cumulative analyte loss versus volume (mL) of washing solution.
671
672
673 Fig. 2. Sketch of the derivatization reaction used. The carboxylate ion produced in the first step
674 attacks the reactive (S_N2 mechanism) to produce the corresponding ester.
675
676
677 Fig. 3. Evolution of the yield (%) of the production of PFB-ester with time (hours) when 20 μL
678 of reactive (PFBBr) and 0.1 M of transfer phase catalyser (NBu_4Cl) in a pH 6.0 buffered solution
679 are used.
680
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682 Fig. 4. SPE//GC-MS-NCI chromatogram (CP-WAX column) of a Pedro Ximenez wine: 163 ng/L 2-
683 methylpentanoic acid (m/z 115), 110 ng/L 3-methylpentanoic acid (m/z 115), 759 ng/L 4-
684 methylpentanoic acid (m/z 115) and 116 ng/L cyclohexanecarboxylic acid (m/z 127) and IS (m/z
685 115). The peaks signalled by the arrows correspond to the derivatized PFB-esters.
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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

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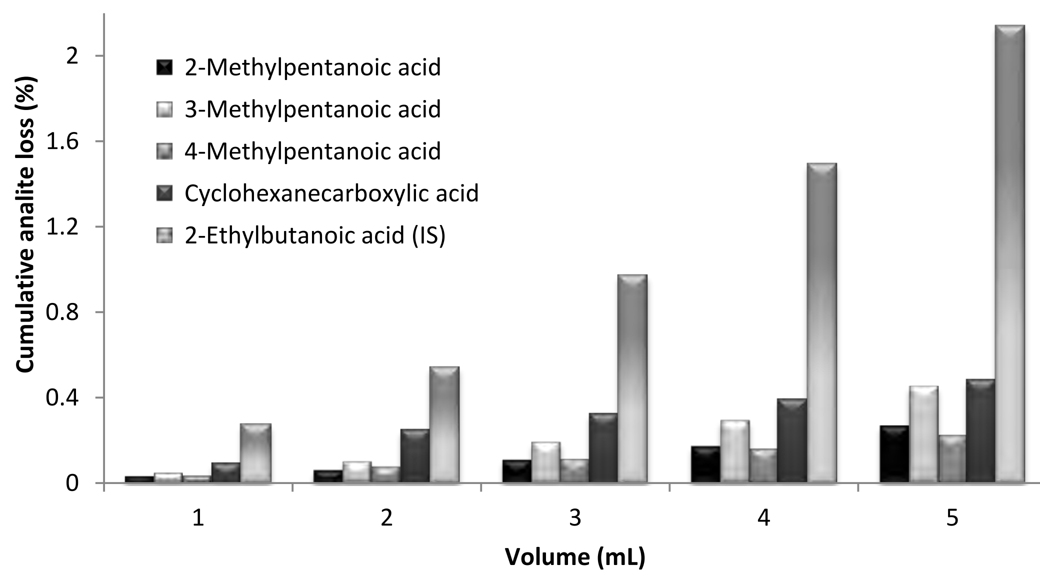

Fig. 1. Effect of an acid washing solution (pH 3.0 water/ 40% methanol) in the retained acids. Cumulative analyte loss versus volume (mL) of washing solution.

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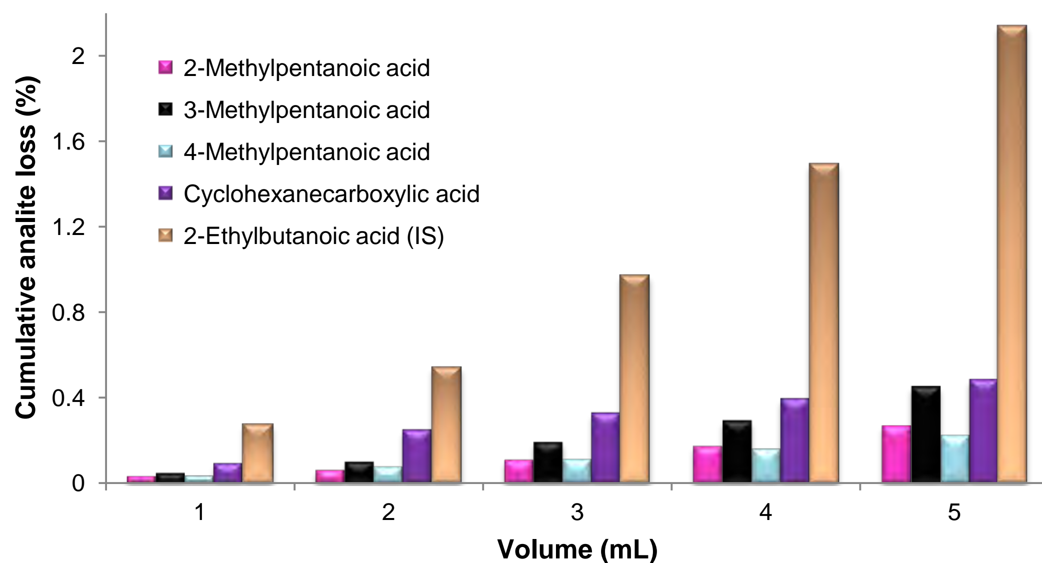

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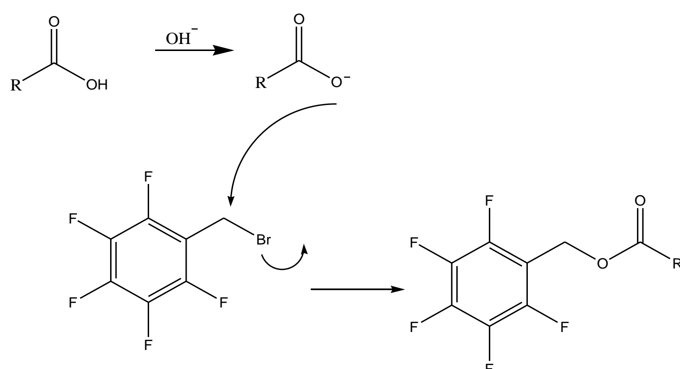

Fig. 2. Sketch of the derivatization reaction used. The carboxylate ion produced in the first step attacks the reactive (S_N2 mechanism) to produce the corresponding ester.

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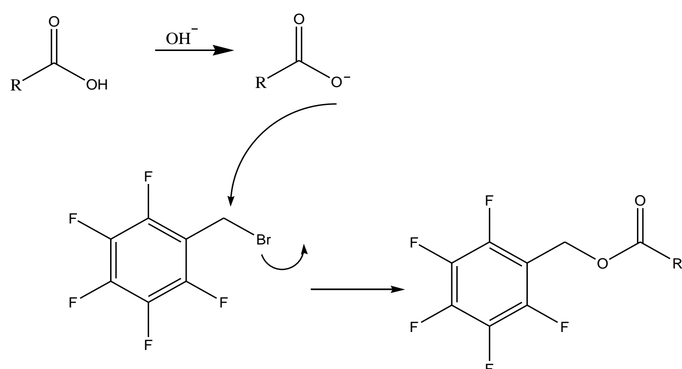

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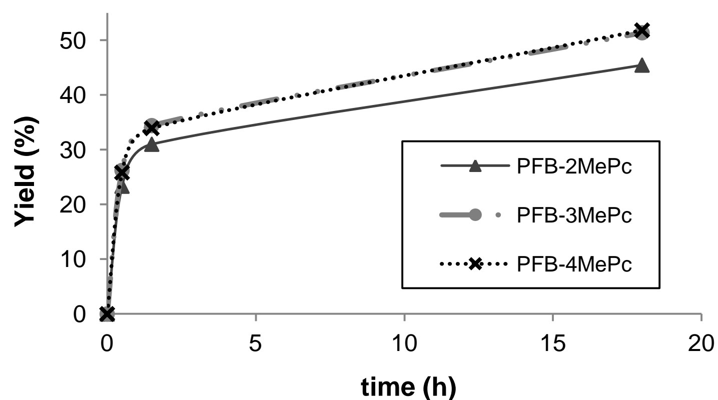

Fig. 3. Evolution of the yield (%) of the production of PFB-ester with time (hours) when 20 μL of reactive (PFBBr) and 0.1 M of transfer phase catalyser (NBu_4Cl) in a pH 6.0 buffered solution are used.

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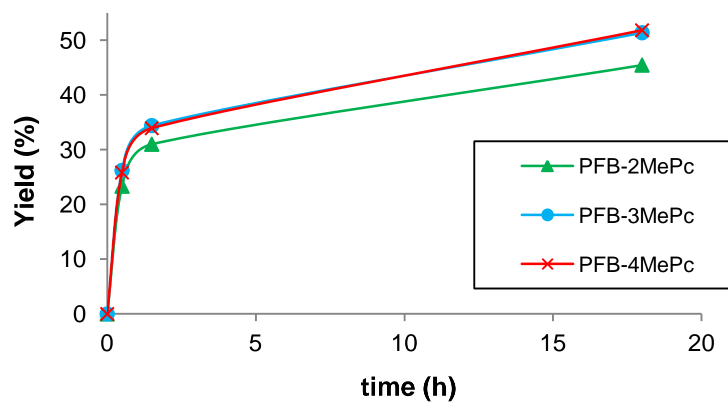

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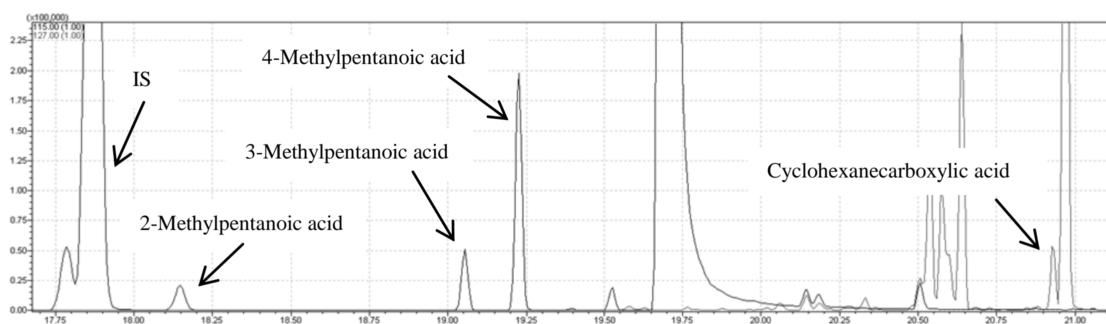

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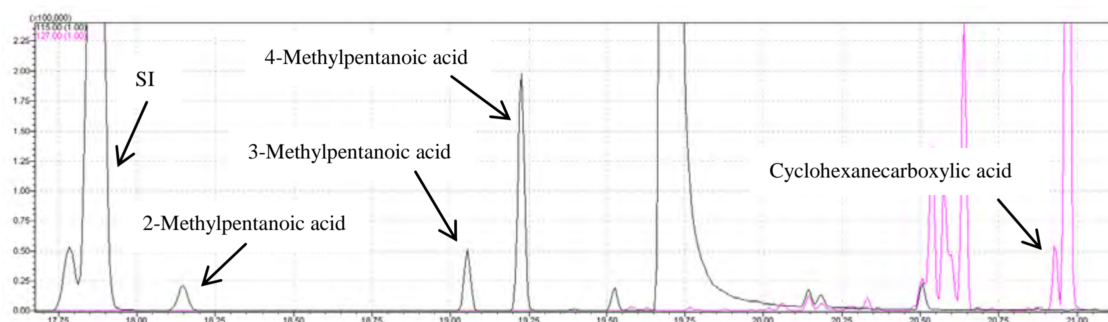

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Table 1
Volume of elution solution needed to completely elute the analytes (mL)

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.

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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.

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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.

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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

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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

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