Straightforward strategy for quantifying rotundone in wine at ng L^{-1} level using solid-phase extraction and gas chromatography-quadrupole mass spectrometry. Occurrence in different varieties of spicy wines

Laura Culleré*, Ignacio Ontañón, Ana Escudero, Vicente Ferreira

[a]*Laboratory for Aroma Analysis and Enology. Instituto Agroalimentario de Aragón (IA2. Associate unit to Instituto de las Ciencias de la Vid y el Vino (ICVV) (UR-CSIC-GR) Department of Analytical Chemistry, Faculty of Sciences, University of Zaragoza, 50009, Zaragoza, Spain*

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* To whom correspondence should be addressed

Phone: 34876553330

Fax: 34 976761292

Email: lcullere@unizar.es

Código de campo cambiado

Abstract

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

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

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

Keywords

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

1. Introduction

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

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

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

The methodology proposed in the literature is based on a SPE followed by SPME-GC-MS strategy (Caputi et al., 2011; Geffroy, Dufourcq, Carcenac, Siebert, Herderich & Serrano, 2014; Mattivi et al. 2011; Siebert et al. 2008) and uses stable isotope dilution analysis with d_5-rotundone as internal standard. These methods gave a limit of detection below the sensory threshold of rotundone in water (Wood et al. 2008), (8 ng L^{-1}), but involved a large number of steps, making it a very tedious analysis.

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

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

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

2. Materials and methods

2.1 Reagents, standards and materials

2.1.1. Solvents

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

2.1.2. SPE material

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

2.1.3. Chemical Standards

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

The volatile compounds assayed as potential internal standards, β-damascone and β-caryophylllene, were supplied by Sigma-Aldrich (Darmstadt, Germany). Methyl octanoate, used as a standard for the estimation of the degree of purity of the rotundone solution, was also purchased from Sigma-Aldrich (Darmstadt, Germany).

2.1.4. Wine samples

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

2.2. Gas chromatography-mass spectrometry

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

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

The solvent cut window was 9.8 min.

2.3. Proposed method

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

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

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

2.4. Method development and validation

2.4.1. Measurement of the rotundone purity

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

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

A volume of 0.5 µL of this mixture was injected in splitless mode. The temperature of the injector was 250 ºC and a pulse of pressure of 20 psi during 3 minutes was used. The splitless time was 5 minutes. This time was chosen to ensure that the rotundone was transferred completely into the gas chromatograph. The mixture containing rotundone and methyl octanoate at 20 mg L^{-1} in ethanol was prepared four times and injected into the chromatograph. The degree of purity of the rotundone was estimated in each injection and finally the average of the purities obtained was calculated.

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

2.4.2. Preliminary experiments

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

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

2.4.3. Method validation

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

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

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

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

2.5. Odor threshold in water

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

3. Results and discussion

3.1. Measurement of the rotundone purity

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

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

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

3.2. Preliminary experiments

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

The second strategy assayed was based on the method developed by Ferreira et al. (2004) to analyze aliphatic lactones in wines, in which 50 mL of wine are percolated in a 200 mg cartridge filled by Bond Elut ENV resins. Interferences were removed with 20 mL of methanol/water (40:60) with 1 % $NaHCO_3$. Elution was carried out with 1.8 mL of dichloromethane and the extract was concentrated to 150 µL and then analyzed by GC-ion trap-MS. This strategy was more successful than the first. The concentration factor of this strategy is 250, while that of the first strategy tests was only 25. This strategy allowed to obtain a better limit of detection, (12 ng L^{-1}), lower than the corresponding odor threshold in red wine. However, it was decided to reduce it further.

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

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

Different washing up composition (40 %, 50 %, 60 % and 70 % of methanol, v/v), containing all of them 1 % $NaHCO_{3,}$ were checked, looking for a large removal of major volatile compounds that could be considered as interferences. As a consequence of this experiment, it can be noted that a washing solution containing 60 % or 70 % of methanol resulted in a cleaner chromatogram profile in comparison with washing solution containing 40 % or 50 % of methanol, as it can be observed in Figure 2. Therefore, a lower detection limit was obtained, (ranged from 5 ng L^{-1}, in the case of using 40 % of methanol to less than 1 ng L^{-1}, when the percentage of methanol increased up to 70 %).

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

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

3.3. Method validation

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

The linearity of the method was obtained by plotting the calibration curve of the corresponding ion peak areas (normalized by that of the internal standard) versus the known amounts of rotundone added to synthetic wine samples and to red wine samples. Standard calibration curve was plotted for concentrations from 3 to 300 ng L^{-1}. The method showed good linearity in both matrices, up to 300 ng L^{-1}, as it can be seen in Table 2.

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

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

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

3.4. Odor threshold determination

In the literature (Wood et al. 2008), 8 ng L^{-1} has been identified as the odor threshold of this sesquiterpene in water.

The levels of rotundone spiked in water solution were: 40 ng L^{-1}, 20 ng L^{-1}, 10 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 detected by the panelists with a significance level higher than 95 %. However, the addition of 5 ng L^{-1} did not produce a significant effect. As a consequence, an intermediate level between 5 and 7.5 was considered as the odor threshold of rotundone, estimated in water. This odor threshold value (6.2 ng L^{-1}) is in agreement with the odor threshold estimated by Wood et al. (2008).

3.5. Analysis of wines

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

Taking into account the odor threshold estimated in red wine, 16 ng L^{-1}, (Wood et al. 2008), it is possible to estimate the odor active value (OAV) for rotundone in each wine sample by dividing the concentration level of this molecule by the red wine odor threshold. It should be emphasized that 18 of the 30 wines analyzed showed aroma values (OAV) higher or equal to the unit. The highest levels were found in a Duras wine and in two wines belonging to the minority variety of La Rioja, named as Maturana tinta, exceeding 100 ng L^{-1} in all these cases. The maximum value, (162.5 ng L^{-1}) was reached in the Duras wine, which equates to an aroma value of 10 units. The rest of Maturana tinta wines exhibited very low levels, being even lower than the odor threshold in wine. These different levels of concentration of rotundone between wines of the same variety could be explained by different process of winemaking. For example, Caputi et al. (2011) demonstrated that long skin contact could contribute to enriching “pepper” notes in wines.

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

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

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

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

4. Conclusion

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

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

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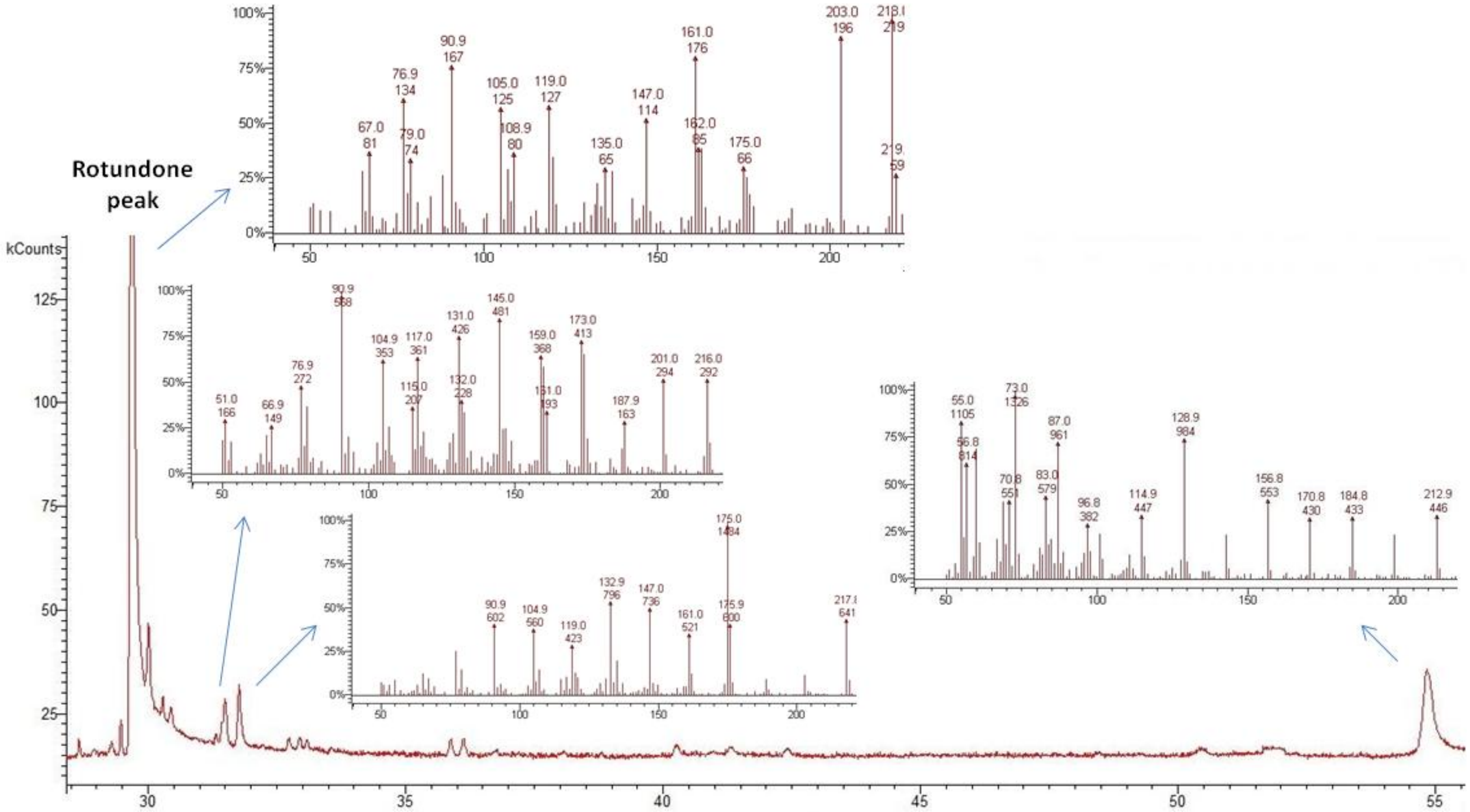
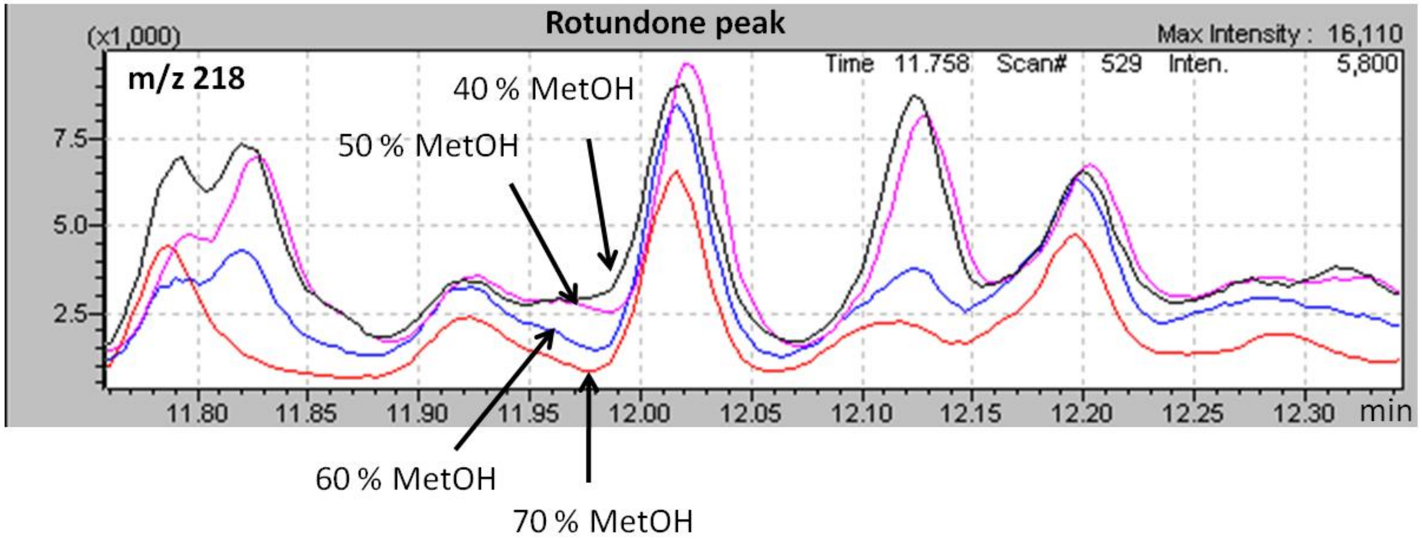
Figure Captions

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

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

Figure 3. Chromatogram signal of rotundone (m/z 218, SIM) in a real wine, containing 11.7 ng L^{-1} of this compound


Rotundone peak
kCounts
125
100
75
50
25
30
35
40
45
50
55
100%
75%
50%
25%
0%
90.9 167
76.9 134
67.0 81
79.0 74
105.0 125
108.9 80
119.0 127
135.0 65
147.0 114
161.0 176
162.0 85
175.0 66
203.0 196
218.0 219
219. 59
51.0 166
66.9 149
76.9 272
90.9 568
104.9 353
115.0 207
117.0 361
131.0 426
132.0 228
145.0 481
159.0 368
161.0 193
173.0 413
187.9 163
201.0 294
216.0 292
90.9 602
104.9 560
119.0 423
132.9 796
147.0 736
161.0 521
175.0 1484
175.9 600
217.0 641
55.0 1105
56.8 814
70.8 551
73.0 1326
83.0 579
87.0 961
96.8 382
114.9 447
128.9 984
156.8 553
170.8 430
184.8 433
212.9 446


Rotundone peak
(x1,000)
Max Intensity : 16,110
m/z 218
Time 11.758 Scan# 529 Inten. 5,800
40 % MetOH
50 % MetOH
60 % MetOH
70 % MetOH
7.5
5.0
2.5
11.80
11.85
11.90
11.95
12.00
12.05
12.10
12.15
12.20
12.25
12.30
min

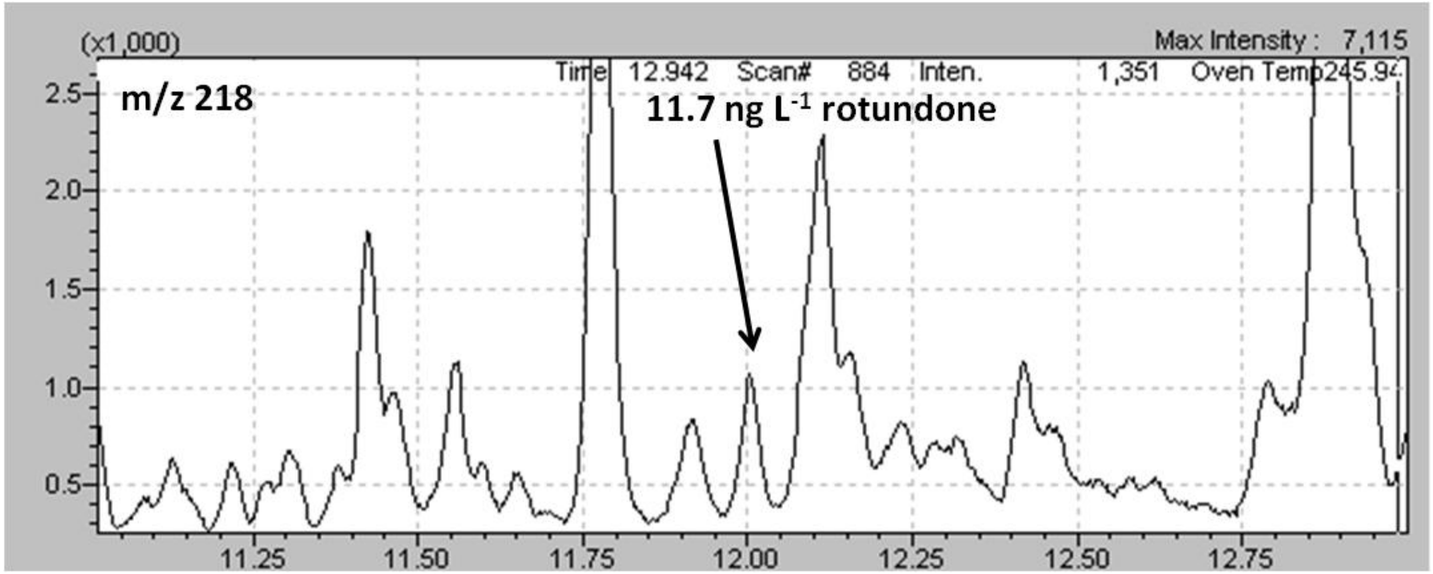

(x1,000)
Max Intensity : 7,115
Time 12.942 Scan# 884 Inten. 1,351 Oven Temp245.94
m/z 218
11.7 ng L-1 rotundone
2.5
2.0
1.5
1.0
0.5
11.25
11.50
11.75
12.00
12.25
12.50
12.75

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 Petrussa, Schioppettino 2010	Sch 2	Schioppettino	Friuli Venezia Giulia, Italy
Arzenton, Schioppettino 2010	Sch 3	Schioppettino	Friuli Venezia Giulia, Italy
Vigna Petrussa, 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 Autralia
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

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]

[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 different red wines.

Tabla 3. Level of rotundone (ng L^{-1}) found in thirty different wines, mostly of them 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	

Gra: Graciano wines; Mat: Maturana wines, Sch: Schioppettino wines, Shi: Shiraz wines; Gam: Gamay wines, Arr: Arrat wines; Malb: Malbec wines; Abou: Abouriou; Dur: Duras; Mou: Mourvédre.

The concentration was expressed with the uncertainty associated to these results (4 %, in terms of RSD). n.e.*: It has not been estimated the average level due to rotundone was not detected in 5 of the 7 wines