An automated Gas Chromatographic-Mass Spectrometric method for the quantitative analysis of the odor-active molecules present in the vapors emanated from wine

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Abstract

An automated dynamic headspace (DHS) method combined with thermal desorption (TD) and gas chromatography-mass spectrometry (GC-MS) has been developed and applied to characterize the composition of the vapors emanating from wine during its consumption. The method provides a snapshot of the contents in the wine vapors of up to 40 relevant aroma compounds, including methanethiol, sulfur dioxide, aldehydes, fusel alcohols or volatile phenols. Leaving aside methanethiol, method repeatability was better than 15%, and better than 11% in 30 cases. Determination coefficients were better than 0.99 and detection limits, ranging from 0.1 to 1200 μg/L, depending on the compound, were below normal ranges of occurrence or odor thresholds of those 40 compounds. The method has been applied to assess the changes in the wine headspaces with time, monitoring the levels of 34 odorants emitted to the headspace by 4 different wines during five consecutive time points. Levels of 15

polar aroma compounds remained constant, while levels of 14 non-polar and highly volatile compounds decayed very fast, which should have strong sensory changes in the odor perceived. The trends followed by methanethiol, dimethyl sulfide, ethyl decanoate, by aldehydes and dicarbonyls were significantly related to the wine, which suggests that prediction of the aroma impact in these cases should include an estimation of the odorant x wine matrix interaction.

Keywords: Odor, headspace, release, aroma profile, aroma perception

1. Introduction

The characteristic odors and flavors elicited by a product are related to the aroma composition of the headspaces that reach the olfactory receptors during the action of smelling or eating the product. In the case of complex aroma mixtures, the qualitative characteristics of the odor perceived are related to the profile of odorants, rather than to the absolute concentrations [1,2]. In the case of wine, there is strong evidence that some aroma compounds can bind to different compounds or structures forming the non-volatile matrix of wine [3-5]. The existence of these interactions suggest that the odor activity of those odorants in a given wine will be related not only to the concentrations of the odorants, but to the amount and type of "aroma-binders" present in that wine. This means that two wines with exactly the same aroma composition could in fact produce headspace vapors differing in composition, depending on the level and type of "aroma-binders" specifically present in each wine [6]. This could explain why the same aroma extract reconstituted in different wine non-volatile matrixes can produce markedly different aroma perceptions [7].

The existence of odorant x matrix interactions potentially responsible for aroma changes has been previously addressed in wine [8] and other products, notably solid or semi-solid food products [9-11]. In these last cases, it is evident that the levels of aroma chemicals released from the product are strongly dependent on the specific composition of the solid or semisolid matrix. It is also evident that the analytically relevant information in these cases is not only the absolute aroma composition, but the rate at which the different aroma compounds are released from the matrix to the headspace.

Several approaches have been proposed for the determination of the aroma compounds present in the headspaces emanated from a given product. The most direct strategy is the continuous monitoring of the composition of the headspace with methods such as direct atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [12,13] or proton transfer reaction mass spectrometry (PTR-MS) [14,15]. These strategies are, however, not sensitive enough for the direct monitoring of aroma compounds present at low levels, which limits their applicability to the study of products containing relatively large amounts of volatile compounds. By contrast, in many natural food products, including wine, aroma properties can be strongly influenced by powerful aroma compounds present at very low concentrations. In the particular case of wine and other alcoholic beverages, selectivity also becomes a problem, since wine headspaces are much enriched in ethanol, fusel alcohols and other major wine volatiles.

A second possibility is trapping the aroma compounds present in the headspace in a sorbent or cold trap in order to gain sensitivity, and to analyze the concentrated odorants by GC-MS, to gain selectivity. The obvious drawback of these strategies is that monitoring will become discontinuous. It should be noted, however, that most reports using these strategies do not really intend to analyze the headspace, but the volatiles present in the product. In this context and because of its simplicity, solid phase microextraction (SPME) is frequently used [16], although other headspace sampling techniques have been also widely applied [17]. In dynamic Headspace (DHS) techniques, a flow of inert gas drags out volatile compounds from the product and is subsequently directed to a sorbent or cryogenic trap, in which volatiles are retained. The vapors produced with these techniques are more similar to those observed in real olfaction

77 than those obtained by using equilibrium methods such as static headspace or
78 headspace SPME sampling [18].

79 There are several reports proposing DHS techniques for wine aroma analysis. In most
80 of them, volatiles are dragged out by an inert gas bubbled through the wine [19-22] or
81 streamed on the wine headspace [23], but as was aforementioned, these methods were
82 designed for the quantitative analysis of the aroma compounds present in the liquid
83 phase of the wine rather than to monitor the changes in concentrations in wine
84 headspaces.

85 In the present work, our main aim is to develop a fast and simple DHS method able
86 to provide a "snapshot" of the headspaces emanated from wine in conditions close to
87 those found during wine tasting. For that, the headspace of unstirred wine will be
88 dragged by a gentle stream of nitrogen during a relatively short time. A second objective
89 is to use the method to make a preliminary assessment about the compositional
90 changes in the wine headspaces potentially experimented during the time that the wine
91 is kept in the glass during consumption.

92

2. Materials and methods

2.1. Reagents and chemicals

Ethanol was supplied by Merck (Darmstadt, Germany) and tartaric acid 99% was obtained from Panreac (Barcelona, Spain). The internal standards (methyl 2-methylbutyrate, 2,6-dichloronisole) and standards of the aroma compounds were obtained from Aldrich, Fluka (Madrid, Spain).

2.2. Wine samples

Four white wines, four red wines and a rosé wine with diverse characteristics (in terms of grape variety, alcoholic content and aging) from Spain were used to validate and develop the method. The synthetic wine contained 5 g/L of tartaric acid, adjusted to pH 3.4 with 1 M NaOH, and an ethanol content of 12% vol.

2.3. Proposed method

Five mL of sample were pipetted into a 20 mL standard headspace vial, then 20 µL of the internal standard solution were added to reach a concentration level of 200 µg/L. The vial was then closed and placed in the Gerstel MPS2 auto-sampler (Mülheiman der Ruhr, Denmark) where the DHS sampling was automatically carried out under the conditions detailed in Table 1. Thermal desorption and cryo-focusing were carried out by means of a Thermo Desorption Unit (TDU) and Cooling Injection System (CIS4) also supplied by Gerstel. Solvent venting mode was used to perform the desorption. Detailed experimental conditions are shown in Table 1.

Gas chromatography-mass spectrometry analysis was performed with a 7890 Agilent GC system coupled with a 5975C Agilent quadrupole mass spectrometer (Santa Clara,

115 CA, USA). A J&W DB-Wax column was used (60 m × 0.25 mm i.d. × 0.25 µm film thickness,
116 Agilent). The temperature program was: initial oven temperature 35°C held for 3 min,
117 then raised to 220°C at 10°C/min, and 7 min of final hold time. The carrier gas was helium
118 at a constant flow of 1mL/min. The chromatograms were collected in both full scan and
119 SIM mode. Ionization was carried out in electronic impact mode at 70 eV. The ion source
120 temperature was 230°C. Spectra were recorded both in scan mode from 33 to 250 m/z
121 and in selected ion monitoring. Selected ions for particular compounds are shown in
122 Table 2.

123 *2.4. Method validation*

124 *2.4.1. Internal standards*

125 Two compounds which potentially should provide a headspace concentration
126 independent of the wine specific composition were tested (methyl 2-methylbutyrate
127 and 2,6-dichloroanisole). For that, a synthetic wine, 4 whites, 2 reds and 1 rosé, all made
128 from different grape varieties were spiked with 200 µg/L of both components and were
129 analyzed in duplicate and on 3 different days.

130 *2.4.2. Precision*

131 Method precision was studied over a four-month period. Four bottles (from the same
132 batch) of a Spanish red Crianza wine from La Rioja were kept refrigerated at 10 ºC. Each
133 month, one bottle was opened in a glove box from Jacomex (Dagneux, France) with
134 oxygen levels under 0.002%. Immediately after opening, each bottle was aliquoted in 4
135 20-mL SPME vials to be analyzed on 4 different days within the same week. The vials
136 were kept in the glove box until the analysis.

137 *2.4.3. Linearity and limits of detection*

138 Method linearity was evaluated using a set of 9 different volatile compounds found
139 in wine representing different chemical families, as detailed in Table 4. These compounds
140 were dissolved in ethanol and were further spiked at five different levels to a Spanish
141 red wine from La Rioja. The ethanol content was adjusted to maintain the same level in
142 all calibration samples. All samples were prepared in duplicate and were analyzed
143 following the procedure described in Section 2.3. The areas of each compound in Table
144 4 were normalized by those of the IS (MBM), corrected by subtracting the relative area
145 obtained for that compound in the unspiked wine and fitted to an unweighted least
146 square regression model.

147 Method sensitivity was assessed by estimation of the limits of detection. These were
148 defined as the amount of analyte in the liquid phase of a wine that produces with the
149 proposed method a peak with a height equivalent to three times the average standard
150 deviation of the baseline in the surrounding area to the ion peak. The concentration of
151 the different compounds in the liquid phase of the wine was estimated by using
152 previously validated methods as is described in section 2.5.

153 *2.5. Quantitative analysis of compounds in the liquid phase*

154 The quantitative analysis of major volatile compounds contained in wine was carried
155 out using the method proposed and validated in our laboratory [24]. In accordance with
156 this method, 3 mL of wine containing the internal standards (2-butanol, 4-methyl-2-
157 pentanol, 4-hydroxy-4-methyl-2-pentanone, and 2-octanol) and 7 mL of water were
158 salted with 4.5 g of ammonium sulfate and extracted with 0.2 mL of dichloromethane.
159 The extract was then analyzed by GC with FID detection. The area of each analyte was

normalized by that of its corresponding internal standard and was then interpolated in the corresponding calibration plot built by applying exactly the same analytical method as that applied to synthetic wines containing known amounts of the analytes covering the natural range of occurrence of these compounds.

The quantitative analysis of minor and trace compounds in the liquid phase of wine was carried out using the method proposed and validated in our laboratory [25] with the following changes in the procedure: standard solid phase extraction (SPE) cartridges (1 mL, total volume) filled with 200 mg of LiChrolut EN resins were placed in the vacuum manifold extraction system (Varian Sample Preparation Products), and the sorbent was conditioned by rinsing the cartridges with 4 mL of dichloromethane, 4 mL of methanol, and, finally, with 4 mL of a water-ethanol mixture (12%, v/v). The cartridges were then loaded with 50 mL of wine sample and 26 µL of a surrogate standards solution (recovery standard) containing 3-octanone, β-damascone, and heptanoic acid (all at 200 µg/g of ethanol). This mixture was passed through the SPE cartridges (2 mL/min), followed by a washing step using 5 mL of 30% methanol in water and 1% NaHCO3 solution. The resins were then dried by letting air pass through them (negative pressure of 0.6 bar, 10 min). Analytes were recovered in a 2-mL vial by elution with 1.6 mL of dichloromethane. Thirty-four µL of an internal standard solution (300 mg/L of 4-hydroxy-4-methyl-2-pentanone and 2-octanol) was added to the eluted sample. The extract was analyzed by GC with ion trap-mass spectrometry (MS) detection (GC-450 gas chromatograph fitted to a Varian Saturn 2200 ion trap-MS).

Total volatile sulfur compounds were quantified by using the method proposed and validated in our laboratory [4]. First, 10 mL of brine was added to a 20 mL standard

183 headspace vial. The vial was then capped and Argon bubbled through the septum for 2
184 min to eliminate oxygen. Next, 200 µL of wine sample and 20 µL of internal standards
185 were added to the vial, and the prepared sample was analyzed immediately by SPME-
186 GC-pFPD.

187 Total and free sulfur dioxide was determined by the aspiration/titration method
188 (Rankine method recommended by the OIV, International Organization of Vine and
189 Wine). All analyses were performed in triplicate.

190 *2.6. Changes in wine headspace with time*

191 For this experiment, 4 Spanish red wines with different ageing times were selected:
192 a 1-year old young red wine without barrel ageing (coded as "YOUN1"), two different 4-
193 year old red wines (coded as "AGED1" and "AGED2") and a 7-year old red wine (coded
194 as "AGED3"). Detailed information about the wines is included in the supplementary
195 data section. The wines were prepared at room temperature and adjusted to 14.5%
196 ethanol content. To assess the changes in the headspace concentrations of the different
197 analytes with time, the headspaces of each wine sample were analyzed with the
198 proposed DHS method five consecutive times. For that, the wines were prepared in the
199 vials as described in the method, analyzed, and after 70 min the same vial was re-
200 analyzed following the procedure. The vials were kept closed in the vials at 25ºC between
201 extractions.
202

3. Results and discussion

3.1. DHS method

The present method seeks to provide a reliable snapshot of the composition of the vapors emanating from wine when it is smelled or consumed at a given time. For this is important to fulfill two conditions:

1st The purging time has to be the smallest possible ensuring acceptable sensitivity.

2nd The purging process has to produce headspaces with compositions equivalent to those produced during real olfaction or consumption.

Regarding this second condition, it should be noted that bubbling through the liquid facilitates mixing and the transport to the headspace of all compounds present in the liquid phase. In those conditions, the stream of vapors produced would have a composition close to those observed in the headspace in equilibrium with the liquid phase [26]. However, such conditions are far from those observed during real tasting and consumption, where the vapor composition is determined by the kinetics of mass transfer from the liquid to the gas [27,28]. If instead, the purging gas is used only to drag the headspace of the unstirred liquid, the headspace is quickly diluted and impoverished in the most volatile compounds which cannot be satisfactorily transferred from the bulk of the unstirred liquid to the headspace.

Regarding the first condition, the total volume of gas used to drag the wine headspace was limited to 100 mL in 4 minutes with the sample thermostated at 30 ºC and without stirring. This relatively short sampling time and gas sampling volume also

ensures that ethanol does not saturate the Tenax trap, which would reduce breakthrough volumes and that even the most volatile compounds are retained.

The optimized experimental parameters of the DHS system are listed in Table 1. A typical GC-MS chromatogram can be seen in Fig. 1. The method allows to study 40 wine aroma compounds in a wide range of volatilities (from methanethiol to 4-ethylphenol), concentrations (from μg/L to >200 mg/L) and polarities (from acetic acid or sulfur dioxide to ethyl decanoate). The other operative conditions, such as the drying volume or solvent split at the TDU were chosen in order to minimize problems with water and column overloading. Once these optimal conditions were found, the method was evaluated for different quality parameters.

3.2. Internal standards

Finding an internal standard whose instrumental response can correct for changes in the instrument sensitivity is of paramount importance for the method. Only with such an internal standard could a comparison between different wines can be achieved. The ideal internal standard for the present method is a compound whose concentration in the headspace is always constant and independent from the wine matrix, implying that it should exert a minimum interaction with the matrix components. According to previous work carried out in our laboratory [5], methyl 2-methylbutyrate (MBM) and 2,6-dichloroanisole (DCA) were suitable candidates. Their potential usefulness was experimentally checked by repeatedly analyzing batches of different commercial wines (n=7) and synthetic wine models containing these compounds at fixed concentrations. The results revealed that both compounds could be used as internal standards. MBM performed better with a global relative standard deviation (RSD) for the absolute ion

247 peak areas of 10%. Additionally, the difference between the average area measured in
248 real wines coincided closely with the average area in synthetic wine (-3.5%), confirming
249 that the volatility of this compound was almost independent of the matrix composition.
250 Therefore, this compound was used to normalize the areas of the analytes and to correct
251 potential variations in the trapping system or in the instrumental response. The DCA
252 performance was slightly worse with a 16% global RSD, but it was retained in the internal
253 standard solution for additional quality controls.

254 *3.3. Precision, linearity and detection limits*

255 Precision was measured in terms of method reproducibility and method
256 repeatability. The repeatability was estimated as the within-batch variability (same
257 sample, different days within the same week), while reproducibility added the inter-
258 month and sample bottle variability and hence is not an appropriate measurement of
259 the method performance. As can be seen in table 3, repeatability was in general
260 satisfactory, particularly taking into account that the measurements took place during
261 one week. Even if wines were kept as stable as possible within an anoxic glove chamber,
262 some inevitable changes will occur during a week, affecting particularly to highly volatile
263 or reactive compounds. This suggests that the values obtained for repeatability in Table
264 3 represent a worst-case scenario. As can be seen, the worst results were obtained for
265 methanethiol. This poor result can be partly attributed to the low levels at which it was
266 present in the wine used in the study (3.5 µg/L) but also to the fact that the
267 concentration of this elusive molecule can change substantially during the experiments
268 because of its high volatility, lability to oxygen and because of the existence of different
269 non-volatile species in equilibrium with the volatile form [4,5]. Relatively poor

repeatabilities obtained for acetaldehyde and methylbutanal could be also related to the ability of these compounds to form stable complexes with SO_2. In the cases of acetaldehyde and DMS, their high volatility and poor retention in the Tenax trap can also explain the outcome. Acetic acid seems to be particularly poorly retained in Tenax. Leaving aside these cases, most compounds can be quantified with a worst-case reproducibility better than 10%, which can be considered acceptable taking into account the conditions of the experiment.

The detection limits were estimated taking into account the concentrations of the compounds in the wine used for validation. These concentrations were determined by different headspace, liquid-liquid or solid phase extraction strategies (see methods). The results are given in table 3. As expected, the detection limits are strongly related to the volatility of compounds in the wine matrix. Accordingly, the lowest detection limits (0.1-0.3 μg/L) were found for various non-polar ethyl esters, such as ethyl -3-methylbutyrate, while the highest were found for the most soluble compounds such as sulfur dioxide, acetaldehyde, acetoin or acetic acid. Fortunately, the method makes it possible to determine many relevant wine aroma compounds at the concentrations at which they are present in normal wines.

Another key validation parameter was linearity. In order to have a realistic estimation of this quality parameter, a red wine was spiked with known amounts of a small group of selected analytes representative of the different chemical families of volatile compounds found in wine. This approach guarantees that the intrinsic volatilities of the compounds do not change as a consequence of changes in the matrix polarity caused by increases in the levels of non-polar compounds. As can be seen in Table 4, in all cases

linear dynamic ranges spanned at least 2 or 3 orders of magnitude with determination coefficients better than 0.99 in all cases. The study of the residuals did not show the existence of any particular trend. These data prove that in the proposed DHS method, any change in the composition of the headspace causes a proportional change in the signal.

In summary, the method showed satisfactory validation parameters and can be used to assess the content of up to 40 relevant aroma compounds in the headspaces emanating from wine and hence to study how these headspaces change in response to different matrix and environmental parameters.

3.4. Changes in wine headspace with time

The method has been applied to study how the headspaces emanated from four different wines change with time as consequence of evaporation, shifts in chemical equilibria or other phenomena that can take place during the time in which the wine is exposed to air in a glass. In this experiment, however, the wines were kept in a closed vial during the experiment (see methods). As will be shown, the levels of nearly a half of the studied aroma compounds decayed with time, and the rates of decay were directly related to the fraction of compound emitted to the headspace, suggesting that evaporation is the major cause of the observed changes. It should be also noted that in the present study decay curves are not used to obtain unbiased estimators of the concentration of compound in the original matrix, as done in previous works [6,29,30], but rather to characterize the specific decay patterns followed by the different aroma compounds and also to assess whether these patterns are general to all wines or if they are dependent on the specific matrix composition of a given wine.

316 Data from each wine were normalized to the level of compound found in the first
317 sampling point, in order to make decay curves independent of the concentration. As the
318 internal standard also decays with time, changes in instrumental sensitivity were
319 corrected by normalizing the areas by those obtained for ethanol, whose levels
320 remained stable during the experiment. Data were then processed by 2-way ANOVA
321 (table 5) to assess the significance of the factors wine, time (injection number) and of
322 their interaction. Results make it possible to classify the 34 aroma compounds which
323 could be monitored in the four wines during the five consecutive injections into four
324 broad categories:

325 1. Compounds whose concentrations in the headspaces remain unchanged
326 2. Compounds whose concentration in the headspace follows irregular wine-
327 dependent trends
328 3. Compounds whose concentration in the headspaces decay. This category can be
329 further subdivided in:
330 a. Those whose decay functions are non-wine-dependent
331 b. Those whose decay functions are wine-dependent

332 The four categories in which compounds can be classified are presented in Table 5, while
333 figures 2a to 2d show five evolution patterns representing illustrative examples.

334 The first category of compounds whose levels in the headspace remain constant with
335 time includes 15 polar or moderately non-polar and not very volatile compounds, as
336 detailed in Table 5. The case of isobutyl alcohol is shown in Figure 2a as example.
337 Compounds in this category are fusel alcohols, volatile phenols, volatile acids, hydroxy
338 esters, aromatic esters, diesters, whiskeylactone, β-damascenone and sulfur dioxide.

The second category includes aldehydes and diacetyl. Levels in the headspaces of these compounds evolved with time differently in each wine, which should be most likely attributed to the different levels of sulfites and of other sulfite binders present in the wines. In the case of acetaldehyde, shown in Figure 2b as example, it can be seen that in samples YOUN1 and AGED1, the content in the wine headspaces increased with time, while in samples AGED2 and AGED3, levels decreased with time.

Nearly a half of the compounds (14 out of 34) followed decreasing trends and are classified in the two last categories, which include non-polar compounds and some polar but very volatile compounds such as dimethyl sulfide and methanethiol. Within the ethyl ester homologous series, the rates at which levels decrease with time increase with molecular size; while the levels of ethyl acetate decay just a 30%, levels of ethyl octanoate and decanoate dropped around 80%. A remarkable observation is that polarity is useful for predicting the decay rate only within a homologous series, since molecular size, which strongly affects volatility, is also relevant. For instance, DMS and methanethiol are lost very quickly in spite of the fact that they have higher polarity than ethyl butyrate.

In most cases, decay trends are not affected by differences in the wine matrix so that over time the levels of those aroma compounds decrease at the same rate in any wine. An illustrative example is shown for the case of ethyl propanoate in Figure 2c. In some few cases however, (isoamyl acetate, and 2 and 3-methylbutyrates) there is a slight effect, close to statistical significance, of the wine matrix. And in the particular case of ethyl decanoate, dimethyl sulfide and methanethiol, the effect of the wine matrix reaches significance so that these compounds are classified into the fourth category. The

362 particular case of ethyl decanoate is shown in Figure 2d. This compound (the same trend
363 observed in isoamyl acetate and 2 and 3-methylbutyrates) is slightly less retained in the
364 youngest wine, and seem to be more retained in one particular aged red wine. The
365 pattern observed in methanethiol and dimethysulfide, is rather the contrary, with the
366 youngest wine showing maxima retention for both compounds. This could be related to
367 the specific levels of metal cations in this wine, which were not measured in the present
368 experiment.

369 The theory of multiple extractions was applied to those compounds following a clear
370 decay [29,31]. According to this theory, if the proportion of compound extracted in each
371 extraction remains constant, and that proportion is represented as a series of areas
372 logarithmically transformed versus the ordinal number of the extraction minus 1, the
373 outcome of this representation is a straight line following the equation:

374 $$\text{Ln } A_i = (i-1) \text{ Ln } \beta + \text{Ln } A_1 \quad (1)$$

375 where *i* denotes the *ith* extraction and A_i refers to the area obtained in the *ith* extraction.
376 The slope of this straight line is by convention named Ln β and it can be demonstrated
377 that Ln β in fact reflects the proportion of compound extracted in each one of the
378 extractions performed in a given sample. A -0.4 value, for instance, means that 40% of
379 the compound is transferred to the headspace in each extraction. The closer to -1 is Ln
380 β, the higher the proportion of compound transferred to the headspace [6].

381 Average Ln β values for the above-mentioned compounds are shown in table 6. These
382 values are in general agreement with those calculated elsewhere [6] even though the
383 instrumental setup and the purpose of the experiment were completely different. Data
384 in the table are arranged in decreasing order of Ln β. The least volatile is ethyl acetate

385 for which 10% its transferred to the headspace in each extraction cycle, and the most
386 volatile is dimethyl sulfide, for which 69% is transferred to the headspace. This implies
387 that wine is depleted from this extremely volatile compound very soon, in agreement
388 with previous results [32].

389 As Ln β values are slopes obtained by regression analysis, the S value provided by the
390 regression model for the slope is an estimation of its uncertainty. The square roots of
391 the average variances obtained for each compound in the four wines is the average
392 within wine uncertainty, and is given in the Table 6. Assuming additivity of variances, the
393 variance of the four Ln β values obtained for each compound in the four wines can be
394 decomposed into within and between wines variability attending to the model:

395 $$S^2_{tot} = S^2_{between\ wines} + S^2_{within\ wine} \quad (2)$$

396 This makes it possible to obtain an estimation of the "between wines" variability (given
397 in Table 6) and also to apply an F test to assess its significance. The results of this test
398 shown in Table 6, where it can be observed that attending to this criterion, only the
399 dimethyl sulfide and ethyl decanoate Ln β values differ significantly between wines. It
400 should be noted, however, that in the case of methanethiol the F quotient is abnormally
401 low because of the huge within wine variability, which should be attributed to its
402 extremely low levels.

403 *3.5.- Potential sensory relevance of these changes*

404 It should be taken into account that the qualitative characteristics of aroma perceptions
405 are essentially linked to the profile of odor volatiles reaching the olfactory receptors
406 located in the nose [33,34]. Although it is outside the scope of the present paper to

407 make a precise assessment on this question, the data presented here indicate that the
408 aroma profiles suffer major changes during the time that the wine is in the glass. As has
409 been previously highlighted, the levels of half of the aroma compounds remained
410 constant with time, while levels of the most volatiles such as DMS, ethyl decanoate or
411 methanethiol quickly dropped to zero. The levels of ethyl esters steadily decreased at
412 rates related to their molecular size, which implies that the profile of volatiles emanated
413 from the wine continuously change which should affect the quality of the odor
414 perceived. Additionally, data indicate that the levels of most aldehydes, many of which
415 have relevant sensory properties, followed matrix-dependent trends as do also dimethyl
416 sulfide, ethyl decanoate, methanethiol and surely other mercaptans. This implies that in
417 all these cases data of concentration in the liquid phase is not enough to accurately
418 interpret the role played by the aroma compound in the product. An estimation of the
419 specific volatility of the odorant in such specific wine should be also provided.

420

421 **4. Conclusions**

422 The proposed HS-TD-GCMS method provides quantitative data of up to 40 different
423 relevant aroma compounds in the vapors emanating from wine and makes it possible to
424 assess how the composition of the vapors change with time. Attending to the pattern of
425 change, aroma compounds have been classified into four categories. Polar and not very
426 volatile compounds (half of the total) are present in the headspaces at levels related to
427 their concentration and do not change during time. On the contrary, non-polar and
428 highly volatile compounds can decay very fast. Additionally, the levels and trends
429 followed by aldehydes, dicarbonyls, methanethiol, DMS or ethyl decanoate are
430 significantly affected by the matrix. This indicates that in these cases the data of

concentration in the liquid phase should be accompanied by an estimation of their volatility in such specific wine in order to make a reliable interpretation of their sensory role. Results confirm that wine headspace continuously changes during time, which should cause relevant changes in the odor qualities perceived.

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563 **Figure captions**

564

565 **Fig. 1.** GC-MS chromatogram (SCAN mode) of a wine sample: (1) acetaldehyde; (2)
566 methanethiol; (3) dimethyl sulfide; (4) isobutanal; (5) ethyl acetate; (6) 2- & 3-
567 methylbutanal; (7) diacetyl; (8) ethyl propanoate; (9) sulfur dioxide; (10) ethyl
568 isobutyrate; (11) methyl 2-methylbutyrate (IS); (12) isobutyl acetate; (13) isobutyl
569 alcohol; (14) ethyl butyrate; (15) 2,3-pentanedione; (16) ethyl 2-methylbutyrate; (17) 1-
570 butanol; (18) ethyl 3-methylbutyrate; (19) isoamyl acetate; (20) isoamyl alcohol; (21)
571 acetoin; (22) ethyl hexanoate; (23) ethyl lactate; (24) cis-3-hexen-1-ol; (25) acetic acid;
572 (26) furfural; (27) benzaldehyde; (28) ethyl octanoate; (29) linalool; (30) butyric acid;
573 (31) γ-butyrolactone; (32) diethyl succinate; (33) 2,6-dichloroanisole (IS); (34) ethyl
574 decanoate; (35) hexanoic acid; (36) phenethyl acetate; (37) β-damascenone; (38) trans-
575 whiskeylactone; (39) β-phenylethanol; (40) cis-whiskeylactone; (41) 4-ethylguaiacol;
576 (42) 4-ethylphenol.

577

578 **Fig. 2.** Evolution patterns of headspace composition after five consecutive extractions
579 for (a) isobutanol, (b) acetaldehyde, (c) ethyl propanoate and (d) ethyl decanoate. Wine
580 codes: YOUN1 was a 1-year old young red wine without barrel ageing, AGED1 and
581 AGED2 were two different 4-year old red and AGED3 was a 7-year old red wine.

582

Figure 1

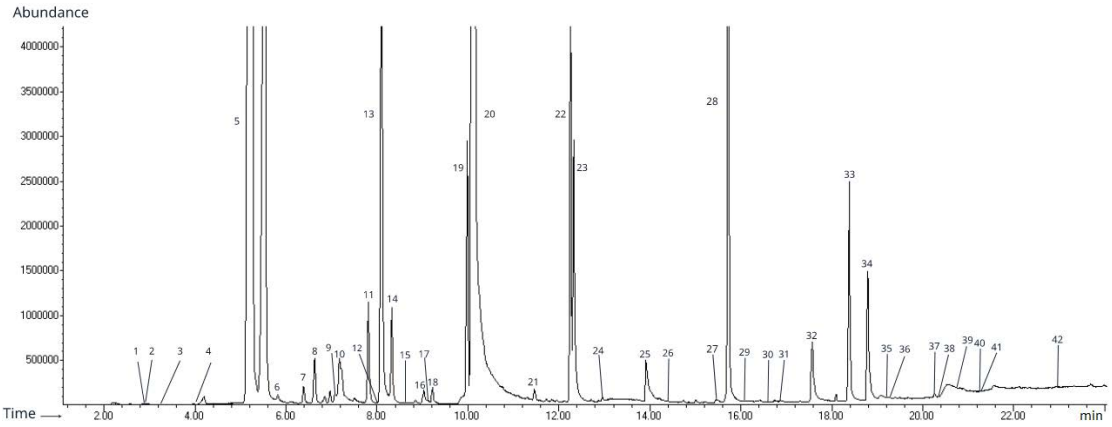
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Figure 2a

Figure 2a

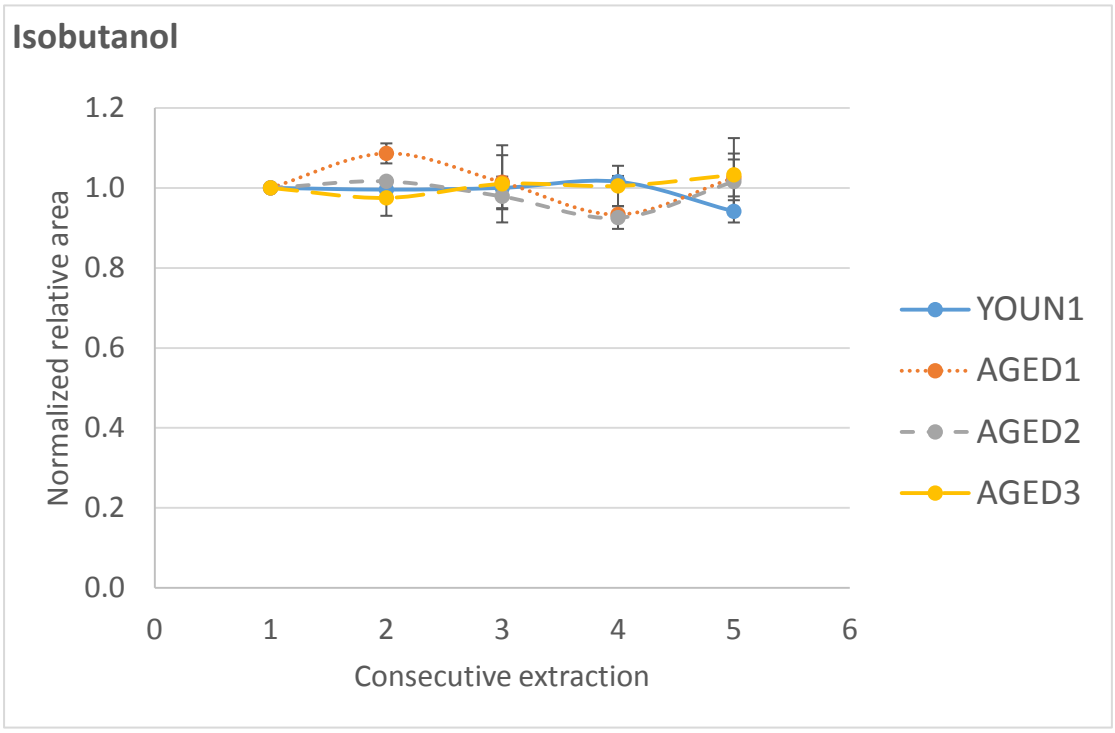

Figure 2b

Figure 2b

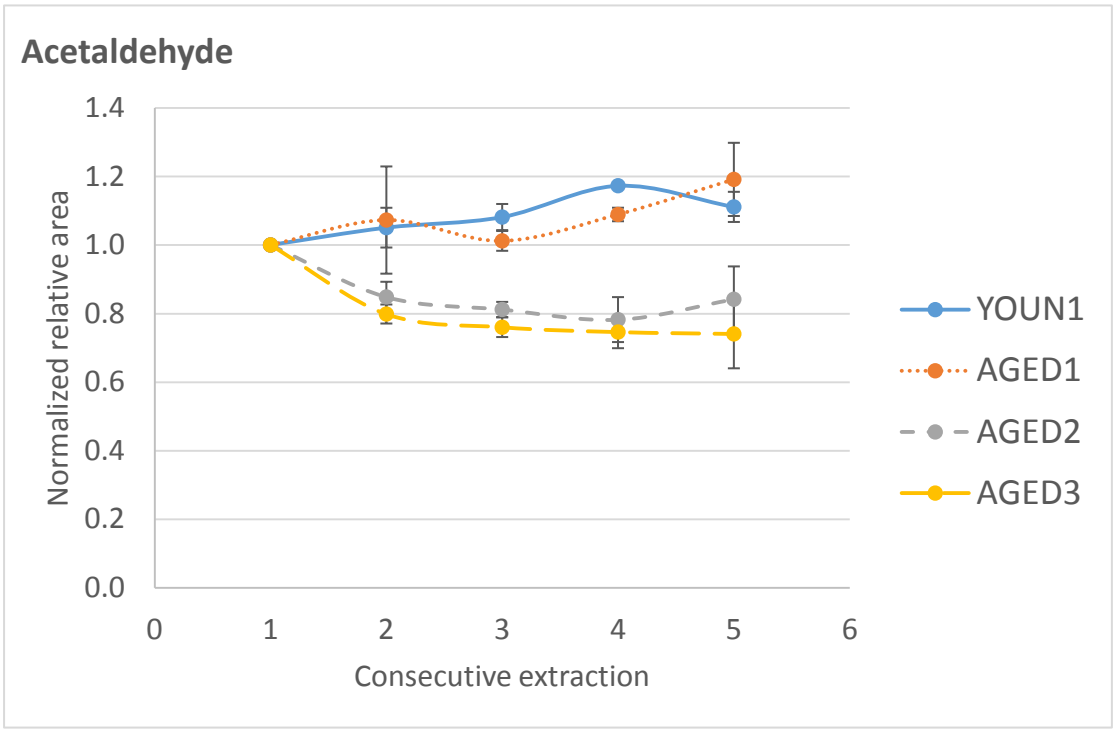

Figure 2c

Figure 2c

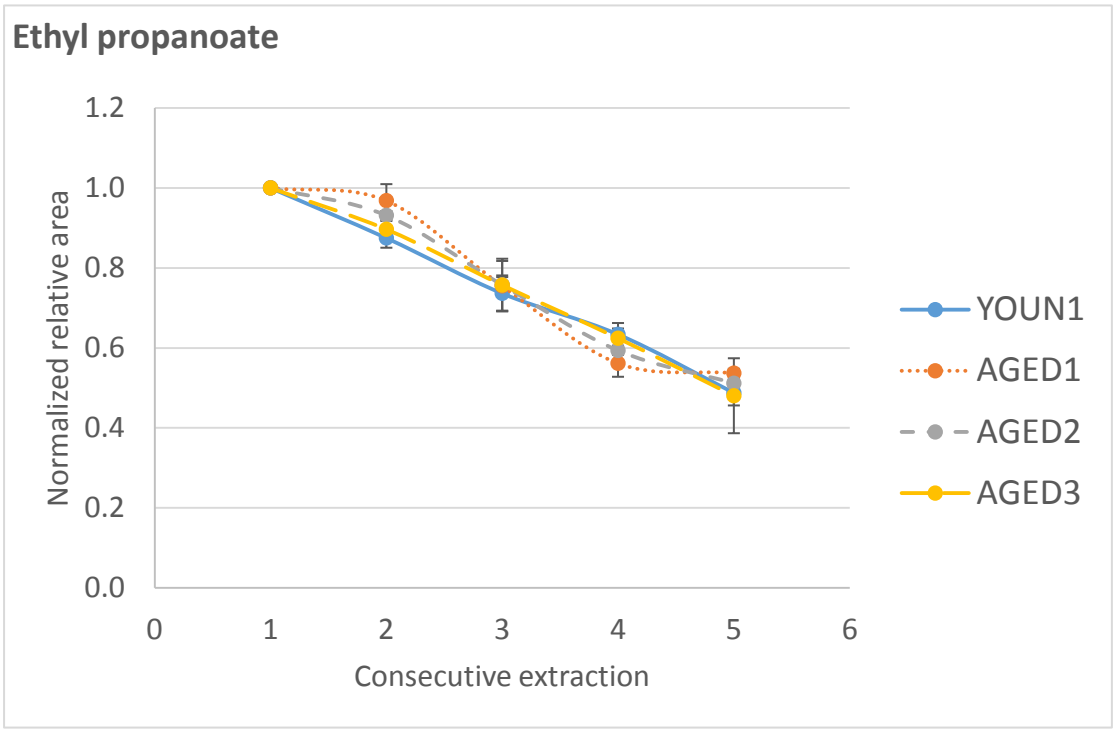

Figure 2d

Figure 2d

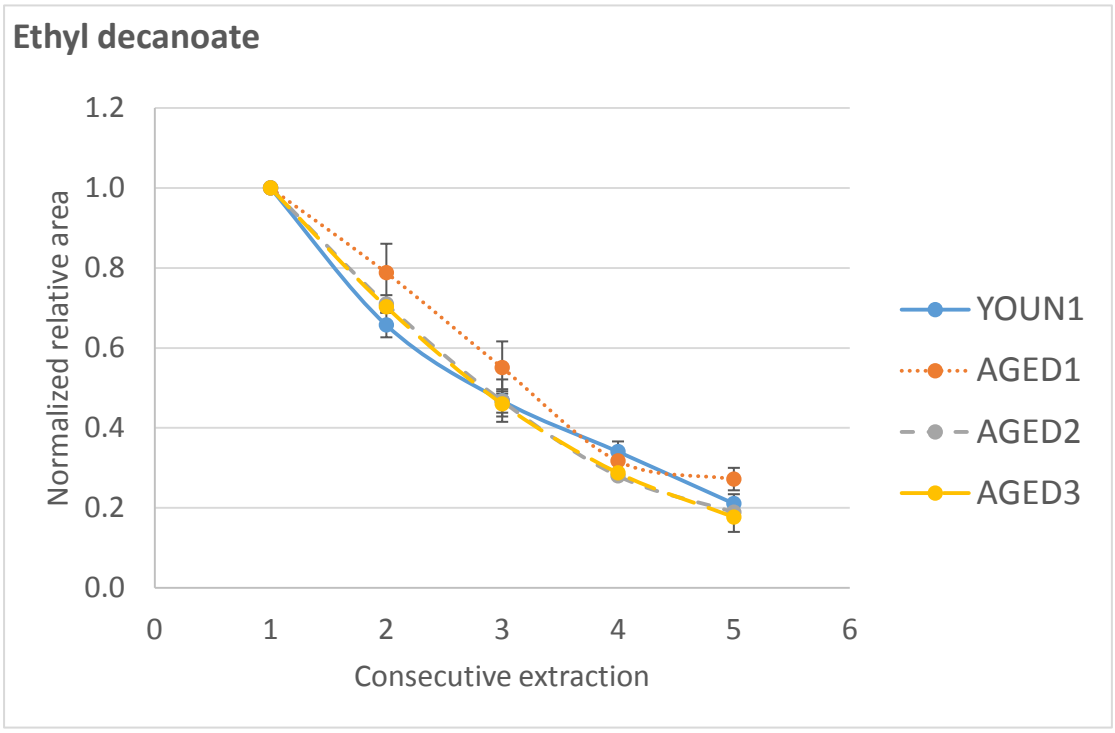

Table 1

Table 1. Experimental parameters of the DHS system.

Parameters			
Incubation time (min)	5	Initial TDU temperature (°C)	20
Incubation temperature (°C)	30	End TDU temperature (°C)	300
Purge volume (mL)	100	Rate TDU (°C/min)	200
Purge flow (mL/min)	25	Initial CIS temperature (°C)	-100
Purge temperature (°C)	40	End CIS temperature (°C)	250
Dry volume (mL)	50	Rate CIS 1 (°C/s)	16
Dry flow (mL/min)	10	Rate CIS 2 (°C/s)	12
Dry temperature (°C)	40	Sample volume (mL)	5
Sorbent material	Tenax TA	No stirring	

Table 2

Table 2. Acquisition mode and selected ions for the determination of target compounds in the study.

Compound	Retention time (min)	Scanning mode[a]	Ions (m/z)
Methanethiol	2.983	SIM	47, 48
Dimethyl sulfide	3.418	SIM	62, 47, 61
Sulfur dioxide	7.151	SIM	64, 48
Ethyl acetate	5.191	full scan	74
Ethyl propanoate	6.632	full scan	102
Ethyl butyrate	8.321	full scan	88
Ethyl hexanoate	12.257	full scan	99
Ethyl octanoate	15.730	full scan	88
Ethyl decanoate	18.799	full scan	101
Ethyl isobutyrate	7.209	full scan	116
Ethyl 2-methylbutyrate	9.029	full scan	115
Ethyl 3-methylbutyrate	9.218	full scan	115
Ethyl lactate	12.332	full scan	45
Diethyl succinate	17.582	full scan	129
Isobutyl acetate	8.057	full scan	73
Isoamyl acetate	9.986	full scan	70
Phenethyl acetate	19.497	full scan	104
Acetaldehyde	2.880	SIM	42, 43, 44
Diacetyl	6.386	full scan	86
isobutanal	4.123	SIM	72, 41
Methylbutanal	5.822	SIM	58, 57, 71
2,3-Pentanedione	8.617	full scan	100
Acetoin	11.466	full scan	88
Furfural	14.216	full scan	96
Benzaldehyde	15.459	SIM	105, 106
β-damascenone	20.246	SIM	121, 190
Isobutanol	8.095	full scan	74
1-Butanol	9.190	full scan	56
Isoamyl alcohol	10.153	full scan	70
cis-3-Hexenol	13.091	full scan	82
2-Phenylethanol	20.473	SIM	122, 91, 92
Linalool	15.827	SIM	121, 93
Acetic acid	13.900	full scan	60
Butyric acid	16.344	SIM	60, 88
Hexanoic acid	19.105	SIM	60, 87
γ-Butyrylactone	16.742	full scan	86
trans-Whiskeylactone	20.281	SIM	99, 71
Cis-Whiskeylactone	21.267	SIM	99, 69
4-Ethylphenol	21.570	SIM	107, 122
4-ethylguaiacol	22.931	SIM	137, 152

[a] SIM: selected ion monitoring

Table 3

Table 3. Precision and detection limits of the DHS method.

	RSD (%)		Concentration in wine (µg/L)[a]	Detection limit (µg/L)
	Repeatability	Reproducibility		
Methanethiol	28	30	3.46 ± 0.08	0.34
Dimethyl sulfide	14	48	20.0 ± 0.7	0.19
Sulfur dioxide	10	12	13900 ± 800	1.83
Ethyl acetate	8	15	87300 ± 3500	15.3
Ethyl propanoate	1	8	220 ± 10	0.74
Ethyl butyrate	5	7	120 ± 6	0.16
Ethyl hexanoate	8	13	357 ± 14	0.11
Ethyl octanoate	6	15	233 ± 9	0.05
Ethyl decanoate	10	17	79.2 ± 1.8	0.07
Ethyl isobutyrate	9	9	141 ± 12	0.35
Ethyl 2-methylbutyrate	6	8	27.4 ± 2.8	0.21
Ethyl 3-methylbutyrate	4	5	51.3 ± 0.9	0.35
Ethyl lactate	9	11	17500 ± 3000	41.3
Diethyl succinate	7	7	19400 ± 600	5.50
Isobutyl acetate	11	14	8.10 ± 0.14	0.33
Isoamyl acetate	7	14	333 ± 12	0.06
Phenethyl acetate	6	11	27.3 ± 1.1	0.23
Acetaldehyde	13	29	6491 ± 410	356
Diacetyl	11	16	990 ± 46	1.97
Isobutanal	8	13	45.5 ± 2.4	0.79
Methylbutanal	13	20	21.0 ± 1.1	0.38
2,3-Pentanedione	11	12	300 ± 17	0.87
Acetoin	10	11	22300 ± 600	4.52
Furfural	11	18	343 ± 79	6.43
Benzaldehyde	10	12	11.1 ± 0.2	0.02
β-damascenone	8	8	1.86 ± 0.04	0.04
Isobutanol	5	12	35400 ± 400	35.9
1-Butanol	7	7	718 ± 17	5.35
Isoamyl alcohol	5	11	245000 ± 4000	17.9
cis-3-Hexenol	7	9	180 ± 4	2.51
2-Phenylethanol	10	10	40900 ± 2400	2.82
Linalool	1	2	6.74 ± 0.35	0.04
Acetic acid	12	17	451000 ± 26000	354
Butyric acid	8	13	968 ± 22	22.9
Hexanoic acid	3	7	2290 ± 130	16.5
γ-Butyrolactone	7	13	17000 ± 400	241
trans-whiskeylactone	4	5	25.2 ± 0.6	0.13
cis-whiskeylactone	10	9	171 ± 3	22.8
4-Ethylphenol	7	9	340 ± 8	0.88
4-Ethylguaiacol	5	6	14.4 ± 0.2	0.75

[a] Uncertainty expressed as the standard error of the mean (n=3)

Table 4

Table 4. Linearity of the proposed DHS method.

Compound	Concentration range (µg/L)	Slope	R^2
Dimethyl sulfide	20 - 566	5.00×10^{-5}	0.9998
Acetaldehyde	1550 - 16400	5.35×10^{-4}	0.9983
Ethyl acetate	2100-41000	1.29×10^{0}	0.9998
Ethyl butyrate	120 - 2450	2.08×10^{0}	0.9945
Ethyl decanoate	80 - 1460	1.51×10^{0}	0.9999
1-Butanol	720 - 14900	6.22×10^{-1}	0.9986
2-phenylethanol	18000-112000	1.10×10^{-2}	0.9971
Butyric acid	970 - 17800	1.05×10^{-2}	0.9952
4-Ethylphenol	340 - 6270	6.85×10^{-2}	0.9995

Table 5

Table 5. 2-way-ANOVA carried out with data from the consecutive sampling of the headspaces of 4 different wines.

compound	wine (p)	injection number (p)	interaction (p)
Constant headspace concentration			
Sulfur dioxide	0.395	0.743	0.871
Acetoin	0.752	0.524	0.986
Furfural	0.925	0.293	0.981
Ethyl lactate	0.898	0.471	0.892
Diethyl succinate	0.427	0.500	0.881
Acetic acid	0.382	0.245	0.994
Butyrolactone	0.676	0.212	0.996
2-Phenylethyl acetate	0.437	0.360	0.931
β-damascenone	0.130	0.975	0.927
Isobutyl alcohol	0.791	0.634	0.677
Isoamyl alcohol	0.928	0.406	0.873
2-phenylethanol	0.746	0.659	0.954
Ethyl guaiacol	0.978	0.630	0.950
4-ethyl phenol	0.865	0.367	0.903
trans-whiskeylactone	0.902	0.197	0.922
Wine-dependent non-decay trends			
Acetaldehyde	**0.000**	0.394	**0.051**
2&3-Methylbutanal	**0.004**	0.868	0.273
Diacetyl	**0.036**	0.181	0.936
Isobutyraldehyde	**0.001**	**0.003**	**0.011**
Benzaldehyde	**0.001**	0.437	0.547
Simple decay trends			
Ethyl acetate	0.688	**0.000**	0.721
Propyl acetate	0.354	**0.000**	0.687
Ethyl propanoate	0.866	**0.000**	0.811
Ethyl butyrate	0.509	**0.000**	0.856
Ethyl isobutyrate	0.214	**0.000**	0.819
Isoamyl acetate	**0.097**	**0.000**	0.598
Ethyl-2-methylbutyrate	**0.062**	**0.000**	0.524
Ethyl-3-methylbutyrate	**0.087**	**0.000**	0.564
Ethyl hexanoate	0.334	**0.000**	0.766
Ethyl octanoate	0.210	**0.000**	0.751
Linalool	-	**0.000**	-
Wine-dependent decay trends			
Dimethyl sulfide	**0.050**	**0.000**	0.934
Methanethiol	**0.048**	**0.002**	**0.007**
Ethyl decanoate	**0.020**	**0.000**	0.586

Table 6

Table 6: Average ln β values and results obtained in the F-test to assess significant differences in Ln β for those compounds following clear decays.

Compound	Ln β [a]	$S^2_{between\ wines}$	$S^2_{within\ wine}$	F
Ethyl acetate	-0.10	0.0000	0.0001	0.54
Ethyl propanoate	-0.18	0.0004	0.0004	1.91
Propyl acetate	-0.18	0.0001	0.0004	0.31
Ethyl butyrate	-0.22	0.0000	0.0005	0.18
Isoamyl acetate	-0.25	0.0005	0.0005	1.84
Ethyl hexanoate	-0.27	0.0002	0.0005	0.81
Ethyl isobutyrate	-0.30	0.0002	0.0003	1.35
Ethyl 3-methylbutyrate	-0.30	0.0003	0.0004	1.40
Ethyl 2-methylbutyrate	-0.31	0.0005	0.0005	2.32
Ethyl octanoate	-0.35	0.0005	0.0005	1.86
Methanethiol	-0.36	0.0025	0.0025	2.07
Ethyl decanoate	-0.38	0.0048	0.0005	**20.75**
Dimethyl sulfide	-0.69	0.0055	0.0017	**6.50**

[a] Ln β value calculated as the average of each of the Ln β values (n=2) obtained from each wine (n=4). $S^2_{between\ wines}$ was calculated with the 4 Ln β values (3 degrees of freedom) and $S^2_{between\ wines}$ was calculated from regression analysis (24 degrees of freedom). Values in bold are significant at $p < 0.05$