Volatile Compound Release from Oak Chips in Model Wine Media: Combined Influence of Toasting Degree, Size, Time of Contact, and Ethanol Content

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Abstract

The effects of size, toasting degree and time of contact on the release of volatile compounds from *Quercus alba* (L.) chips during a simulated fermentation and post-fermentative process were studied. The results obtained indicated that the large size chips favoured the releasing of furfural and furfuryl alcohol, while the small ones increased the concentration of cyclotene and maltol. The interaction between chips size and time of contact showed that the small size chips are more sensible to the increase of ethanol concentration for the extraction rate of some compounds (furfural, vanillin, maltol, cyclotene, whiskey lactones and eugenol) compared to the large size ones, increasing their concentrations at the end of maceration. The toasting degree of oak chips had a different influence on the volatile compounds studied. Cyclotene and guaiacol concentrations increased with the toasting intensity whereas the extracted concentration of all compounds increased from light to medium toasted chips, excepting for eugenol, and then it decreased by further increasing the toasting level for 5-methylfurfural, whiskey lactones, eugenol, and only using high-level toasted chips for furfuryl alcohol, maltol and vanillin. A possible protection effect of the chips size toward the possible degradation or volatilization losses of furfural for high toasting degree was observed.

Key words: wine, oak chips, alcoholic fermentation, volatile compounds, LLE-GC-MS

1. Introduction

The use of wood in wine elaboration is a well-established practice: the physical structure and chemical composition of this material can offer some interesting opportunities to modify the grape juice and wine composition with a possible improvement of their sensory features and colour stability.[1] (Traditionally the most used wood species in the oenological field are *Quercus robur* (L.), *Quercus petraea* (Mattuschka) and *Quercus alba* (L.), but it is also possible to use the wood from *Robinia pseudoacacia* (L.), *Castanea sativa* (Miller) and *Prunus avium* (L.).[2].

To date, wine is often matured in different types of barrels such as "caratello", "barrique", "tonneau" or wood casks with greater volume capacity [3,4]. In more recent times, the use of wood pieces (i.e. chips, cubes, staves) in wine elaboration was authorized in the European Union (Council Regulation CE 2165/2005): this type of addition, combined with micro-oxygenation, allows to modify the organoleptic features of wines with a less expensive investment compared to the barrel aging.[2,5,6].

With the use of wood in oenology, it is possible to manage the chemical composition of the product improving the organoleptic features of the final wine. These modifications can be sorted into two groups: the evolution effect of the wine polyphenolic fraction (especially for red wines) and the enrichment in volatile compounds.[7].

The effect of wine treatment with wood is the result of different factors: botanical species, tree growing area, abiotic and abiotic factors, age of the trees and technological operation for the barrel and chips production. [8,9]. The mass/mass macro-composition of a mature oak wood is about 40% cellulose, 20% hemicellulose, 25% lignin, 10% hydrolysable tannins and a little part of lignans, lipids and carotenoids.[10,11]. One of the most important operations in wood preparation is the toasting, which strongly affects the chemical composition of wood due to the reaction of wood macro-components to heating conditions.[9].

From the pyrolysis, dehydration and multiple rearrangement of the hemicellulose fraction, furfural, 2,3-furaldehyde and 2,5-furaldehyde are formed, while 5-hydroxymethyl-2-furaldehyde (hydroxymethylfurfural) is originated from the glucose units of cellulose. The greater thermal stability of cellulose compared to hemicellulose is explained by the presence of crystalline structures.[11]. These furanic aldehydes are recognised as responsible for the "toasty" nuance.[12].

In angiosperm, lignin is an heteropolymer formed by co-polymerization of especially three phenylpropenoic alcohols: coniferyl alcohol, sinapyl alcohol and p-coumaric alcohol. From the heating of this wood fraction, different benzenic compounds are formed.[11] such as eugenol, guaiacol, syringol and 4-allyl-2,6-dimethoxyphenol[11]. For the compounds derived from the pyrolysis of lignin, different olfactory descriptors were described: vanillin can give vanilla hint, eugenol is described as "clove" scent, guaiacol as "smoke" nuance, syringol as "smoke" and "medicinal" scents.[13].

The level of wood toasting is also able to regulate the concentration of the unsaturated aldehyde (E)-3-nonenal, responsible for the "sawdust" aroma found in some wines. It was hypothesized that this compound is formed during the seasoning of the oak staves by the auto-oxidation of the linoleic acid contained in wood. Also an enzymatic origin was supposed with the intervention of lipoxygenase and hydroperoxide cleavage enzymes.[14]. Chatonnet and Dobourdieu in 1998 showed that a very significant reduction of unsaturated aldehyde extraction in the wine is observed when oak wood is heated to 200 °C for 5 min.[14].

From the lactonization of 3-methyl-4-hydroxyoctanoic acid, available in oak wood as the glycosylated precursor forms (galloylglucoside, glucoside, rutinoside), and after thermal degradation caused by toasting or acid-catalysed hydrolysis in the wine, the oak lactones are formed (*trans*-whiskey lactone and *cis*-whiskey lactone). These compounds have a strong coconut scent, and the *cis* isomer is more olfactive active than *trans* isomer.[15-18]. Oak lactones are also recognised as off-flavours when their concentrations exceed the preference limit of 240 $\mu g \cdot L^{-1}$ in red wines and 380 $\mu g \cdot L^{-1}$ in white wines (as racemic mixture of the two

isomers), with "woody" and "resinous" sensory descriptors [12]. The oak botanical origin plays a fundamental role at level of wine molecular influence: *Q. petraea* and *Q. alba* have shown an oak lactone concentration more than 15 times higher than *Q. robur.*[19,20].

The use of oak wood during the alcoholic fermentation, conducted in oak barrels or with chips added, leads to different results at level of aroma profile compared to the use of oak wood during wine aging. It was hypothesized that the compound furfurylthiol can be formed from the reaction between H_2S produced by the yeasts during the alcoholic fermentation and furfural.[21]. Furfurylthiol is a powerful compound with an olfactive threshold of 0.4 ng $\cdot$ L^{-1}. [16]. Yeasts during the alcoholic fermentation can reduce furfural into furfuryl alcohol, 5-methylfurfural into 5-methylfururyl alcohol and vanillin into vanillyl alcohol.[22-24]. The ethyl ethers of these alcohols were found in the wine.[24]. Also, vanillylthiol was discovered in red wines aged in barrel, with the putative precursor vanillin, although the formation mechanism remains unclear.[25].

Few studies have been published on the effect of using oak chips during alcoholic fermentation on the volatile composition of wines and most of them evaluate the effect of the addition at different doses and stages of the winemaking process and/or the biological origin of oak chips.[2,5,25,26,27]. To the best of our knowledge, the present study is the first to evaluate the effect of the oak chip size and the interaction of this factor with toasting intensity and contact time. The main goal of this study is to understand the volatile compounds release dynamics from oak chips in model solutions, simulating the ethanol increase as it occurs during alcoholic fermentation, as well as to assess the influence of chips size, toasting degree and time of contact on the volatile composition of these solutions. The use of model solutions allows to avoid the side-effects of the fermentative carbon dioxide production and the enzymatic activities of yeasts but just the oak chip addition and the ethanol concentration. To reach this objective, a specific liquid-liquid extraction combined with gas chromatography-mass spectrometry (LLE-GC-MS) method was developed. The outcomes of this study are of practical relevance to winemakers, allowing them to better manage the extraction of volatile compounds from oak chips and therefore to improve the sensory profile of wines.

2. Material and methods

2.1. Reagents and standards

All volatile compound standards, absolute ethanol, dichloromethane, tartaric acid, sodium hydroxide and sodium chloride were purchased from Sigma-Aldrich (Milan, Italy). The solutions were prepared in deionized water produced by a Milli-Q system (Merck Millipore, Darmstadt, Germany).

2.2. Oak chips material

All oak chips used were obtained from *Q. alba* wood species and they were provided by AEB (Brescia, Italy) as products specifically designed for wine contact. Two oak chip sizes were used (small and large) and four toasting degrees for each size were tested: light, medium, medium-plus and heavy.

Small size average dimensions (30 measurements): length 10.2 mm, depth 3.8 mm, height 1.3 mm. Large size average dimensions: length 13.2 mm, depth 8.1 mm, height 1.8 mm.

2.3. Simulated alcoholic fermentation and volatile compound concentration correction

The model solution was prepared with 4% v/v ethanol and 5 g $\cdot$ $L^{-1}$of tartaric acid, and the pH was adjusted to 3.4 with 4 M NaOH. One liter of model solution was placed in a 1 L glass jar (Bormioli Rocco, Fidenza, Italy) and 4 g $\cdot$ L^{-1} of oak chips were added together with a magnetic stir-bar. The oak chips were kept in immersion

thanks to a blocking net placed into the jar and the containers headspace was saturated with N_2. The solutions were then incubated at 28 °C in a laboratory oven. Sixteen jars were prepared (4 toasting factors × 2 size factors, these variables were completely crossed and tested in duplicate).

The samplings were performed at 4, 11, 18, and 33 days (final point) of maceration. At each intermediate point, the solution was stirred (with a magnetic bar) for 30 s, then an aliquot of 50 mL was taken and at the 4 and 11-day points replaced with 50 mL of absolute ethanol, therefore increasing by 5% ethanol by volume. Then, the headspace was saturated with N_2, and the glass jar was sealed and kept at 28 °C till the next sampling point.

The resulting alcohol concentration during the kinetics was therefore as follows: initial concentration 4 % v/v, at 4 days increased to 9 % v/v of ethanol, at 11 days increased to 14 % v/v of ethanol, at 18 days kept at 14 % v/v of ethanol until the end of the experiment (33 days).

Due to the subtraction of volatile compounds, from the second sampling onwards a correction to the concentration detected was applied for each analyte, as indicated in Equation 1, where: C_c = concentration corrected; C_D = concentration determined in the actual sampling; C_{CP} = concentration corrected of the previous sampling.

$$C_C = C_D + \frac{C_{CP} \cdot 50\ mL}{1000\ mL}$$

[equation 1]

The volume contraction due to the mix of absolute ethanol and a solution with a different ethanol concentration was considered negligible.

2.4. Extraction and determination of volatile compounds

To 50 mL of solution, the internal standard (3,4-dimethylphenol 60 mg · L^{-1} in 10 % v/v of absolute ethanol) and 5 g of NaCl were added, and volatile compounds were extracted three times with CH_2Cl_2 (10 mL × 3) in a 200 mL conical flask with stopper kept in agitation with a magnetic stirrer (VELP Scientifica, Usmate Velate, Italy) and a stir bar (10 min × 3 times). The dichloromethane fraction was recovered in a 100 mL round bottom flask, dehydrated with anhydrous Na_2SO_4 and evaporated using a gentle N_2 flow until approximately 75 µL volume measured through a sign on the vial's volume adaptor.

One µL of extract was injected in splitless mode in a 7890A gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a split/splitless injector set to 250 °C. The carrier gas was He (5.5 purity), with a purge flow of 3 mL · min^{-1} and a column flow of 1 mL · min^{-1}. The column used was a polyethylene glycol film capillary column DB-WAX (Agilent Technologies, Santa Clara, California, USA, 30 m, 0.25 mm, 0.25 µm). The oven program was 35 °C for 1 min, increased until 190 °C with a rate of 3 °C/min and kept for 0 min, finally increased until 230 °C with a rate of 4 °C/min and kept for 15 min[28].

The gas chromatograph was coupled with a 5975C mass spectrometer (Agilent Technologies, Santa Clara, California, USA), the transfer line was set to 280 °C, the acquisition mode was in scan with a *m/z* range of 35-350. Data integration was performed with the software MSD ChemStation (Agilent Technologies, Santa Clara, California, USA). Quantitative determination was carried out through linear regression using external standards.

2.4.1. Method validation

The sample extraction and analysis were validated through evaluation of ethanol interference, linearity, accuracy, repeatability and the limits of detection and quantification. The influence of ethanol concentration on analyte quantification, in particular on the area ratio between each analyte studied and internal standard (3,4-dimethylphenol), was evaluated in model solutions containing 4, 9 and 14% v/v of ethanol. The samples were extracted and analysed as previously described.

For the assessment of linearity, three different linear regressions were fitted for model solutions (pH 3.4) at 4, 9 and 14% v/v of ethanol spiked with analyte at seven concentration points, from 0 $\mu g \cdot L^{-1}$ to the maximum concentration of linear range for each compound. The samples were extracted and analysed as previously described. The semi-concentration value (expressed in $\mu g \cdot L^{-1}$ equivalent of 3,4-dimethylphenol) was chosen as the dependent variable, calculated as shown in Equation 2.

$$Semiconcentration = \frac{Analyte\ area}{Internal\ standard\ area} \cdot Internal\ standard\ concentration$$

[equation 2]

The method accuracy was assessed by the recovery calculation of analyte in two different situations. The first assessment was performed by adding 10 $g \cdot L^{-1}$ of small chips (medium toasted) to 1 L of model solution containing 14% v/v of ethanol, and subjecting them to 1 week of maceration at 28 °C, to create a matrix effect like that present in our experimental condition. After a week, the samples were analysed in triplicate as previously described to determine the native concentration, and an analyte concentration corresponding to the 50% of the maximum calibration range of each compound was added in three samples. These latter were extracted and analysed to calculate the recovery value (Equation 3), where: C_D = concentration detected in the sample spiked; C_N = native concentration without analyte addition; C_A = concentration added of analyte.

$$Recovery\ \% = \frac{C_D - C_N}{C_A} \cdot 100$$

[equation 3]

The recovery was also calculated using model solution without chips in triplicate at 4, 9 and 14% v/v of ethanol, as reported above.

The method repeatability was evaluated by analysing six times the same sample previously prepared for the recovery (model solution containing 14% v/v of ethanol, macerated at 28 °C for 1 week with small chips and spiked with analyte). The repeatability value was reported as residual standard deviation expressed as percentage.

The limits of detection and quantification (LOD and LOQ, respectively) were obtained from the linear regression model as described by ICH (1998), following Equation 4, where: σ = response standard deviation; b = slope of the regression equation.

$$LOD = \frac{\sigma \cdot 3.3}{b}$$

197 $$LOQ = \frac{\sigma \cdot 10}{b}$$

198 [equation 4]

199

200 *2.5. Statistical treatment of the data and graphical outputs*

201 For the evaluation of ethanol interference, the distribution of the three ethanol levels was checked for their
202 variance homogeneity through the Levene's test on median and for the ANOVA residual normal distribution
203 through the Shapiro-Wilk's test on residuals. Then, their means were compared through ANOVA (with type
204 III sum of squares) and, in the case of null hypothesis (F test, p-value < 0.05) rejection, Tukey's HSD test with
205 α -value of 0.05 was performed. For the linearity test, the linear regressions were fitted with the software R
206 3.5.1 (R statistical foundation, Austria): a first complete model was adapted and the intercept was submitted
207 to Student's t test with no statistical difference from zero as null hypothesis (α Student's t test of 0.05), in
208 the case of p-value > 0.05 the model was re-fitted without intercept (y = bx).

209

210 *2.5.1.Three-way repeated measure linear model with interaction*

211 To take into consideration the random factor generated by the longitudinal nature of the study, a linear
212 mixed effect model (lme) has been adopted using the software R 3.5.1 (R statistical foundation, Austria) and
213 fitting the model with the package nlme (Linear and Nonlinear Mixed Effects Models).[30]. For each analyte
214 considered, a three-way model with interaction was fitted, the independent variables were: time, size and
215 toasting degree, the interactions among these factors were included, the dependent variable was the
216 concentration of analyte, the random factor was the sample on which the longitudinal study was performed.

217 The data were checked for the model assumption with the procedure described by Pinheiro & Bates (2006).[31],
218 which is included in the package nlme.[30]: in the case of assumption violation, a natural logarithmic
219 transformation of x_i plus one was applied (plus one to overcome the presence of zero value, data below the
220 limit of quantification were considered zero for the statistical purpose). The data after the transformation
221 were newly checked as previously described. Only in the case of guaiacol no assumption requirement was
222 achieved and therefore, for this compound, a linear mixed model was not fitted.

223 After the linear mixed model fit, a type three ANOVA was calculated on the previous model. In the case of F
224 ratio p-value < 0.05 the marginal means of different levels of the factors and their interactions were tested
225 using the Bonferroni's α correction (total α-value of 0.05), this test was performed using the package
226 emmeans.[32].

227

228 *2.5.2.Principal component analysis*

229 The principal component analysis (PCA) was performed on scaled to unit data (single value divided by the
230 variable standard deviation) with the software R 3.5.1 (R statistical foundation, Austria) using the package
231 FactoMineR. [33]. Sample and variable coordinates on the two first principal components were plotted thank
232 to the packages Factoextra[34] and ggplot2.[35].

233

234 # 3. Results

235 *3.1. Influence of ethanol concentration on analyte determination*

236

As reported in Table 1, it is possible to observe a significant variation (F test, < 0.05) among different ethanol contents of the model solution on the analyte area/internal standard area ratio (A_M/A_{STD}) for most of the analytes under consideration. Only eugenol and 4-vinylguaiacol showed no statistical differences. This ratio represents the basis for a real quantification of analytes because it can be used as predictor (as such or multiplied by the concentration of internal standard) in a regression model.

For maltol and whiskey lactones, a negative relationship was observed between the A_M/A_{STD} ratio and ethanol concentration; for all other compounds the effect was opposite. This variation does not allow to use a single regression model on the 4-14 % v/v ethanol range.

3.2. Method linearity, limit of detection and limit of quantification

The regression model parameters derived from the 3 different matrices are shown in Table 2. Good linear regressions were obtained for the extraction and quantification method proposed with the determination coefficient (R^2) values higher than 0.989856 (furfural evaluation in model solution containing 14% v/v ethanol) while the R^2 coefficients exceeded 0.99 for all of the other studied analytes. The R^2 value is a useful indicator of the regression quality.

In most cases, the intercepts of the models were not significantly different from zero (for the Student's t-test). In these cases we chose to re-calculate the linear model without the intercept. With this operation it was also possible to increase the values of the determination coefficient (data not shown) and to avoid a possible mathematical conflict between the intercept value and the limits of quantification.

Sensitivity is intrinsically related to the slope of the regression model and it is defined as the change in the response corresponding to a change in the analyte quantity. Response factors, calculated as the inverse of the regression model slope, between 0.5 and 5 (good sensitivity) were obtained for most of the analytes determined, excepting for 4-vinylguaiacol with a response factor of about 12 (lower sensitivity).

The minimum amount of analyte that can be detected and quantified was determined through the calculation of the limit of detection (LOD) and limit of quantification (LOQ), as shown in Table 2. For all compounds studied, LOQ values ranged from 0.02 $\mu g \cdot L^{-1}$ to 0.46 $\mu g \cdot L^{-1}$. For all solutions tested, furfural and maltol showed the highest LOQ values.

3.3. Method accuracy

The accuracy of the method was evaluated by calculating the recoveries both in solution with chips added and in a model solution spiking the analytes of interest (Table 3). Regarding recoveries with added chips (Table 3), we can observe an error range in absolute value ranging from 31.09% for vanillin to 0.90% for furfuryl alcohol. We can also note a predominant tendency to underestimate the concentration of the analysed compounds. Also, for the recoveries determined without the use of chips we can note a preponderant tendency to underestimate the analytes concentration.

As shown in Table 3, error values less than 15% were obtained for all analytes, excepting for 4-vinylguaiacol in all the tested solutions, for vanillin in model solution containing 9% v/v ethanol, and for maltol in model solution containing 14% v/v ethanol. When the solution with chips added was used, furfural, 5-methylfurfural, whiskey lactones, guaiacol, vanillin and 4-vinylguaiacol achieved error values between 15 and 31%.

3.4. Method precision

The relative standard deviation (RSD) of the method with chips added (Table 4) in all cases is below 15% and ranged from 3.11% (eugenol) to 12.11% (4-vinylguaiacol). Excluding 4-vinylguaiacol, all the relative standard deviations calculated for analysis of spiked model solutions without chips (Table 4) are lower than 10%. At the same ethanol concentration (14% v/v), it is possible to note a relevant reduction in RSD for the model solution without chips added (9 cases on 10), only an increase in RSD was found for 4-vinylguaiacol The RSD value is lower than 15% and therefore the method can be considered precise, although the precision is less satisfactory for 4-vinylguaiacol.

3.5. Determination of volatile compounds released from oak chips

Using three-way model for chips maceration with interactions, the data obtained from the analysis of extracts subjected to oak chips maceration was statistically analysed according to the different characteristics of the used oak material: chips size (small, large), toasting degree (light, medium, medium-plus, heavy), and the sampling point (1-4). The results of the factorial model are reported as follows.

Regarding the first main effect, chips size affects only some molecules. The large size seems to favour the presence of furfural and furfuryl alcohol (Figure 1 A1 and C1); while the small size seems to increase the concentration of cyclotene and maltol (Figure 1 D1 and E1). No statistical differences were observed for all other analytes between chips size (Figure 1 B1; Figure 2 A1, B1, C1 and D1).

Regarding toasting degree of oak chips, a dual effect was observed for the majority of volatile compounds analysed. When a medium toasting intensity was used, the concentration of all compounds in model solution increased, and only for eugenol it did not differ from that corresponding to the light toasting degree (Figure 1 and Figure 2). Increasing the toasting intensity, a negative effect was observed for the extracted concentration of 5-methylfurfural, whiskey lactones and eugenol, from the medium-plus to heavy class (Figure 1 B2; Figure 2 A2, C2). A similar dynamic was observed for furfuryl alcohol, maltol and vanillin, whose concentration decreased when high toasting degree chips were used (Figure 1 C2, D2; Figure 2 D2). Lastly, cyclotene and guaiacol showed a generally continuous positive relation between toasting degree and extracted concentration (Figure 1 E2; Figure 2 B2).

From the interaction between chip size and toasting degree it is possible to note a “protection effect” of the chips size toward the possible compound degradation or volatilization losses due to an excess of heating for furfural (Figure 1 A3). The analysis of the interaction between these two factors allows to individuate the best option to maximize the concentration of a single compound; however, for a better approach, it is necessary to consider the entire model wine volatile profile, for example using a dimensional reduction technique as follows.

In Figure 3 it is possible to observe the results of the principal component analysis of all the combinations among size, toasting degree and time of maceration. The first two components together explained the 73.5% of the total variance. The first dimension (48.3% of explained variance) was strongly and positively correlated (coefficient ≥ 0.70) with guaiacol, vanillin, maltol and cyclotene; while the second dimension (25.2% of explained variance) was strongly and positively correlated with whiskey lactones, 5-methylfurfural and eugenol.

The light toasting group was characterized by a low amount of guaiacol, vanillin, maltol and cyclotene. The medium toasted chips group was marked by the greater concentration of whiskey lactones, eugenol, 5-methylfurfural and an intermediate presence of guaiacol, vanillin, maltol and cyclotene. A separation of the samples along the first dimension based on their toasting degree, especially for what concerns the light toasting group (in green colour) and medium group (in blue colour), was found (Figure 3). It resulted that light toasted chips and medium toasted chips are different based on the t multivariate 95% confidence

interval, while medium-plus and high toasted chips are similar between them but separated from other groups. All the samples belonging to these toasting degree groups (medium-plus, heavy) intersected their 95% confidence interval area, excluding the small medium plus-sample at 33 days of maceration (SM+4), which was located in the more positive point along the second PCA dimension and hence characterized by a greater concentration of compounds such as 5-methylfurfural, furfural, furfuryl alcohol, maltol and vanillin when compared to the other samples.

A double effect of toasting degree on the extracted concentration of whiskey lactones, eugenol and 5-methylfurfural is visible (Figure 3). Applying a medium toasting intensity, it was possible to note an increase of the compounds highly correlated to the second component, but when a medium-plus or a higher toasting level was applied the concentration of these compounds showed a decrease. Within high toasting group, the large size chips showed a systematic higher extracted concentration of whiskey lactones, eugenol and 5-methylfurfural compared to the small size chips. Finally, it is also possible to note from the Figure 3 that the effect of the maceration time on volatile compounds concentration increased with the toasting degree in a positive way.

4. Discussion

Analyzing the main published methods for the determination of volatile compounds derived from wood, we encounter various analytical techniques. The solid-phase microextraction (SPME) methods, such as those published by Díaz-Maroto et al., 2004.[36], Bozalongo et al., 2007.[7], and Chatonnet et al., 1999.[37], have the advantage of not using solvents for compound extractions. They also offer the benefit of easy automation of a significant part of the sample preparation process. However, they have the disadvantage that compounds with lower vapor pressure (high-boiling compounds) are difficult to detect or have a relatively high limit of quantification.

On the other hand, the method reported by Bosso et al., 2008.[38], is based on cartridge fractionation with a solid phase of silica functionalized with C18 polymers. It allows generating two fractions for each analyzed sample: a hydrophilic fraction (not retained by the cartridge), where it is necessary to add sodium chloride and an internal standard for liquid-liquid extraction and recovery of more hydrophilic compounds such as furans and most of the benzenoids. The elution from the cartridge with dichloromethane allows the recovery of whisky lactones and a portion of eugenol, isoeugenol, and vanillin. These latter benzenoids are generally distributed between the two generated fractions. The distribution of compounds between the two fractions depends largely on the ethanol concentration, but adequate dilution allows for excellent repeatability with the possibility of constructing reliable calibration curves. This fractionation can be both an advantage and a disadvantage. It represents an advantage in the analysis of wines, where simultaneous determination of fermentative compounds, varietal compounds, and wood-derived compounds is made possible without signal saturation of the detector and without loss of volatile compounds at low concentrations. However, it represents a disadvantage in specific cases when working with a model solution that does not contain fermentative compounds. Injecting such compounds in splitless mode would overload the column and saturate the mass spectrometer signal, requiring the preparation and injection of two fractions for each sample used.

Another type of technique developed is Stir Bar Sorptive Extraction (SBSE), as reported by Tredoux et al., 2008.[39]. It also has the advantage of not using solvents and being highly automatable, allowing for the determination of fermentative, varietal, and wood-derived compounds in a single analysis. However, this type of technology was not available in our specific case.

In terms of accuracy in determining the compounds studied in our work, various approaches are found among the methods reported in the literature. Díaz-Maroto et al., 2004.[36], maximized the analytical response for each target compound through optimization of sample preparation phases but did not evaluate the recoveries. Similarly, Bozalongo et al., 2007.[7], maximized the analytical response through optimization of sample preparation variables using the central composite design (CCD) technique but did not determine the analytical accuracy. As for Chatonnet et al., 1999.[37], who conducted a preliminary investigation on the use of SPME as a rapid discriminatory analysis of wood toasting levels, comparing it to a liquid-liquid extraction similar to the one validated in our study, but there is no evidence of the accuracy of the methods. Bosso et al., 2008.[38], relied on a method by Gianotti & Di Stefano, 1991.[40], which reported the untargeted semi-quantification technique. In the case of Tredoux et al., 2008.[39], the analytical responses are maximized through optimization of analysis variables, but the compound recoveries were not reported.

Regarding the precision of the method, the RSD% values of the applied method are similar to those of Díaz-Maroto et al., 2004.[36], generally slightly higher than those of Gómez García-Carpintero et al., 2014.[26], and Tredoux et al., 2008.[39], and generally lower than the methods reported by Chatonnet et al., 1999.[37].

Moving on to linearity, the applied method exhibited excellent coefficient of determination values for each established alcohol level, which are fully compatible with the adopted calibration range.

Regarding analytical data from the chips experiment, it is possible to assess different dynamics linked to the size and toasting degree of American oak chips. Starting from the chips size factor, large size seems to favour the release of furfural and furfuryl alcohol. For the compound furfural it was hypothesized in literature its volatilization at high temperature.[16]. Due to the fact that wood has a low coefficient of thermal conductivity, it is conceivable that the inner part of large chips reaches a lesser temperature with the same level of toasting compared to the small ones, avoiding the volatilization of this compound and limiting its loss in high toasted chips. Hale and co-workers (1999).[9], studying the toasting effect on oak wood carbohydrates and lignin derivatives, have hypothesized that a high energy provided to wood during the toasting phase can “destroy” some volatile products, while the use of a lower energy (i.e. in case of light toasting) can be insufficient to create a “large pool” of newly formed substances. In the same study the authors reported an important observation that can explain our “protection effect” hypothesis: analysing the oak wood structure they noted that the carbohydrates and lignin derivatives are mostly located at the wood surface, and their presence decreases rapidly in the inner wood material. In agreement with this last study, a “protection effect” towards the degradation/volatilization of oak chips volatile compounds was seen also for whiskey lactones and eugenol: when a high toasting intensity was applied, the large chips showed a greater contribution in these compounds compared to small chips.

The small size oak chips favoured the extraction of cyclotene and maltol. These compounds were described as products of Maillard’s reactions between glucose (originated from the pyrolysis of oak carbohydrate fraction) and proline [41,42]. It is conceivable a promoting effect for these compounds given by a lesser ratio between volume and area of the small chips compared to the large size ones. Regarding carbohydrate derivatives and lactones, Fernández de Simón et al. 2010.[43] comparing the effect of using toasted oak chips and staves have highlighted that the concentrations of maltol, cyclotene, 5-methylfurfural and lactones were higher in chips whereas furfural was higher in staves. Therefore, the oak response to the toasting conditions is determined by the chip size, probably due to physical and structural properties. The formation of vanillin is usually favoured from finer toasted oak pieces, which are more combustible, but vanillin losses by evaporation have been also detected for small oak chips, usually about 5 mm.[44].

The strong dual effect observed in the present study by increasing the toasting degree, with a first phase increasing all volatile compounds released in model solution and later one during which most of volatile compounds decreased, agrees with the results reported by Chatonnet (1998).[12] for vanillin, eugenol and whiskey lactones. In the same way, the generally continuous increase of guaiacol and cyclotene with the

toasting degree is in agreement with the trend observed by Chatonnet (1998).[12] for guaiacol. In fact, Campbell et al. (2005).[44] have detected low quantities of guaiacol for toasting temperatures below 230 °C.

The difference in concentration between the first sampling (after 4 days of maceration) and the last one (33 days) seemed to increase with the toasting degree. As a hypothesis, this disparity may be ascribed to the varying levels of extractable volatile compounds present in oak wood, which are in some cases correlated with the intensity of toasting. Through the evaluation of different oak chips parameters and interactions among them, it was possible to note a preponderant effect of toasting degree on the size of chips.

5. Conclusions

To reach the goal to study the combined influence of toasting degree, size, time of contact and ethanol content on a volatile compound releasing in wine media a suitable and rapid LLE-GC-MS method was validated based on the assessing the effect of ethanol concentration and evaluating its accuracy, repeatability and linearity.

Based on the compounds analysed in this study it is possible to indicate the typical volatile profiles for different toasting degrees: light toasted chips are marked by whiskey lactones and eugenol, medium toasted chips are distinguished by the presence of furfural, 5-methylfurfural, eugenol, and more whiskey lactones than light toasted ones. Medium plus chips until 18 days of maceration are distinguished by a high presence of vanillin, maltol and furfuryl alcohol, and after 33 days of maceration it is possible to also add a relevant concentration of whiskey lactones, vanillin, maltol and furfuryl alcohol. High toasted chips are marked by a high concentration of guaiacol and cyclotene.

The large size factor seemed favour the releasing of furfural and furfuryl alcohol probably due to the physical properties of low thermal conductivity of the wood.

Based on the scientific design of this experiment, it was possible to observe a preponderant effect of oak chips toasting on chip size in the definition of the volatile profile of model wines obtained. Furthermore, the time factor can affect the concentration of volatile compounds released from chips with the same toasting degree but this effect is more significant when the toasting is more intense.

The ethanol concentration influenced in a positive way the rate of extraction of more lipophilic compounds like whiskey lactones and eugenol, especially in small size chips probably due by ethanol nature and the greater exposed area for mass of wood added.

The study of the factors implied in the production of oenological chips can help to understand how to manage in a proper way the volatile profile of wines according to the features desired.

6. Supporting Information

Table S1: extracted compounds grouped by the main factor size.

Table S2: extracted compounds grouped by the main factor toasting degree.

Table S3: extracted compounds grouped by the interaction between size and time of maceration.

Table S4: extracted compounds grouped by the interaction between toasting degree and time.

Table S5: extracted compounds grouped by the interaction between size and toasting degree.

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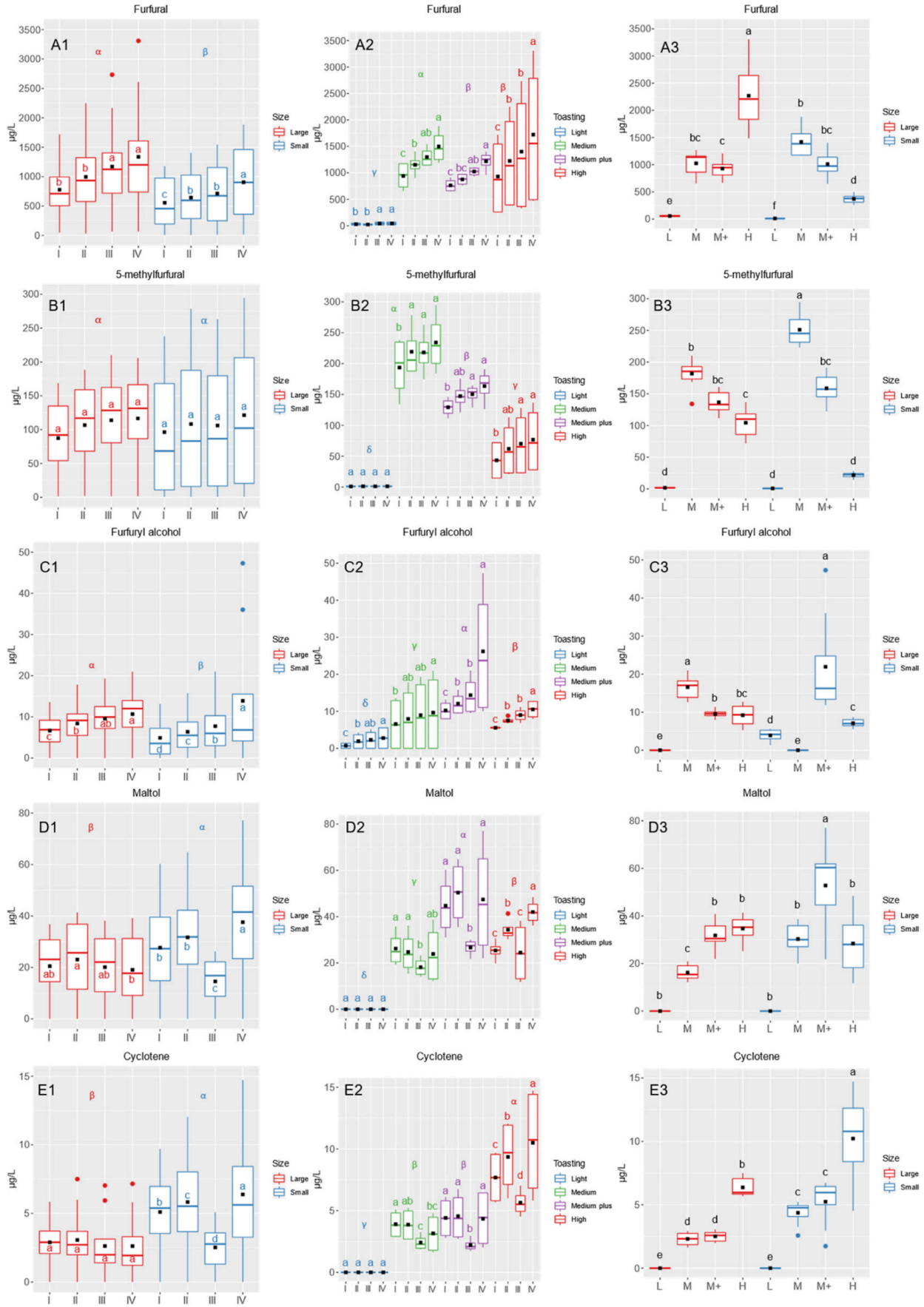
Figure captions and figures

Figure 1. Carbohydrate-derived volatile compounds (rows) extracted from oak chips: separation by sampling point and size (column 1), sampling point and toasting (column 2), toasting and size (column 3); different letters represent significantly different means for the t-test multiple comparison with Bonferroni's p-value correction based on marginal means; Greek letters were used to compare the different levels of main factor, Latin letters were used to compare the different level of interaction. I = 4 days and 4 % v/v of ethanol; II = 11 days and 9 % v/v of ethanol; III = 18 days and 14 % v/v of ethanol; IV = 33 days and 14 % v/v of ethanol. L = light toasting; M = medium toasting; M+ = medium-plus toasting; H = high toasting.

Figure 2. Whiskey lactone- and lignin-derived volatile compounds (rows) extracted from oak chips: separation by sampling point and size (column 1), sampling point and toasting (column 2), toasting and size (column 3); different letters represent significantly different means for the t-test multiple comparison with Bonferroni's p-value correction based on marginal means; Greek letters were used to compare the different levels of main factor, Latin letters were used to compare the different level of interaction. For guaiacol was not possible to calculate the statistics due to assumptions not respected. I = 4 days and 4 % v/v of ethanol; II = 11 days and 9 % v/v of ethanol; III = 18 days and 14 % v/v of ethanol; IV = 33 days and 14 % v/v of ethanol. L = light toasting; M = medium toasting; M+ = medium-plus toasting; H = high toasting.

Figure 3. PCA biplot: each individual sample is codified as follows: first letter, chips size (S small, L large); second letter (and plus sign), toasting degree (L light, M medium, M+ medium-plus, H heavy); last digit, sampling point (1 after 4 days, 2 after 11 days, 3 after 18 days, 4 after 33 days).



588
589 Figure 1

590

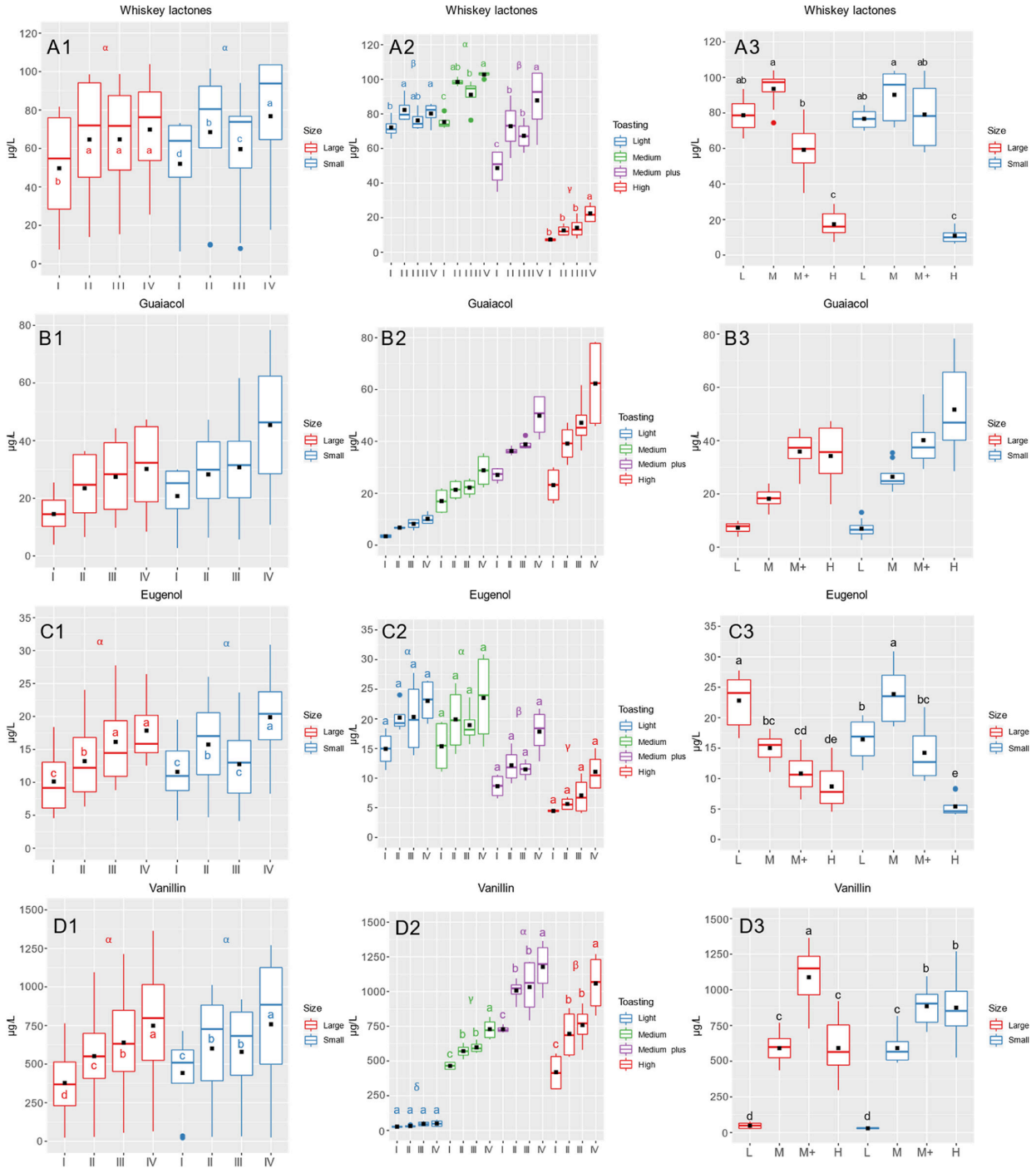

Whiskey lactones
A1
A2
A3
Guaiacol
B1
B2
B3
Eugenol
C1
C2
C3
Vanillin
D1
D2
D3
µg/L
Size
Large
Small
Toasting
Light
Medium
Medium plus
High
I II III IV
L M M+ H

Figure 2

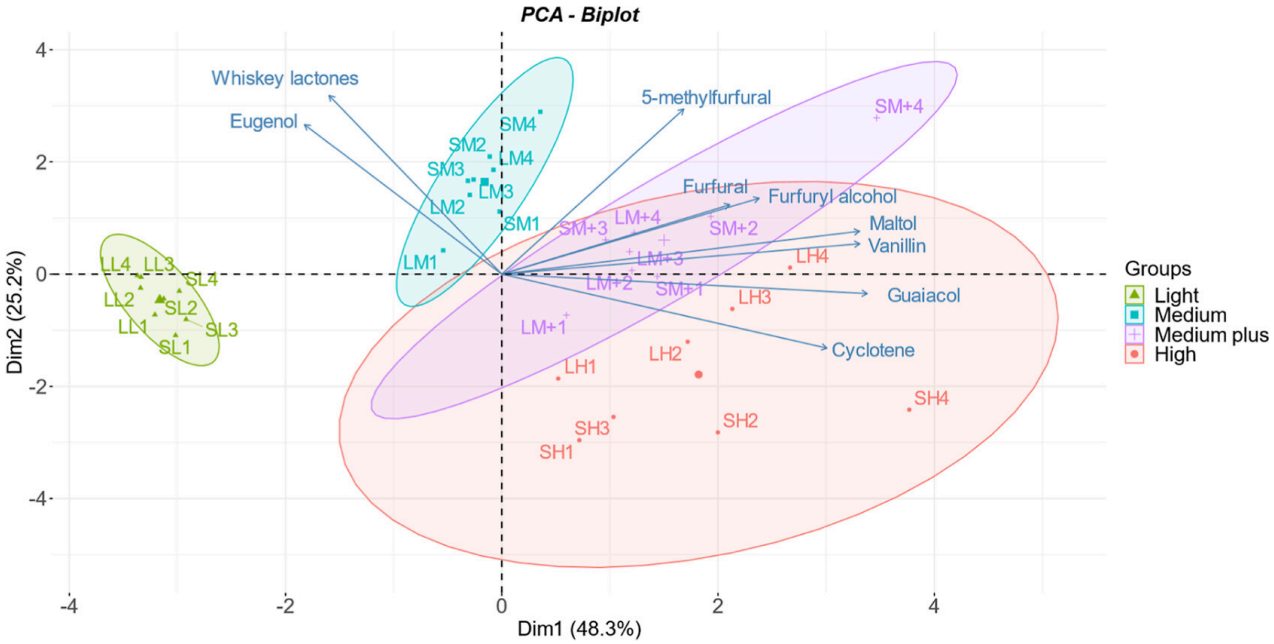

PCA - Biplot
Whiskey lactones
Eugenol
5-methylfurfural
Furfural
Furfuryl alcohol
Maltol
Vanillin
Guaiacol
Cyclotene
LL4
LL3
SL4
LL2
SL2
LL1
SL3
SL1
SM4
SM2
LM4
SM3
LM3
LM2
SM1
LM1
SM+4
SM+3
LM+4
SM+2
LM+3
LH4
LM+2
SM+1
LH3
LM+1
LH2
LH1
SH4
SH3
SH2
SH1
Groups
Light
Medium
Medium plus
High
Dim2 (25.2%)
Dim1 (48.3%)
4
2
0
-2
-4
-4
-2
0
2
4

Figure 3

Tables

Table 1. Influence of different ethanol concentrations on analyte area/internal standard area

Compound	4% v/v ethanol	9% v/v ethanol	14% v/v ethanol	Sign.
Furfural	3.17 b	3.22 ab	3.49 a	*
5-Methylfurfural	1.09 b	1.13 ab	1.19 a	**
Furfuryl alcohol	0.13 c	0.14 b	0.16 a	***
Maltol	0.35 a	0.34 a	0.30 b	**
Cyclotene	0.37 b	0.38 ab	0.40 a	*
Whiskey lactones	1.65 a	1.59 b	1.59 b	**
Guaiacol	0.64 b	0.66 ab	0.68 a	*
Eugenol	0.27	0.27	0.26	n.s.
Vanillin	3.94 a	3.65 b	4.12 a	**
4-vinylguaiacol	0.04	0.04	0.05	n.s.

Data are expressed as ratio between analyte area and internal standard area; Sign.= ANOVA significance; n.s. = p-value > 0.05; *0.01 ≤ p-value < 0.05; ** 0.001 ≤ p-value < 0.01; *** p-value < 0.001. Different letters within the same row indicate significant differences among treatments according to Tukey's HSD test.

640 *Table 2. Parameters from ordinary least square regressions, x=concentration, y= semi-concentration (3,4-dimethylphenol*
641 *equivalents), LOD and LOQ are expressed in µg · L^{-1}; n.s.= intercept not different from zero by Student's t test (α= 0.05)*

Compound	Slope	Intercept	R^2	Sign. intercept	LOD	LOQ
4% v/v ethanol						
Furfural	0.468 ± 0.014	0	0.994700	n.s.	0.10	0.30
5-methylfurfural	0.660 ± 0.007	0	0.999300	n.s.	0.04	0.11
Furfuryl alcohol	0.196 ± 0.001	0	0.999700	n.s.	0.02	0.07
Maltol	0.399 ± 0.015	0	0.992100	n.s.	0.12	0.36
Cyclotene	0.379 ± 0.002	0	0.999800	n.s.	0.02	0.06
Whiskey lactones	1.864 ± 0.005	0	0.999953	n.s.	0.01	0.03
Guaiacol	0.740 ± 0.006	0	0.999564	n.s.	0.03	0.09
Eugenol	0.617 ± 0.002	-0.730 ± 0.275	0.999947	*	0.01	0.03
Vanillin	0.683 ± 0.010	0	0.998625	n.s.	0.05	0.15
4-vinylguaiacol	0.082 ± 0.000	0	0.999839	n.s.	0.02	0.05
9% v/v ethanol						
Furfural	0.422 ± 0.014	0	0.993333	n.s.	0.11	0.33
5-methylfurfural	0.615 ± 0.004	0	0.999768	n.s.	0.02	0.06
Furfuryl alcohol	0.200 ± 0.001	0	0.999770	n.s.	0.02	0.06
Maltol	0.403 ± 0.014	0	0.992656	n.s.	0.12	0.35
Cyclotene	0.376 ± 0.004	0	0.999351	n.s.	0.03	0.10
Whiskey lactones	1.819 ± 0.010	0	0.999814	n.s.	0.02	0.06
Guaiacol	0.704 ± 0.002	0	0.999947	n.s.	0.01	0.03
Eugenol	0.605 ± 0.005	0	0.999521	n.s.	0.03	0.09
Vanillin	0.658 ± 0.016	0	0.996250	n.s.	0.08	0.25
4-vinylguaiacol	0.078 ± 0.001	0	0.999746	n.s.	0.02	0.07
14% v/v ethanol						
Furfural	0.418 ± 0.017	0	0.989856	n.s.	0.14	0.41
5-methylfurfural	0.658 ± 0.014	0	0.997302	n.s.	0.07	0.21
Furfuryl alcohol	0.228 ± 0.001	0	0.999765	n.s.	0.02	0.06
Maltol	0.442 ± 0.014	0	0.994161	n.s.	0.10	0.31
Cyclotene	0.414 ± 0.002	0	0.999816	n.s.	0.02	0.06
Whiskey lactones	1.869 ± 0.019	0	0.999368	n.s.	0.03	0.10
Guaiacol	0.735 ± 0.008	0	0.999334	n.s.	0.03	0.11
Eugenol	0.603 ± 0.001	0	0.999976	n.s.	0.01	0.02
Vanillin	0.648 ± 0.012	0	0.997815	n.s.	0.06	0.19
4-vinylguaiacol	0.084 ± 0.000	0	0.999929	n.s.	0.01	0.03

642 Intercept: expressed in µg · L^{-1}; Sign. intercept: decision on Student's t test null hypothesis; n.s.= p-value > 0.05, * 0.01
643 ≤ p-value < 0.05; LOD: limit of detection expressed in µg · L^{-1}; LOQ: limit of quantification expressed in µg · L^{-1}. Parameters
644 of the linear regression are reported ± standard error.

645

646 *Table 3. Method recovery for model solutions without chips at three ethanol levels, and with chips maceration*

Compound	C added	C calculated	Recovery %	Error %
4% v/v ethanol, without chips				
Furfural	2312.00	2044.96	88.45	-11.55
5-methylfurfural	548.50	500.21	91.20	-8.80
Furfuryl alcohol	226.80	198.41	87.48	-12.52
Maltol	268.50	261.53	97.40	-2.60
Cyclotene	318.50	298.19	93.62	-6.38
Whiskey lactones	259.00	267.93	103.45	3.45
Guaiacol	285.75	260.86	91.29	-8.71
Eugenol	133.75	133.45	99.78	-0.22
Vanillin	2020.00	1740.32	86.15	-13.85
4-vinylguaiacol	253.58	148.20	58.44	-41.56
9% v/v ethanol, without chips				
Furfural	2312.00	2307.95	99.83	-0.17
5-methylfurfural	548.50	556.78	101.51	1.51
Furfuryl Alcohol	226.80	218.99	96.56	-3.44
Maltol	268.50	253.99	94.59	-5.41
Cyclotene	318.50	308.24	96.78	-3.22
Whiskey Lactones	259.00	263.45	101.72	1.72
Guaiacol	285.75	282.69	98.93	-1.07
Eugenol	133.75	133.26	99.63	-0.37
Vanillin	2020.00	1675.97	82.97	-17.03
4-vinylguaiacol	253.58	163.78	64.59	-35.41
14% v/v ethanol, without chips				
Furfural	2312.00	2524.15	109.18	9.18
5-methylfurfural	548.50	548.04	99.92	-0.08
Furfuryl Alcohol	226.80	216.35	95.39	-4.61
Maltol	268.50	201.90	75.20	-24.80
Cyclotene	318.50	291.07	91.39	-8.61
Whiskey Lactones	259.00	256.25	98.94	-1.06
Guaiacol	285.75	280.79	98.26	-1.74
Eugenol	133.75	132.29	98.91	-1.09
Vanillin	2020.00	1920.05	95.05	-4.95
4-vinylguaiacol	253.58	190.51	75.13	-24.87
14% v/v ethanol, with chips				
Furfural	915.55	660.83	72.18	-27.82
5-methylfurfural	217.21	163.44	75.25	-24.75
Furfuryl Alcohol	88.91	89.71	100.90	0.90
Maltol	107.40	109.38	101.85	1.85
Cyclotene	124.85	126.18	101.06	1.06
Whiskey Lactones	101.53	85.80	84.51	-15.49
Guaiacol	114.30	93.19	81.53	-18.47
Eugenol	52.97	51.40	97.04	-2.96
Vanillin	399.96	275.60	68.91	-31.09
4-vinylguaiacol	101.43	78.66	77.55	-22.45

647 C added = concentration in $\mu g \cdot L^{-1}$L added; C calculated = concentration in $\mu g \cdot L^{-1}$ calculated

Table 4: Method repeatability for model solutions without or with chips maceration

Ethanol percentage	***4% v/v***	***9% v/v***	***14% v/v***	***14% v/v***
Chips presence	***Without chips***	***Without chips***	***Without chips***	***With chips***
Compound	**RSD (%)**	**RSD (%)**	**RSD (%)**	**RSD (%)**
Furfural	2.24	5.51	1.56	7.33
5-methylfurfural	1.60	4.56	1.36	6.78
Furfuryl Alcohol	1.68	6.12	1.25	3.46
Maltol	4.56	4.55	3.58	5.40
Cyclotene	2.96	2.59	1.34	3.22
Whiskey Lactones	0.38	0.82	1.40	6.26
Guaiacol	0.42	3.40	0.67	10.33
Eugenol	1.18	1.21	0.52	3.11
Vanillin	1.96	3.01	1.46	4.16
4-vinylguaiacol	25.92	21.25	23.03	12.11

RSD % = relative standard deviation, also named variation coefficient (standard deviation on mean, expressed in percentage).