Recovery of aromatic aglycones from grape pomace winemaking by-products by using liquid-liquid and pressurized-liquid extraction

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ABSTRACT

The potential of winemaking grape pomace by-products as a source of glycosidic aroma precursors that under enzymatic hydrolysis might release aroma compounds has been evaluated. Two different extraction methodologies, Liquid Liquid and Pressurized Liquid Extraction (LLE and PLE) were employed. Solid Phase Extraction (SPE)-GC-MS analysis of the hydrolyzed LLE glycosidic extract revealed 22 aroma compounds belonging to different chemical families (terpenes, C13 norisoprenoids, vanillines, etc). Response surface methodology was employed to study the effect of the most significant PLE experimental variables (temperature and solvent composition) on the extraction of aromatic aglycones. The parameters of the model were estimated by multiple linear regressions. Most of the aroma compounds showed an adequate fit to the calculated model (18 compounds from 22 with R^2>0.8). The application of the optimized PLE conditions (50% of ethanol in the hydroalcoholic solution) and 90 ºC showed higher extraction yield of aglycones when comparing with the extraction yield obtained by LLE.

Key words: winemaking grape pomace by-products, glycosidic aroma precursors, varietal aroma compounds, Pressurized Liquid Extraction, SPE-GC-MS

Introduction

Grape pomace consists in the skin, stems and seeds of grapes that remain after processing in the wine and juice industry. Recently, it has been stated that 10 million tons of grape pomace was produced in 2005 from 66 million tons of harvested grapes (*Vitis vinifera* L.) in the world [1]. Grape processing wastes can be an important economical problem to producers besides the environmental impact caused by the large amount of these types of residues generated during the harvest season. The majority of this pomace is discarded as natural waste or distilled to produce alcohol and other distilled beverages. However, as Fernández and collaborators have recently pointed [2] the new regulation in the reform of the Common Organization Market (OCM) of wine eliminates the subsidy to distillation in 2013. Therefore, the wineries will have new economic difficulties with winery waste management.

Besides some traditional applications of grape pomace for animal fed formulations [3] or compost production [4] in the latest years, the scientific works carried out on the characterization of the chemical components of waste grape by-products has allowed looking for different applications in trying to obtain high added value ingredients. Some of these applications are the production of grape seed oil [5] or biodiesel from it [2], obtaining dietary fibre [6-8] and mainly in the last years, the extraction of polyphenols with antioxidant properties [9-14] for food, cosmetic or pharmaceutical applications.

Some other potential applications, such as the use of grape pomace to recover aroma compounds have been less explored. Ruberto and collaborators [15] explored this possibility, but they focused on the free volatile profile of grape pomace coming from the processing of different grapes varieties (Nero d'Avola, Nerello Mascalese, Frappato

and Cabernet Sauvignon), showing a volatile profile mainly dominated by carboxylic acid derivatives with relatively high odor thresholds.

However, grape aroma compounds can be present both as free volatiles and in much higher concentrations, as non-volatile sugar-bound glycoside conjugates [16]. The occurrence of glycosidically bound volatiles is typically two to eight times greater that of their free counterparts [17] and, although their distribution in the grape berry might change during ripeness [18] they are present in the largest amount in the skin [19]. In spite that grape glycosides are non-volatile odorless flavor precursors, under enzymatic or acid hydrolysis during winemaking they can release the corresponding odorant aglycones, which are generally potent flavor compounds (monoterpenes, norisoprenoids, benzenoids compounds, etc) characterised by low aroma thresholds and interesting sensory properties [17].

In spite of the evident interest of using grape glycosides as a source of aroma compounds, the works focused on the characterization of glycosides in grape pomace are scarce in the literature. Only Vasserot and collaborators [20] carried out pioneer studies, in which they evidenced the presence of monoterpenol glucosides in Muscat grape by-products. Nonetheless, in their study, the characterization of the released odorant aglycones, which are the interesting compounds as a source of natural flavors, was not performed, since they quantified the total amount of monoterpenols using a colorimetric assay.

For the extraction of grape aroma glycosides, most of the works in the literature use liquid- liquid extraction employing hydroalcoholic solutions letting the sample macerate in the darkness during long extraction times (24 hours at least) [21-23]. However, other technologies, such as the use of supercritical CO_2 extraction has also been successfully used [24], although in the above mentioned work the characterization of the corresponding

odorant aglycones was not performed. The use of pressurized liquid extraction (PLE) is a relatively new extraction approach that is being applied for the extraction of different types of phytochemicals from plants [9, 10, 25, 26]. The use of high pressure-high temperature extraction might increases the contact with the solvent facilitating solvent penetration into complex matrices such as grape pomace. In addition, other advantages are the relatively short extraction times and the possibility of using GRAS solvents or even water (subcritical water), which makes PLE a "green" extraction methodology [26, 27]. Different procedures using PLE have been optimized for the extraction of some phytochemicals from grape pomaces [10, 25] in recent years. However, as far we now, none scientific work has evaluated the use of PLE for the extraction of glycoside aroma precursors.

Therefore, the objective of this work has been firstly to check the potential of grape pomace (*Verdejo* white grape variety) as a source of glycosides that under enzymatic hydrolysis might release aroma compounds, and secondly, to know the feasibility of PLE for the extraction of these glycosides comparing it with the more conventional liquid-liquid extraction (LLE).

Materials and Methods

Grape Pomace Samples

Grape pomace from Verdejo white grape variety was provided by a winery from the O.D Rueda (Spain). Fresh pomace from pressed grapes (pneumatic pressing) previously submitted to a maceration process (without fermentation), was immediately recovery, and placed into plastic bags in absence of oxygen, sealed and stored at -20ºC. Frozen

120 grape pomace was dried in a lyophiliser (Labonco, Kansas City, MO, USA) and ground
121 into a fine and homogenous powder using a commercial coffee grinder. The powder was
122 stored at -20ºC in absence of oxygen till it was used for the analyses.
123
124 Extraction of glycosidic aroma precursors from grape pomace by liquid-liquid
125 extraction (LLE)
126
127 The procedure for the extraction of aroma precursors from grape pomace was based on
128 that described by Hernandez-Orte and co-authors [21] with some modifications. One
129 hundred grams of the grape pomace powder were suspended in 500 mL of a buffer
130 solution (0.1 M Na_2HPO_4/NaH_2PO_4) at pH 7 and 13% (v/v) ethanol (Scharlau Chemie S
131 A., Barcelona, Spain) allowing macerating in the darkness in absence of oxygen (60 h,
132 20ºC in a nitrogen atmosphere). This solution was centrifuged at 16770 g for 15 min at
133 20 ºC, and the supernatant was filtered through filter paper. Ethanol was removed from
134 the sample by using a Rotavapor R-200 (Buchi Labortechnik AG, Flawil, Switzerland)
135 at 25 ºC.
136
137 Extraction of glycosidic aroma precursors from grape pomace by pressurized-liquid
138 extraction (PLE)
139
140 Aroma precursors were extracted from the grape pomace by using an accelerated
141 solvent extractor (ASE 200, Dionex Corporation, Sunyvale, CA) equipped with a
142 solvent flow controller. Two solvents of different polarity, ethanol (Scharlau Chemie
143 S.A.), and purified water by using a Milli-Q system (Millipore, Inc., Bedford, MA)
144 were employed. Freeze dried grape pomace (9 g) was dispersed thoroughly with 9 g of

145 sea sand (Panreac, Barcelona, Spain). The homogeneous mixture was loaded into a 33
146 ml extraction cell with a cellulose paper filter at the bottom of the cell. PLE
147 experimental variables were pressure (1500 psi), three extraction cycles, flush volume
148 (60 %), nitrogen purge time (60 sec), static time (8 min) and preheat time (5 min).
149 Ethanol was removed from the collected sample by using a Rotavapor R-200 (Buchi
150 Labortechnik AG, Flawil, Switzerland) at 25 ºC. The experiment was repeated until the
151 complete extraction of 50 g of grape pomace.
152
153 Solvent and Temperature Optimization in the PLE method
154
155 The effect of two factors, solvent type (*S*) and temperature (*T*) on the relative peak area
156 of each aroma compound (response variable) obtained after the hydrolysis of the grape
157 glycoside aroma precursors recovered from the grape pomace was evaluated by using a
158 central composite circumscribed (CCC) design [28]. A total of 10 assays: four points of a
159 full factorial design (combination of levels -1 and +1), four star points (at levels ± α, α =
160 start distance = 1.414), and two centre points to estimate the experimental error, were
161 carried out in randomized run order. By using this design, the two factors were tested at
162 five different experimental levels: the concentration of ethanol employed in the
163 hydroalcoholic mixture as solvent (*S*) at 0, 15, 50, 85 and 100 (% v/v EtOH); and the
164 temperature (*T*) at 48, 60, 90, 120 and 132 (ºC); in correspondence with the coded
165 levels: -1.414, -1.000, 0, +1.000, +1.414, respectively. **Table 1** shows the experimental
166 matrix design, with the experimental levels of the independent variables (factors).
167 The quadratic polynomial model proposed for the response variable (Y_i) for each
168 selected volatile compound was:

$$Y_i = \beta_o + \beta_1 S + \beta_2 T + \beta_{1,1} S * S + \beta_{2,2} T * T + \beta_{1,2} S * T + \varepsilon \qquad \text{(Equation 1)}$$

Where β_o is the intercept, β_i the linear coefficients, $\beta_{i,i}$ the quadratic coefficients, $\beta_{1,2}$ the interaction coefficient, and ε is the variable error. The parameters of this model were estimated by Multiple Linear Regression (MLR) using the Statgraphics Centurion XV program (StatPoint Inc., www.statgraphics.com) that permits the creation and analysis of experimental designs. The effect of each term and their statistical significance for each of the response variables (aroma compounds released from the corresponding glycosides) were analysed from the standardized Pareto chart. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the residual standard deviation (RSD). The terms not significantly different from zero ($p>0.10$), were excluded of the model and the mathematical model was re-fitted by MLR. From the fitted model, the estimated surface plot and the optimum conditions that maximized the response variable were obtained.

Isolation of glycosides aroma precursors from the grape pomace extracts by using Solid Phase extraction (SPE)

The glycosides aroma precursors contained in the extracts obtained by LLE or PLE were isolated by adsorption onto an Amberlite XAD-2 (Supelco, Bellefonte, USA), column. A 10 cm length glass column (Pobel, Madrid, Spain), filled with 40 g of Amberlite XAD-2 was prepared by sequentially conditioning it with 120 mL of dichloromethane, methanol and water. The sample extract was introduced into the

column which was afterward rinsed it with 100 ml of water and 150 ml of pentane/dichloromethane (2:1 v/v) to remove any residual of free volatiles. Elution of the glycosides aroma precursors was performed with 150 mL of ethyl acetate/methanol (9:1 v:v). This fraction was collected and solvent was evaporated by using a rotavapor (Buchi Labortechnik AG). The dried extract was reconstituted in 4 mL of water, extracted twice with 1 mL of dichloromethane and 1 ml of pentane to ensure the complete removal of free volatiles, aliquoted and stored at -20ºC. The absence of free volatiles in the aroma precursor extract was further tested.

Release of aromatic aglycones from the glycosidic extracts by enzymatic hydrolysis

Previous to the GC-MS analysis, the glycoside extracts from grape pomace were submitted to enzymatic hydrolysis to release the corresponding free aroma compounds (aglycones). Enovin® (Agrovin, Ciudad Real, Spain), a commercial oenological enzymatic preparation of several *Aspergillus niger* (GMO free) with β-glucosidase activity was used to release the odorant aglycones. The enzymatic preparation was dissolved in a citrate/phosphate buffer (pH=5; 51.5% 0.2 M sodium phosphate and 48.5% 0.1 M citric acid) and 500 μL of a 20 mg/mL of the enzyme solution were added to the glycosidic precursors extract. The amount of enzyme was previously optimised to provide the maximum hydrolysis yield. After the addition of 50 μL of a 90 mg/mL solution of n-octylglucoside in ethanol as internal standard, the mixture contained in a tube was closed and placed in a bath at 40 º C for 16 h. The hydrolyzed was cooled over ice, and the released aglycones were analysed by SPE following the procedure described as following.

218 Analysis of the aroma compounds released from the glycosidic aroma precursors by
219 SPE-GCMS
220
221 The SPE was carried out using the method proposed and validated by Loscos and
222 collaborators [23] with slight modifications. The total volume of the glycoside hydrolisate
223 containing 20 µL of a solution of β-damascone from Sigma-Aldrich (0.25 mg/ml in
224 ethanol) as internal standard (previously, it was checked its absence in the hydrolysed
225 extract) was passed through a 50 mg LiChrolut EN cartridge (Merck KGaA, Darmstadt,
226 Germany) previously pre-conditioned (2 mL of dichloromethane, 2 mL of methanol and
227 2 mL of a 12% ethanol solution). The sorbent was washed with 5 mL of 40% (v/v)
228 methanol solution and dried by letting air pass through (0.6 bar, 10 min). Aglycones
229 were recovered by elution with 1 mL of dichloromethane. Twenty µL of an internal
230 standard solution (4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone and 2-octanol
231 at a concentration of 465.5, 598.5 and 665 µg in 10 ml of dichloromethane) were added
232 to the eluted sample. The extract was concentrated under a gentle stream of nitrogen to a
233 final volume of 100 µL and then analyzed by GC-MS under the conditions described
234 below.
235
236 Two µL of the aroma extracts were directly injected in splitless mode into the GC-MS.
237 (Agilent 6890) provided with an Agilent MSD ChemStation software to control the
238 system. For separation, a Supra-Wax fused silica capillary column (60 m × 0.25mm i.d.
239 × 0.50 µm film thickness) from Konik (Barcelona, Spain) preceded by a 50 cm x 0.25
240 mm uncoated and deactivated precolumn from Quadrex (Woodbridge, CT, USA) was
241 used. Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was

initially held at 40 ºC for 5 min, then increased at 4 ºC/min to 240 ºC and held for 20 min.

For the MS system (Agilent 5973N), the temperatures of the transfer line, quadrupole and ion source were 270, 150 and 230 ºC respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and Sim modes for some specific compounds. The signal corresponding to a specific ion of quantification was calculated by the data system. The identification of compounds was carried out by comparison of retention times and mass spectra of the references compounds with those reported in the mass spectrum library NIST 2.0. Quantitative data were obtained by calculating the relative peak area in relation to that of the corresponding internal standard. To calculate the concentration of each aroma compound, calibration curves of each reference compound at different concentrations covering the concentration ranges expected in the samples were prepared in dichloromethane and analysed in the same conditions that the samples. To do so, standards of volatile compounds of the maximum purity available (>98%) were purchased from different providers: Aldrich (Steinheim, Germany); Fluka (Buchs, Switzerland); Merck (Munchen, Germany) and Firmenich (Geneve, Switzerland). These compounds are shown in **Table 2**.

Results and Discussion

Aroma compounds released after the hydrolysis of aroma precursor glycosides recovered from grape pomace using LLE.

Aroma compounds were released from the aroma glycosides extracted from Verdejo grape pomace by using commercial fungal glycosidases, therefore in trying to obtain a more natural flavour profile compared to the acidic hydrolysis, which has been indicated it might induce a molecular rearrangement and the transformation of some of the liberated aglycones [17]. The aroma composition after the enzymatic hydrolysis of the glycoside extracts recovered by LLE was shown in **Table 2**. A total of 22 varietal aroma compounds belonging to different chemical families (terpenes, C13 norisoprenoids volatiles phenols, benzenoids, vanillines and lipid derivatives) were identified based on their characteristic gas chromatography and mass spectra data. **Figure 1** shows a chromatogram of the typical GC-MS volatile profile of the varietal compounds released from the glycosidic aroma precursors. All the compounds identified in the extracts came from the hydrolysis of the glycosides extracted from grape pomace, since the chromatogram of the no hydrolyzed precursor extract, did not show any significant peak (data not shown). As can be seen in **table 2**, the most represented class of aroma compounds were terpenes, volatile phenols and lipids derivatives, followed by benzenoids and vanillins. Most of these compounds can be interesting on the basis of their aroma characteristics. For instance, the monoterpenes limonene, nerol, geraniol and two linalool related compounds such as 8-hydroxylinalool and linalool oxide were detected in the grape pomace in an average concentration of 10 µg of monoterpenes / Kg dry pomace, in which geraniol, was the monoterpene extracted at the highest amount. In wines, this compound presents a *floral* aroma. Moreover, geraniol and linalool are compounds associated to the pleasant Muscat like odor [29]. Many monoterpenoids have been associated to pleasant floral aroma attributes and it is important to notice that in general, they present very low odor thresholds (100-400 µg/L) [16]. Another poly-oxygenated terpene identified in the pomace extract was the

291 compound 8-hydroxylinalool. Although by their own, poly-oxygenated terpenes might
292 have small sensory relevance, they can be transformed into odorant monoterpenols by
293 hydrolysis at acid pH [30]. Linalool, one of the most common odorant aglycones released
294 from some floral grape varieties such as Muscat, Riesling and Gewürztraminer, was
295 absent in the hydrolyzed grape pomace extract, which might be due to its oxidation via
296 the formation of an epoxide into different types of linalool oxides. In fact, linalool oxide
297 was also identified in the LLE extract (**table 2** and peak nº 6 in **Figure 1**). The presence
298 of other types of hydroxylated linalool derivatives has been described in the bound
299 fraction of other white grape varieties such as Muscat and Melon B grape varieties [31].
300 The compound oxo-α-ionol was the only C13 norisoprenoid identified in the hydrolyzed
301 extract. However, it was one of the quantitatively most abundant compounds extracted
302 from the grape pomace (28.6 µg / Kg dry pomace). This compound has been associated
303 with a *spicy* aromatic note and as opposite to terpenes, it is normally found in the same
304 quantities in all the grape varieties, aromatics or neutral [17]. **Table 2,** also shows the four
305 volatile phenols and three related compounds (vanillins) that were identified in the
306 pomace extract. In wines, these compounds might contribute to wine flavor because of
307 their low odor thresholds. Their presence in the pomace extract is likely due to the
308 hydrolysis of the corresponding glycosidic precursors [30]. However, some vanillins could
309 have been formed from ethanolysis of lignin [29], which forms part of the stem and seeds
310 present in the pomace, and the use of ethanol employed as extracting solvent during the
311 extraction. Among the volatile phenols, the compound 2-methoxy-4-vinylphenol, was
312 present at the highest amount (176.76 µg/Kg dry pomace). This compound exhibits a
313 very low odor threshold (10 µg/L in water) [29], and it has been related to *clove-like*,
314 *balsamic*, *peppery-woody* aroma nuances [32]. Among the vanillins, acetovanillone, was
315 the quantitatively most important compound detected in the pomace extract (9.77 µg/Kg

316 pomace extract). The three vanillins identified in the extracts (vanillin, methyl vanillate,
317 acetovanillone) have been associated with pleasant vanilla aromatic notes in wines [33, 34].
318 In addition, three benzenoids compounds (benzyl alcohol, β-phenylethyl alcohol and
319 benzaldehyde) were also identified. Taking into account their interest for their aroma
320 characteristics, β-phenylethyl alcohol could be the most interesting one, which has been
321 related to *rose-like* odor. This compound was detected in the extract in a relatively large
322 amount compared to other aglycones (above 136 µg/Kg dry pomace) (**Table 2**). The
323 amount of this compound in the pomace extract was even higher than that reported by
324 Gómez and co-authors [19] in the skin of other non-aromatic grape varieties, such as
325 Monastrell, Cabernet Sauvignon and Tempranillo (43, 72 and 73 µg / Kg grape
326 respectively). Although the origin of β-phenylethyl alcohol in many fermented
327 beverages is from the catabolism of amino acids during the alcoholic fermentation, this
328 compound can occur in the fruit berry (e.g grape) in a rather high concentration as a non
329 volatile precursor bound to an uncharacterised glycoside residue [35]. **Table 2** also shows
330 some lipids derivatives identified in the pomace extract corresponding, in general, to
331 some C6 aliphatic alcohols and the lactone γ-nonalactone. It has been shown, that some
332 C6 aliphatic alcohols might be in the grape as odorless β-D-glucosides [31]. In fact, it has
333 been reported that while in aromatic grapes monoterpenols are important aglycones, in
334 the case of non aromatic grapes, instead of monoterpenols, the C6 aliphatic alcohols are
335 the most preponderant varietal alcohols [19]. Most of them are associated to *green-herbal*
336 aroma nuances [36, 37]. The only lactone identified in the extracts, was γ-nonalactone,
337 although its concentration was relatively low (1.6 µg / Kg dry pomace). Nonetheless, it
338 could be interesting because of its aroma characteristics, since it has been shown it
339 possess a lower odor threshold (30 µg/L) and a pleasant odor described such as *coconut-*
340 *like* [38].

Therefore, the hydrolyzed extract from Verdejo grape pomace showed different types of varietal aroma compounds mainly characterised by very low detection thresholds and many of them associated to pleasant aromatic notes. Taking into consideration their aroma characteristics, this aroma extract seems more interesting for different types of industrial applications, than the remaining free volatiles fraction present in the grape pomace previously considered for the valorisation of this type of wine by-products [15].

Optimization of a procedure based on Pressurized Liquid Extraction (PLE) for the recovery of aroma precursor glycosides from grape pomace

Once it was proven that grape pomace contained glycosides that after hydrolysis can release a wide spectrum of aroma compounds, the next step in the work was looking for an extraction method allowing the maximum glycoside extraction yield. To do so, pressurized liquid extraction (PLE) was chosen for this objective. This technique has been recently and successfully used for the recovery of other grape phytochemicals from red grape pomaces [10, 25]. For the optimization of the best extraction conditions, we focused on the effect of the extracting solvent (different hydroalcoholic solutions) and temperature, since they are outstanding variables in the PLE extraction procedure [26, 27]. The relative peak areas of the aromatic aglycones released after the hydrolysis of the extracts obtained by PLE were calculated in the different analysis conditions provided by the experimental matrix of the factorial design (**Table 1**). These ranges were chosen on the basis of previous works based on the extraction of other grape phytochemicals [10, 25]. All the experiments were randomly performed to minimize the effect of uncontrolled factors that might introduce bias in the measurements. MLR was applied to estimate the parameters of the proposed model in **Equation 1** for all the aglycones identified in the

366 extracts (response variables). The effect of each parameter in the model and their
367 statistical significance were analyzed from the Pareto chart. **Figure 2a** shows an
368 example, in which the effect of each term of the model divided by its standard error is
369 shown. The terms not significantly different from 0 ($p<0.10$) were excluded of the
370 model and the mathematical model was refitted. The regression coefficients, for
371 unscaled factors and the statistics of the fitting for each response variable (determination
372 coefficient, and residual standard deviation RSD) are also shown in **Table 3.** As can be
373 seen, most of the aroma compounds released from the pomace glycosides, showed an
374 adequate fit to the calculated model (18 compounds from 22 with $R^2>0.8$). Only four
375 compounds, 2-methoxy-4-vinyl-phenol, 8-hydroxylinalool, oxo-α-ionol and 4-
376 vinylphenol showed an inadequate fit to the proposed model. In the table, it can be seen
377 that the linear terms with the strongest influence on the recovery of odorant aglycones
378 after the hydrolysis were both the extracting solvent composition (*S*) and the
379 temperature (*T*) having in general, a negative and a positive influence respectively. Only
380 four compounds were negatively affected by the temperature: limonene, γ-nonalactone,
381 8-hydroxylinalool and oxo-α-ionol, although the two latter ones also showed inadequate
382 fits to the model. It seemed clear that solvent composition (% of ethanol/water) affected
383 the glycoside extraction from grape pomace as has been also shown for the extraction of
384 other grape phytochemicals [9, 39, 40]. Considering the temperature, the significant effect of
385 this factor during the PLE extraction, might be explained because it provokes an
386 increase in mass transfer favoring the solubility of the metabolites of interest [26, 27]. The
387 quadratic terms (S^2 and T^2) seemed to be less important for the model, although T^2
388 showed a significant and negative effect for many compounds, confirming the large
389 effect of temperature in the extraction. On the contrary, the interaction term ($S*T$) did

not seem very significant, and only five compounds (γ-nonalactone, 2,6-dimethoxyphenol, methyl vanillate and 4-vinylphenol) were affected.

When comparing the optimum values (maximum values of relative peak area) for the extraction of each aroma compounds, there were not an ideal solvent/temperature conditions valid for all of them likely due to the structural differences and complexity of the different types of glycosides present in the grape pomace [17, 35]. This has been already stated when optimizing the extraction conditions of other structurally complex grape phytochemicals such as anthocyanins [10, 25]. In addition, some of the extraction conditions essayed, specifically those not involving the use of ethanol (extraction with subcritical water), gave a lot of operational and technical issues during the extraction procedure (clogging valves and tubes of the ASE device), possibly because of the extraction of other polar compounds from the grape pomace (peptides, proteins, pectines, polyphenols) that made unviable the use of low ethanol hydroalcohlic mixtures. Therefore, the optimal extraction conditions were chosen taking into consideration those which provided the highest extractions (µg/Kg grape pomace) of the majority of aromatic aglycones, which were obtained during the assay number 2 and 10 (**Table 1**) using 50% of ethanol in the hydroalcoholic solution and 90 ºC as extraction temperature. **Figure 2b** shows an example of the surface plot for the optimal extraction conditions calculated for one of the aromatic aglycones. In this case, as can be shown, although the best extraction yield was obtained at lower ethanol concentration, as it was stated before, compromise conditions were used in order to obtain higher extraction yield, but avoiding technical and operational problems in the extractor device.

Therefore, the optimized PLE conditions (50 % ethanol and 90 ºC) were applied for the extraction of glycosidic aroma precursors from grape pomace. The compounds

identified and their concentrations after the enzymatic hydrolysis are also shown in **Table 2**. As can be seen in the table, these data were compared to those previously obtained by using LLE (ethanol 13% v/v at room temperature during 60 hours in the darkness). Compared to the most conventional extraction procedure (LLE), the extraction efficacy of the PLE was higher. The hydrolyzed extracts obtained by PLE had considerably higher amounts of the majority of varietal aglycones whatever the chemical family considered. Only the amounts of lipid derivatives were more or less similar indistinctly of the extraction method used. It is worth to notice that almost 50% more terpenes derivatives were found in the PLE extracts. However, nerol was not detected in this extract, which might be due to a minor conversion rate from its precursor, geraniol [18], because of the shorter extraction time applied during the PLE procedure compared to the LLE method. The compound 2,6-dimethoxyphenol was not identified in the PLE extract either, although its concentration was also very low by using LLE (**Table 2**). On the contrary, the three other volatile phenols, eugenol and mainly 2-methoxy-4-vinylphenol and 4-vinylphenol, were higher extracted by using PLE (3.7, 1872.9 and 590.8 µg/dry pomace respectively). In addition, very important differences between both extraction methods were observed in the extraction of vanillines, and for example, vanilline was above 90 percent more extracted using PLE than LLE (only about 10% extracted using LLE) (**Table 2**). Benzenoids compounds were only between 24 and 54% extracted using LLE compared to the PLE. These results showed that PLE was more effective in the extraction of glycosides from grape pomace than the more conventional LLE method. This higher effectiveness can be linked to the advantages associated of using an ethanolic mixture at high pressure and high-temperature compared to a conventional method also using a hydroalcoholic mixture but in static conditions during longer extraction times. However, it is important

to consider, than is spite of the higher extraction rate of glycosides (therefore, of the corresponding aromatic aglycones) associated to the PLE method, some drawbacks of this procedure have also been noticed during this work. First of all, the limited amount of sample that can fit in the extraction cell (using a conventional ASE device), which makes necessary many repeated extraction cycles, and secondly, some operational problems when using higher proportion of water in the hydroalcoholic solution, which could be of interest lowering the solvent cost and making possible the use of more environmental friendly solvents, because of the high extraction of other grape-polar compounds.

Conclusions

The results of this work show that grape pomace by-products can be a source of glycosidic aroma precursors that after hydrolysis can release interesting odorant compounds based on their aroma quality and low odor thresholds. The use of PLE working in the optimised conditions (50% ethanol/water, 90 ºC) greatly improves the extraction compared to the more conventional LLE. Considering the large amount of grape pomace produced every year in the world, the extraction of aroma glycosides can be an interesting alternative for the recovery and valorisation of grape by-products with potential applications in different industrial sectors (agro-food, cosmetic, perfumery, etc) and besides, reducing their environmental consequences.

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Compliance with Ethics Requirements

Carolina Muñoz-González declares that she has no conflict of interest. Juan J. Rodríguez-Bencomo declares that he has no conflict of interest; Pedro J. Martín-Álvarez declares that he has no conflict of interest; M. Victoria Moreno-Arribas declares that she has no conflict of interest; M. Ángeles Pozo-Bayón declares that she has no conflict of interest.

This article does not contain any studies with human or animal subjects.

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549

550 **Figure captions**

551 **Figure 1.** Chromatogram corresponding to the aroma compounds released after the
552 enzymatic hydrolysis of the aroma precursors glycosides extracted by LLE from grape
553 pomace. Peak identities are shown in Table 2. ISa, ISb, ISc, ISd and ISe correspond to
554 the internal standards 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanol, 2-octanol,
555 1-octanol and β-damascone.

556

557 **Figure 2**. **a**) Standardized Pareto Chart plot with the effect of each term the model
558 divided by its standard error for the response variable eugenol (μg/Kg dry pomace). The
559 vertical line tests the significance of the effects at the 90% confidence level. Legend for
560 the bars corresponds to the terms in the model of Equation 1. **b**) Surface plot of the
561 estimated response variable (eugenol, μg/Kg dry pomace) as a function of the extraction
562 temperature, T(ºC) and solvent, S (% of ethanol in the hydroalcoholic mixture).

563