

1 **Straightforward strategy for quantifying rotundone in**
2 **wine at ng L⁻¹ level using solid-phase extraction and**
3 **gas chromatography-quadrupole mass spectrometry.**
4 **Occurrence in different varieties of spicy wines**

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Código de campo cambiado

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28 **Abstract**

29 This paper presents a straightforward methodology to quantify rotundone in
30 wine at ng L^{-1} level. This compound, responsible for the black pepper aromatic note,
31 may have sensorial relevance in some wines due to its low odor threshold, estimated at
32 only 16 ng L^{-1} in red wines. The proposed strategy is based on solid phase extraction
33 and analysis by GC-MS.

34 The detection limit value was 0.6 ng L^{-1} , which is more than one order of
35 magnitude below its odor threshold in wine. Matrix effects have not been found and a
36 synthetic wine calibration was proposed. The precision of the method was evaluated in
37 reproducibility terms, obtaining a very acceptable value (RSD 4%).

38 The optimized and validated strategy was applied to quantify this molecule in
39 thirty wines belonging to different varieties as Graciano, Maturana tinta, Schioppettino,
40 Shiraz, Duras and Gamay. Two of these wines exhibited levels higher than 100 ng L^{-1} .

41

42 **Keywords**

43 Rotundone; wine; black pepper; Maturana tinta; Schioppettino; Gamay; Graciano;
44 aroma

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53 **1. Introduction**

54 Rotundone is a bicyclic sesquiterpene very potent from an aromatic point of
55 view, and responsible for a potent aromatic note of black pepper. Its odor threshold has
56 been estimated in red wine and in water, being these values 16 ng L⁻¹ and 8 ng L⁻¹
57 respectively (Wood et al. 2008). However, it has been noticeable that approximately 20
58 % of the population is anosmic for this pepper attribute (Wood et al. 2008).

59 In spite of its huge aromatic potential, rotundone was discovered by Siebert,
60 wood, Eelsey and Pollnitz (2008). The reason why rotundone had not been identified
61 before is this compound is relatively non volatile. In fact, this compound elutes
62 afterwards the vanilla zone and in GC-O studies, sniffers usually smell until vanillin
63 appears.

64 Very few papers focusing on the aromatic role of rotundone have been published
65 (Caputi et al. 2011; Mattivi et al. 2011; Scarlett, Bramley & Siebert, 2014; Siebert et al.
66 2008; Wood et al. 2008). The main difficulty for analyzing this compound is related to
67 the lack of a commercial standard of rotundone, which it requires synthesizing it.
68 Mattivi et al., (Mattivi et al. 2011) proposed an alternative process of synthesis which
69 was reported previously (Siebert et al. 2008; Wood et al. 2008).

70 The methodology proposed in the literature is based on a SPE followed by
71 SPME-GC-MS strategy (Caputi et al., 2011; Geffroy, Dufourcq, Carcenac, Siebert,
72 Herderich & Serrano, 2014; Mattivi et al. 2011; Siebert et al. 2008) and uses stable
73 isotope dilution analysis with d₅-rotundone as internal standard. These methods gave a
74 limit of detection below the sensory threshold of rotundone in water (Wood et al. 2008),
75 (8 ng L⁻¹), but involved a large number of steps, making it a very tedious analysis.

76 The peppery aroma, attributed to rotundone, is characteristic in some wines of
77 the Shiraz variety (very common in Australia). Furthermore, this aroma is also
78 characteristic of other Australian wines such as Mourvèdre and Durif, in which this
79 molecule can reach levels up to 145 ng L⁻¹ (Wood et al., 2008). Another grape variety
80 that produces high-quality wines with a clear “peppery” aromatic note is Vespolina
81 (Caputi et al. 2011; Mattivi et al. 2011). This red grape is autochthonous to
82 northwestern region of Italy. Recently, this compound has also been found in Duras
83 wines from the southwest of France (Geffroy et al. 2014). Schioppettino variety is an
84 autochthonous variety from the northeast of Italy characterized by spicy fragrances with
85 a special pepper flavor. Recent studies, not published yet, carried out by the Institut
86 Français de la Vigne et du Vin, have found this powerful molecule in French grape
87 cultivars, such as Pineau d’Aunis (up to 200 ng L⁻¹) and Gamay (up to 88 ng L⁻¹) and in
88 other varieties from La Rioja (Spain), as Graciano (≈ 15 ng L⁻¹) or Maturana Tinta (≈ 50
89 ng L⁻¹). Herderich et al. (2012) have already found this molecule in some Graciano
90 wines. Recently, it has been published that vineyard topography, vine vigor, vine
91 orientation, and bunch orientation together influence the microclimate in the grape
92 bunch zone, and therefore, influence grape quality parameters, including rotundone
93 levels (Zhang, Barlow, Krstic, Herderich, Fuentes & Howell, 2015).

94 In this paper, the development and validation of a faster and simpler
95 straightforward method for the determination of this molecule faster and simpler than
96 other methodologies previously proposed is presented. This method is based on the
97 isolation using a solid phase extraction, (SPE) strategy, followed by GC-MS quadrupole
98 analysis, which allows the quantification of this aromatic compound at levels of a few
99 ng L⁻¹.

100 Another goal of this paper consists in evaluate the importance of this aromatic molecule
101 in different spicy wines such as some Gamay from Côtes d’Auvergne (France), Shiraz
102 from France and Australia, Schioppettino from Italy and Graciano and Maturana tinta,
103 from La Rioja, (Spain), for which black pepper is a frequent aroma descriptor. Other
104 five monovarietal wines have been also analyzed, in order to evaluate if in these
105 samples this molecule is relevant from an aromatic point of view.

106 **2. Materials and methods**

107 **2.1 Reagents, standards and materials**

108 **2.1.1. Solvents**

109 Dichloromethane and methanol were purchased from Merck (Darmstadt, Germany).
110 Hexane was obtained from Fisher Scientific (Loughborough, UK) and ethyl ether from
111 VWR Chemicals (Leuven, Belgium). Ethanol and sodium hydrogencarbonate were
112 purchased from Panreac (Barcelona, Spain). Water was purified in a Milli-Q system
113 supplied by Millipore (Bedford, Germany).

114 **2.1.2. SPE material**

115 Bond Elut PPL 200 mg cartridges were supplied by Agilent Technologies (Las
116 Rozas, Madrid, Spain). Semiautomated solid phase extraction was carried out with a
117 VAC ELUT 20 station supplied by Varian (Walnut Creek, USA).

118 **2.1.3. Chemical Standards**

119 The rotundone standard was kindly supplied by the Australian Wine Research
120 Institute, AWRI, Adelaide, Australia.

121 The volatile compounds assayed as potential internal standards, β -damascone
122 and β -caryophyllene, were supplied by Sigma-Aldrich (Darmstadt, Germany). Methyl

123 octanoate, used as a standard for the estimation of the degree of purity of the rotundone
124 solution, was also purchased from Sigma-Aldrich (Darmstadt, Germany).

125 **2.1.4. Wine samples**

126 Table 1 provides information relating to the thirty wines analyzed in this study
127 belonging to different grape varieties. Rotundone has been determined in six Graciano,
128 five Maturana tinta wines, six Gamay, four Schioppettino and four Shiraz wines.
129 Moreover, only one sample from other varieties such as Arrat, Malbec, Abouriou, Duras
130 and Mourvèdre has been included in this study.

131 **2.2. Gas chromatography-mass spectrometry**

132 GC-MS analysis was carried out using a Shimadzu QP-2010 gas chromatograph
133 with a quadrupole mass spectrometric detection system. The injection was carried out in
134 the large volume mode typical of the PTV (Programmable Temperature Vaporizing)
135 injector. The initial injector temperature was 65 °C during 0.40 minutes, then raised to
136 300 °C at 400 °C min⁻¹, remaining at that temperature for 32 minutes. After this, a rate
137 of - 400 C° min⁻¹ was applied to return to the initial temperature (65 °C). The injection
138 was in splitless mode and after 4.35 min the split valve was opened. Four microliters
139 were injected. The carrier gas was He at a constant linear velocity of 44 cm/s (1.50 mL
140 min⁻¹ flow rate). The column was a DB-5 capillary column from Agilent J&W
141 (Paloalto, California, USA), 30 m x 0.32 mm i.d., with 1.00 µm film thickness. The
142 chromatographic oven was held at 40 °C for 4.00 min, then raised to 300 °C at 8 °C min⁻¹
143 ¹, remaining at that temperature for 10 min. The acquisition time is only 35 minutes.
144 The ion source was operated in EI mode. The temperature of the ion source was 220 °C
145 and the transfer line was kept at 300 °C. The mass analyzer was operated in single ion
146 monitoring (SIM) mode, selecting the following ion mass: m/z 218 for rotundone, m/z

147 177 for β -damascone and m/z 204 for β -caryophyllene. Apart from m/z 218, fragment used to
148 quantify rotundone, m/z 203 was monitored and employed as qualifier ion to confirm the presence of
149 this molecule in real wines.

150 The solvent cut window was 9.8 min.

151 **2.3. Proposed method**

152 Rotundone was extracted using SPE as an isolation step and quantified with a
153 GC-MS analysis using a variation of the method proposed by Ferreira, Jarauta, Ortega
154 and Cacho (2004) to analyze aliphatic lactones in wine.

155 In the proposed method for analyzing rotundone, 30 μL of internal standard
156 solution (β -damascone $\approx 100 \text{ mg L}^{-1}$ in ethanol) were added to 50 mL of wine, and then
157 this volume was loaded into a 200 mg Bond ELUT PPL cartridge. The cartridges were
158 previously conditioned with 4 mL of dichloromethane, 4 mL of methanol and 4 mL of a
159 hydro-alcoholic solution containing 12 % (v/v) ethanol. After loading the wine, the bed
160 was washed with 5 mL of water and with 20 mL of an aqueous solution containing
161 methanol (70 % (v/v)) and 1 % NaHCO_3 . After the cartridges were dried, the elution
162 was carried out with 2 mL of hexane containing 25 % (v/v) of ethyl ether. Then, this
163 extract was concentrated to 200 μL by a nitrogen stream.

164 The area of the peak corresponding to the m/z fragment chosen for the rotundone
165 was normalized to the β -damascone. These relative areas were interpolated in the
166 calibration curve, prepared by the SPE-GC-MS analysis of synthetic wine samples
167 containing known amounts of rotundone and the internal standard. The methodology
168 used for the analysis of synthetic wine samples is exactly the same that the proposed
169 method for real wine samples. Synthetic wine used in this experiment consisted of a 12
170 % water/ethanol (v/v) solution containing 5 g L^{-1} tartaric acid with the pH adjusted to
171 3.5.

172 **2.4. Method development and validation**

173 **2.4.1. Measurement of the rotundone purity**

174 To evaluate the degree of purity of the rotundone standard kindly provided by
175 the AWRI, the methodology proposed by Tissut, Rochat, Debonneville and Chaintreau
176 (2012) was applied. This strategy is based on making a model mixture with
177 approximately the same constituent proportions of the target compound and the methyl
178 octanoate (employed as an internal standard).

179 A solution containing rotundone and methyl octanoate was made, being the
180 concentration of both compounds around 20 mg L⁻¹ in ethanol. This mixture was
181 homogenized and analyzed by GC-FID, using a Varian CP 3800 chromatograph. The
182 column was a DBWAX (J&W Scientific), 30 m x 0.32 mm i.d., with 0.5 µm film
183 thickness. The chromatographic oven was held at 40 °C for 5.0 minutes, raised to 100
184 °C at 15 °C min⁻¹, and then raised to 220 °C at 10 °C min⁻¹, remaining at that
185 temperature for 10 minutes.

186 A volume of 0.5 µL of this mixture was injected in splitless mode. The
187 temperature of the injector was 250 °C and a pulse of pressure of 20 psi during 3
188 minutes was used. The splitless time was 5 minutes. This time was chosen to ensure that
189 the rotundone was transferred completely into the gas chromatograph. The mixture
190 containing rotundone and methyl octanoate at 20 mg L⁻¹ in ethanol was prepared four
191 times and injected into the chromatograph. The degree of purity of the rotundone was
192 estimated in each injection and finally the average of the purities obtained was
193 calculated.

194 Furthermore, one more concentrated solution was prepared (300 mg L⁻¹ of each
195 substance in ethanol) and injected in split mode (1:20). This mixture was repeated again
196 several times and the average purity was calculated.

197 **2.4.2. Preliminary experiments**

198 **Methodology:** Two different previously developed and published methods were
199 assayed (Ferreira et al. 2004; Lopez, Aznar, Cacho & Ferreira, 2002). Both of them
200 were based on solid phase extraction (SPE) isolation, although different resins were
201 used. Some changes (resins, elution solvent) were introduced to the method developed
202 originally to analyze aliphatic lactones (Ferreira et al. 2004) in order to optimize a
203 strategy for quantifying rotundone in wines at ng L⁻¹ level.

204 **Choice of internal standard:** Two different aromatic compounds were tested as
205 possible internal standards: β-damascone and β-caryophyllene. Their chemical
206 structures are similar to rotundone and they are not present in the usual composition of
207 wines.

208 **2.4.3. Method validation**

209 Method linearity was evaluated in synthetic wine and in a real red wine up to
210 300 ng L⁻¹ through seven calibration points (0, 10, 25, 50, 100, 200, 300 ng L⁻¹).

211 Evaluation of the existence of matrix effects depending on the characteristics of
212 each wine was carried out. For this task, recovery data was estimated by comparing the
213 increase in the relative area observed upon addition of a known amount of rotundone (≈
214 35 ng L⁻¹) in fifteen different wines with the relative area obtained as a consequence of
215 the addition of the same amount of this compound in a synthetic wine matrix.

216 A precision parameter was evaluated through its reproducibility, analyzing eight
217 wines spiked with rotundone ($\approx 35 \text{ ng L}^{-1}$) in duplicate on different days.

218 The sensitivity of the proposed method was evaluated in terms of the limit of
219 detection (L.D.), which was defined as the concentration which gives a signal to noise
220 ratio of 3. This limit was estimated at synthetic wine and different red wines. For this
221 last calculation, the s/n ratios of four wines containing low amounts ($< 10 \text{ ng L}^{-1}$) of this
222 compound were used.

223 **2.5. Odor threshold in water**

224 Sensory analysis was carried out following standard procedures (AENOR,
225 1997). The sensory panel was formed by eight judges aged 23 – 40. All the judges have
226 had previous experience in sensory analysis. The tastings were carried out in a
227 conditioned tasting room. In all cases, samples (20 mL) were served in black tulip-
228 shaped wine coded glasses covered with a Petri-dish top after an equilibration time of
229 30 min at 21 °C. The determination of odor thresholds was carried out according to the
230 Spanish Norm (AENOR 87-006-92) by means of triangle tests, presenting to the
231 panelists solutions containing the tested odorant progressively diluted (the dilution
232 factor was 1:2). The odor threshold was determined in water matrix, via triangle tests
233 following a decreasing-concentration order, and was defined as the minimum amount of
234 odorant which must be added to a water sample to cause a sensory difference
235 (significant at $p < 0.05$) between the reference and the spiked sample.

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239 **3. Results and discussion**

240 **3.1. Measurement of the rotundone purity**

241 For an analytical laboratory, checking the purity of a target compound once its
242 standard is opened requires having a fresh standard to measure its relative response
243 factor (RRF) and then calculating the purity of the sample in concerned. In addition,
244 there are no defined GC peaks corresponding to the degradation products. For the same
245 reasons, quantifying a compound in a mixture such as a natural source can be difficult.
246 To solve this dilemma, a simulated RRF can be used.

247 The reproducibility associated to the results obtained was very high (3 % in
248 terms of relative standard deviation). The purity degree obtained for the standard
249 assayed was 72 %. Figure 1 shows the chromatogram corresponding to the rotundone
250 solution, analyzed by GC-MS. Appearance of several peaks can be observed, probably
251 being products formed during the synthesis of the rotundone.

252 As consequence, this degree of purity has been taken into account in the various
253 mathematical operations used in the validation and application of this analytical
254 method.

255 **3.2. Preliminary experiments**

256 Firstly, a method developed for the determination of minor compounds in wines
257 was assayed (López et al. 2002). This strategy was based on a solid phase extraction
258 (SPE) in which 50 mL of wine was percolated through LiChrolut EN resins (Merck)
259 and subsequently eluted with a small volume of dichloromethane. However, this
260 methodology did not provide a satisfactory detection limit because the value obtained
261 (25 ng L^{-1}) was slightly higher than the odor threshold estimated in red wine (16 ng L^{-1}).

262 The second strategy assayed was based on the method developed by Ferreira et
263 al. (2004) to analyze aliphatic lactones in wines, in which 50 mL of wine are percolated
264 in a 200 mg cartridge filled by Bond Elut ENV resins. Interferences were removed with
265 20 mL of methanol/water (40:60) with 1 % NaHCO₃. Elution was carried out with 1.8
266 mL of dichloromethane and the extract was concentrated to 150 µL and then analyzed
267 by GC-ion trap-MS. This strategy was more successful than the first. The concentration
268 factor of this strategy is 250, while that of the first strategy tests was only 25. This
269 strategy allowed to obtain a better limit of detection, (12 ng L⁻¹), lower than the
270 corresponding odor threshold in red wine. However, it was decided to reduce it further.

271 Thus, some important improvements were implemented in this last methodology
272 in order to reach lower detection limit and to obtain a cleaner chromatographic profile.
273 The main changes were related to the type of sorbent, the composition of the washing
274 up step and the solvent to carry out the elution.

275 Originally, this method used Bond Elut ENV resins. In this work, Bond Elut
276 PPL resins were also tested and were finally chosen because the extracts provided were
277 slightly cleaner than the previous ones.

278 Different washing up composition (40 %, 50 %, 60 % and 70 % of methanol,
279 v/v), containing all of them 1 % NaHCO₃, were checked, looking for a large removal of
280 major volatile compounds that could be considered as interferences. As a consequence
281 of this experiment, it can be noted that a washing solution containing 60 % or 70 % of
282 methanol resulted in a cleaner chromatogram profile in comparison with washing
283 solution containing 40 % or 50 % of methanol, as it can be observed in Figure 2.
284 Therefore, a lower detection limit was obtained, (ranged from 5 ng L⁻¹, in the case of

285 using 40 % of methanol to less than 1 ng L⁻¹, when the percentage of methanol
286 increased up to 70 %).

287 Extracts obtained using dichloromethane as eluent solvent gave high background
288 signal and some peaks could be an interference to quantify rotundone. To reduce this
289 dirtiness, different solvents were tested: hexane, hexane/ether 10 % v/v, hexane/ether 25
290 % v/v and pentane/ethyl acetate 25 %. Finally, hexane/ether 25 % v/v was selected as a
291 solvent and a consequent cleaner chromatogram profile was acquired together with a
292 slight improvement of the detection limit.

293 In order to find an optimum internal standard, two different aromatic
294 compounds, β -damascone and β -caryophyllene, were tested. These compounds have a
295 molecular weight similar to that of rotundone. β -Caryophyllene is a bicyclic
296 sesquiterpene with 15 atoms of carbon (like rotundone), while β -damascone is a
297 norisoprenoid that present a similar structure to rotundone. Both β -damascone and
298 rotundone contain a ketone group. Experimentally, the reproducibility parameter was
299 evaluated using both compounds as internal standards. Eight wines were analyzed in
300 duplicate and the relative areas were calculated. As a result of this experiment, β -
301 damascone was chosen because when this compound is used as an internal standard,
302 only 4 % relative standard deviation (RSD) was obtained. However, the use of β -
303 caryophyllene provided worse reproducibility (15 % in terms of RSD)

304 **3.3. Method validation**

305 Quality parameters such as linearity, limit of detection and precision were
306 evaluated after the optimum conditions were established.

307 The linearity of the method was obtained by plotting the calibration curve of the
308 corresponding ion peak areas (normalized by that of the internal standard) versus the

309 known amounts of rotundone added to synthetic wine samples and to red wine samples.
310 Standard calibration curve was plotted for concentrations from 3 to 300 ng L⁻¹. The
311 method showed good linearity in both matrices, up to 300 ng L⁻¹, as it can be seen in
312 Table 2.

313 Recovery degree was calculated by comparing the ion peak area increments
314 observed between the analysis of spiked (35.4 ng L⁻¹) and non spiked wine samples,
315 with the ion peak areas corresponding to the spiked mass of rotundone in synthetic
316 wine. The experiment was carried out with fifteen different red wines. These results
317 showed recovery data nearly of 100 % in all cases. Therefore, the average recovery was
318 estimated as 96 %, with a relative standard deviation of 4 %. These results demonstrated
319 that the methodology developed was free from matrix effects and made it possible to
320 use the calibration graph constructed with synthetic wine.

321 The reproducibility of the proposed method was evaluated by the analysis of
322 eight wines spiked with rotundone level of 35.4 ng L⁻¹ carried out in two different days.
323 As a result of these experiments, the method precision was quite satisfactory in terms of
324 reproducibility, whose relative standard deviation (RSD, %) was only 4 %.

325 The sensitivity of the proposed method was evaluated in terms of limit of
326 detection (L.D.) and limit of quantification (L.Q.) These parameters were
327 experimentally calculated from the signal to noise ratio obtained in the analysis of six
328 wines, all of them contained levels of rotundone lower 10 ng L⁻¹. The limit of detection
329 obtained was 0.6 ng L⁻¹, and the limit of quantification was 2.0 ng/L⁻¹. Both values were
330 considered very satisfactory limits. These values are lower than the limits provided by
331 Mattivi et al., (2011), but slightly higher than the values obtained by Siebert et al.,
332 (2008).

333 **3.4. Odor threshold determination**

334 In the literature (Wood et al. 2008), 8 ng L⁻¹ has been identified as the odor
335 threshold of this sesquiterpene in water.

336 The levels of rotundone spiked in water solution were: 40 ng L⁻¹, 20 ng L⁻¹, 10
337 ng L⁻¹, 7.5 ng L⁻¹ and 5 ng L⁻¹. The first four additions, including the 7.5 ng L⁻¹, were
338 detected by the panelists with a significance level higher than 95 %. However, the
339 addition of 5 ng L⁻¹ did not produce a significant effect. As a consequence, an
340 intermediate level between 5 and 7.5 was considered as the odor threshold of rotundone,
341 estimated in water. This odor threshold value (6.2 ng L⁻¹) is in agreement with the odor
342 threshold estimated by Wood et al. (2008).

343 **3.5. Analysis of wines**

344 The proposed method has been applied to the analysis of thirty wines produced
345 from different grape varieties such as Graciano and Maturana tinta grapes from La
346 Rioja, Gamay grapes from Côtes d'Auvergne in France and other varieties such as
347 Shiraz, Arrat, Abouriou and Duras, as is shown in Table 1. Figure 3 displays the
348 chromatographic signal provided by a real wine containing a level of 11.7 ng L⁻¹ of
349 rotundone. Table 3 shows the concentration, expressed as ng L⁻¹, of rotundone found in
350 all these wines. As it can be seen, this compound has been quantified in all the samples
351 except for four of the six Graciano wines, in which the level was found to be lower than
352 the detection limit of the method (0.6 ng L⁻¹).

353 Taking into account the odor threshold estimated in red wine, 16 ng L⁻¹, (Wood
354 et al. 2008), it is possible to estimate the odor active value (OAV) for rotundone in each
355 wine sample by dividing the concentration level of this molecule by the red wine odor
356 threshold. It should be emphasized that 18 of the 30 wines analyzed showed aroma
357 values (OAV) higher or equal to the unit. The highest levels were found in a Duras wine

358 and in two wines belonging to the minority variety of La Rioja, named as Maturana
359 tinta, exceeding 100 ng L⁻¹ in all these cases. The maximum value, (162.5 ng L⁻¹) was
360 reached in the Duras wine, which equates to an aroma value of 10 units. The rest of
361 Maturana tinta wines exhibited very low levels, being even lower than the odor
362 threshold in wine. These different levels of concentration of rotundone between wines
363 of the same variety could be explained by different process of winemaking. For
364 example, Caputi et al. (2011) demonstrated that long skin contact could contribute to
365 enriching “pepper” notes in wines.

366 The wine ranked in fourth place according to its high level of rotundone
367 correspond to one of the Gamay wines analyzed (Gamay 1). This wine had a
368 concentration level of 84.7 ng L⁻¹, but the rest of Gamay wines showed concentration
369 between 19 and 50 ng L⁻¹. Schioppettino wines presented very similar concentration of
370 this compound, which were between 19 and 35 ng L⁻¹. These levels are enough to set
371 the black pepper aromatic note of this compound both in the nose and the mouth.
372 Concentration of rotundone in Arrat and Abouriou wines was around 50 ng L⁻¹,
373 however only one wine of each variety was analyzed and therefore it cannot be
374 confirmed if this compound is characteristic of these kind of wines. On the other hand,
375 Malbec and Moúrvadre wines have levels of rotundone below its odor threshold (16 ng
376 L⁻¹), although they are very close to it.

377 However, amazingly low levels of rotundone were found in the four Shiraz
378 wines analyzed. Only one of them had a concentration higher than its odor threshold.
379 Anyway, it is widely known that the flavor of Shiraz, is commonly described as “spicy”,
380 “dark fruit” and “berry”- like, but it may present very different styles depending on the
381 region of origin and the characteristics of the winemaking process. Considering

382 Australian Shiraz wines, it can be distinguished two different styles: one from a
383 relatively cooler climate in Western Australia (Margaret River) and the other from a
384 warmer region in the South Australia (Barossa Valley). Mayr, Geue, Holt, Pearson,
385 Jeffery and Francis (2014) reported this molecule in levels above its odor threshold
386 concentration in the Shiraz wines from cool zones, whereas the concentration in the
387 Barossa Valley Shiraz was only 1 ng L^{-1} . Apart from this difference related to the origin
388 region, it is known that a same Shiraz wine can vary its level of rotundone in nearly one
389 order of magnitude depending if belong to a “peppery” or “not peppery year” (Wood et
390 al. 2008). The fact is that one of the Shiraz Australian wines analyzed in this work
391 presented levels above its odor threshold, although it did not belong to a region with an
392 appropriate climate to stimulate the presence of rotundone in wines.

393 As summary, some of the results presented here are consistent with recently
394 published results (Geffroy et al. 2014), in which authors found the highest levels of this
395 compound in a Duras wines (240 ng L^{-1}) and reported a level of 88 ng L^{-1} in a Gamay
396 wine. However, levels found in Schioppettino wines are one order of magnitude lower
397 than those reported in bibliography, which reach up to 560 ng L^{-1} in some wines from
398 the years 2001 and 2005 (Mattivi et al. 2011).

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406 **4. Conclusion**

407 This paper proposes a SPE strategy to determine rotundone in wine faster and
408 simpler than the methodologies reported in the literature (Caputi et al. 2011; Mattivi et
409 al. 2011; Siebert et al. 2008; Wood et al. 2008), requiring half of the volume of the
410 original methodology. The isolation step for 10 samples of wine takes approximately
411 one hour. The most time-consuming step is the concentration of each one of the extracts
412 obtained. However, the chromatographic analysis takes only 35 minutes.

413 This alternative strategy was applied to the analysis of rotundone in 30 wines
414 from different grape varieties, most of them characteristic for their peppery aroma. As a
415 result of these analyses, data about the concentration of rotundone in a wide range of
416 wines has been provided. In addition, it has been demonstrated that Gamay and
417 Schioppettino wines exhibit important levels of rotundone, which is responsible for the
418 characteristic peppery note of these kinds of wines. Graciano wines have shown low
419 levels of rotundone, except for one of them, which has exceeded very slightly its odour
420 threshold in red wines. It is noticeable the high level of this compound found in two
421 Maturana tina wine, both of them elaborated in 2010.

422

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479 **Figure Captions**

480 Figure 1. Chromatographic profile corresponding to the solution of rotundone with a
481 purity degree of 72 %. Mass spectrum of each chromatographic peak is shown

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483 Figure 2. Effect of the percentage of methanol present in the wash solution in the
484 chromatographic profile of rotundone, using the mass 218 for quantifying

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486 Figure 3. Chromatogram signal of rotundone (m/z 218, SIM) in a real wine, containing
487 11.7 ng L⁻¹ of this compound

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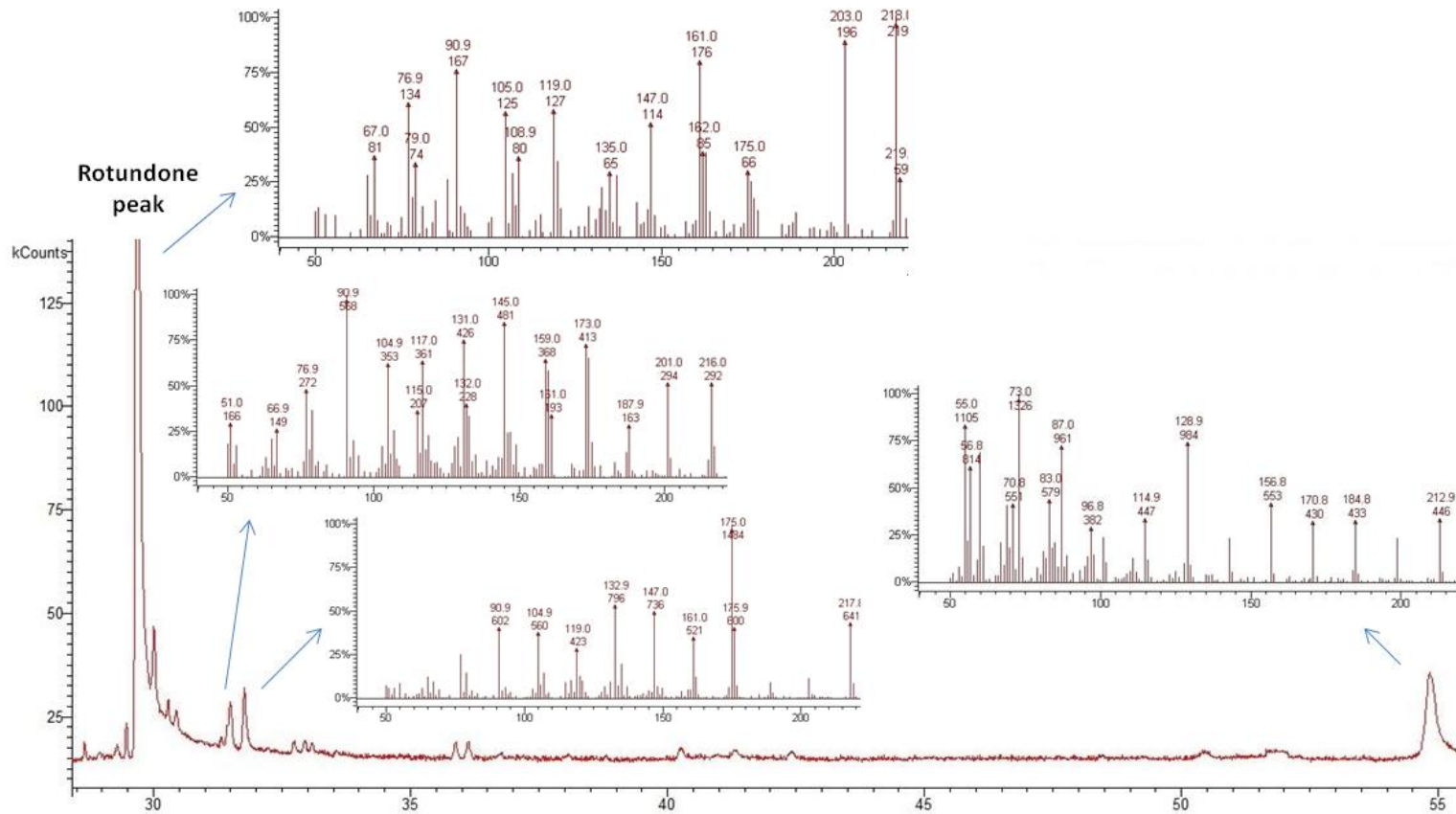
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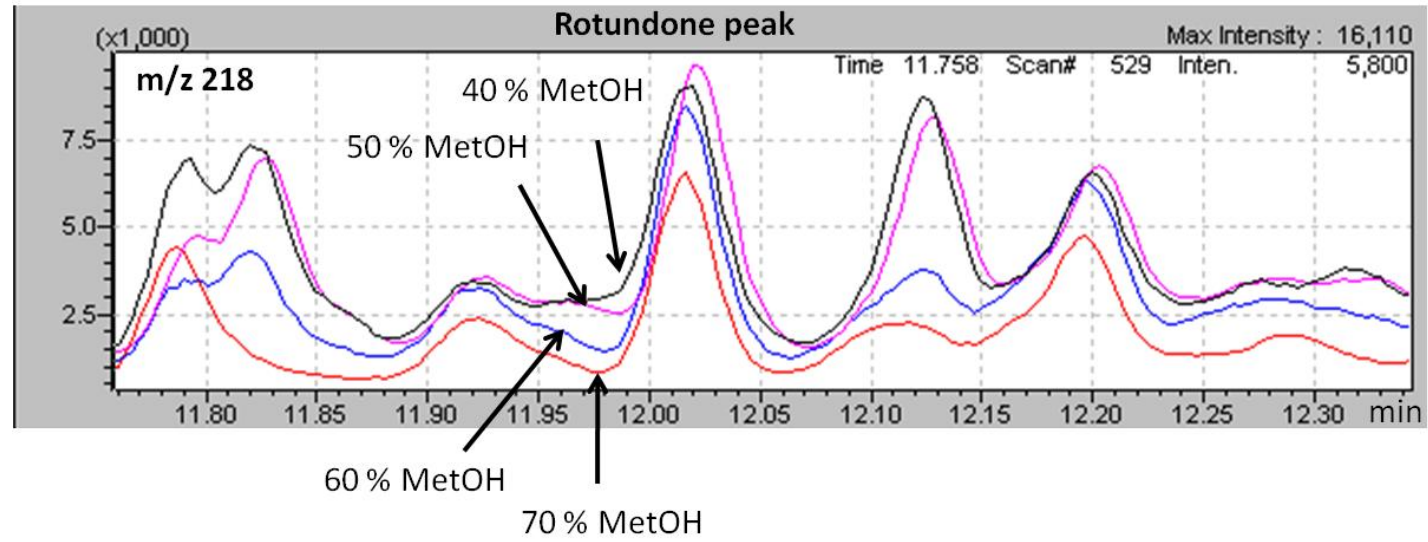
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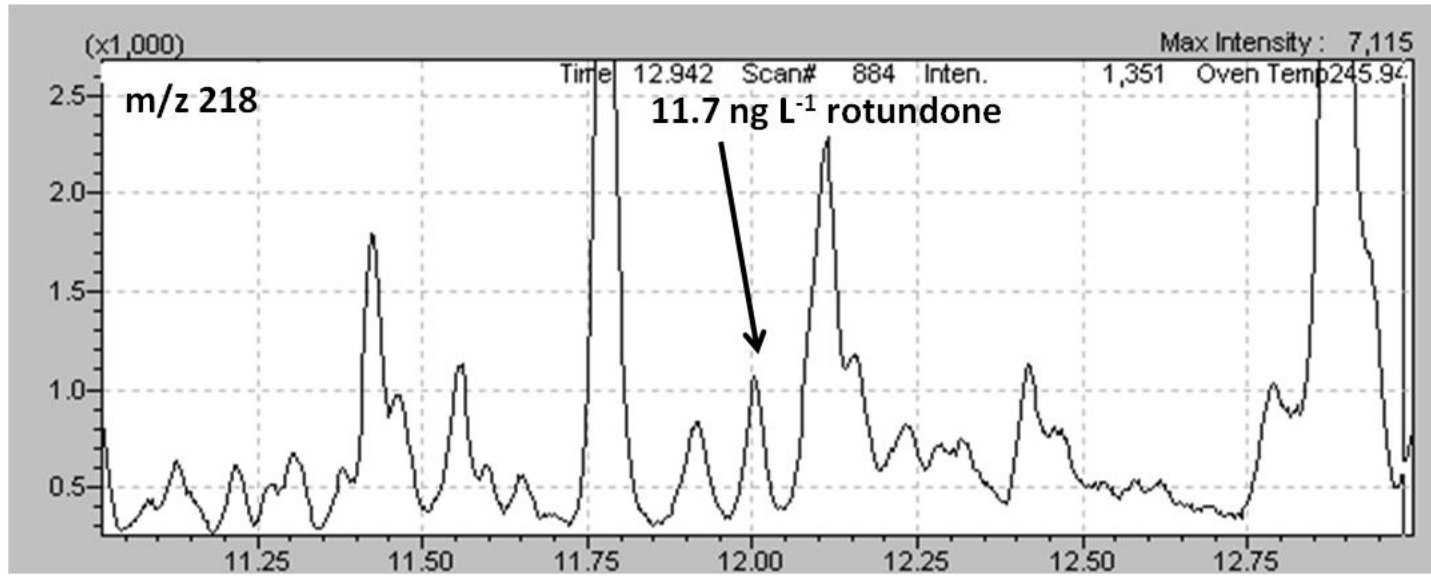
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Table 1. List of the thirty wines analyzed

Wines	Code	Grape variety	Origin
Beronia Graciano 2007	Gra 1	Graciano	La Rioja, Spain
Amaren Graciano 2006	Gra 2	Graciano	La Rioja, Spain
Ijalba Graciano 2005	Gra 3	Graciano	La Rioja, Spain
Graciano 2010, (non commercial)	Gra 4	Graciano	La Rioja, Spain
Coronado Graciano 2005	Gra 5	Graciano	La Rioja, Spain
Ilurce 2007 Graciano	Gra 6	Graciano	La Rioja, Spain
Maturana (Dionisio Ruiz Ijalba) 2010	Mat 1	Maturana tinta	La Rioja, Spain
Barón de Ley Varietales Maturana 2010	Mat 2	Maturana tinta	La Rioja, Spain
Ad Libitum Maturana tinta 2011	Mat 3	Maturana tinta	La Rioja, Spain
Ad Libitum Maturana tinta 2012	Mat 4	Maturana tinta	La Rioja, Spain
Nada que ver-Maturana tinta-Martínez Alessanco 2010	Mat 5	Maturana tinta	La Rioja, Spain
Bourrassol, Châteaugay 2013	Gam 1	Gamay	Côtes d'Auvergne, France
Cotes d'Auvergne Gamay 2013	Gam 2	Gamay	Côtes d'Auvergne, France
Gamay, boudes, Les Rivaux, 2013	Gam 3	Gamay	Côtes d'Auvergne, France
Domaine du Clos de la Parre, Le Clos, Gamay 2013	Gam 4	Gamay	Côtes d'Auvergne, France
Madargues, Gamay 2013	Gam 5	Gamay	Côtes d'Auvergne, France
Amnolium, Gamay 2013	Gam 6	Gamay	Côtes d'Auvergne, France
Schioppettino Dario Coos, Venezia Giulia 2012	Sch 1	Schioppettino	Friuli Venezia Giulia, Italy
Vigna Petrusa, Schioppettino 2010	Sch 2	Schioppettino	Friuli Venezia Giulia, Italy
Arzenton, Schioppettino 2010	Sch 3	Schioppettino	Friuli Venezia Giulia, Italy
Vigna Petrusa, Ribolla Nera 2013	Sch 4	Schioppettino	Friuli Venezia Giulia, Italy
Cape Mentelle 2011	Shi Aus 1	Shiraz	Margaret River, Southwest Australia
Chapoutier Domaine Tournon Mahtilda 2012	Shi Aus 2	Shiraz	Victoria, Southeast Australia
Domaine de Nidolères, La Pierroune 2010	Shi Fr 1	Shiraz	Côtes du Roussillon, France
Domaine de Majas Ravin des Sieurs 2012	Shi Fr 2	Shiraz	Côtes du Roussillon, France
Arrat 2010 (non commercial)	Arr	Arrat	Gascona, South-West France
Iri 2013 (non comercial)	Malb	Malbec	Nord oest, France
Just Abouriou 2011	Abou	Abouriou	Côtes du Marmandais, South-West France
Resdu 2013 (non commercial)	Dur	Duras	Gaillac, South France
Mourvedre 2014 (non comercial)	Mou	Mourvèdre	Nord oest, France

543 **Table 2. Linearity data of the proposed method**

Matrix	Slope	Intercept	r ²	Range (ng L ⁻¹)	n ^a	I.S.	L.D. (ng L ⁻¹)	L.Q. (ng L ⁻¹)
Synthetic wine	0.00095	0.0028	0.9937	≤ 300	7	β-damascone	0.5	1.7
Red wine	0.00092	0.0053	0.9974	≤ 300	7	β-damascone	0.6 ^b	2.0 ^b

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 545 ^a Seven calibration points were used. ^b The values of limit of detection and limit of quantification in wine were calculated as the average of these limits estimated in six
 546 different red wines.
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569 **Tabla 3. Level of rotundone (ng L⁻¹) found in thirty different wines, mostly of them**
 570 **characterized by spicy aromatic notes**

		C (ng/L)			
GRACIANO		average	desvest	OAV min	OAV max
Gra 1	< 0.6	n.e. *		< 0.04	1.1
Gra 2	17.2 ± 0.7				
Gra 3	11.8 ± 0.5				
Gra 4	< 0.6				
Gra 5	< 0.6				
Gra 6	< 0.6				
MATURANA TINTA		average	desvest	OAV min	OAV max
Mat 1	111.8 ± 4.5	48.0	50.5	0.5	7.0
Mat 2	15.1 ± 0.6				
Mat 3	10.7 ± 0.4				
Mat 4	8.5 ± 0.3				
Mat 5	94.0 ± 3.8				
SCHIOPPETTINO		average	desvest	OAV min	OAV max
Sch 1	34.6 ± 1.4	26.8	7.1	1.2	2.2
Sch 2	19.3 ± 0.8				
Sch 3	22.7 ± 0.9				
Sch 4	30.8 ± 1.2				
SHIRAZ		average	desvest	OAV min	OAV max
Shi Fr 1	18.0 ± 0.7	8.9	7.5	0.3	1.1
Shi Fr 2	4.1 ± 0.2				
Shi Aus 1	1.6 ± 0.1				
Shi Aus 2	11.7 ± 0.5				
GAMAY		average	desvest	OAV min	OAV max
Gam 1	84.7 ± 3.4	42.8	23.8	1.2	5.3
Gam 2	46.7 ± 1.9				
Gam 3	47.4 ± 1.9				
Gam 4	37.0 ± 1.5				
Gam 5	21.6 ± 0.9				
Gam 6	19.1 ± 0.8				
OTHER VARIETIES				OAV	
Arr	46.7 ± 1.9			2.9	
Malb	16.0 ± 0.6			1.0	
Abou	58.2 ± 2.3			3.6	
Dur	162.5 ± 6.5			10.2	
Mou	11.5 ± 0.5			0.7	

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572 Gra: Graciano wines; Mat: Maturana wines, Sch: Schioppettino wines, Shi: Shiraz wines; Gam: Gamay wines,
573 Arr: Arrat wines; Malb: Malbec wines; Abou: Abouriou; Dur: Duras; Mou: Mourvèdre.
574 The concentration was expressed with the uncertainty associated to these results (4 %, in terms of RSD). n.e.*: It
575 has not been estimated the average level due to rotundone was not detected in 5 of the 7 wines
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