Development of a new strategy for studying the aroma potential of winemaking grapes through the accelerated hydrolysis of phenolic and aromatic fractions (PAFs)

Yohanna Alegre, Ignacio Arias-Pérez, Purificación Hernández-Orte and Vicente Ferreira*

Laboratorio de Análisis del Aroma y Enología (LAAE), Department of Analytical Chemistry, Universidad de Zaragoza, Instituto Agroalimentario de Aragón (IA2) (UNIZAR-CITA), c/ Pedro Cerbuna 12, 50009 Zaragoza, Spain

**corresponding author:* Vicente Ferreira (*vferre@unizar.es*); phone: 34 976 762067; Fax: 34 976 761292

Abstract

Current methods for assessing grape aroma potential are based on the fast hydrolysis of precursor fractions but provide hydrolyzates of poor aromatic quality. A new strategy based on the accelerated hydrolysis of reconstituted phenolic and aromatic fractions (PAFs) extracted from grapes is herein developed. PAFs are obtained by solid phase extraction on 10g-C18 sorbents of partially dealcoholized "mistelle", obtained from grapes treated with ethanol. Under optimal conditions, PAFs contain all aroma precursors but the most polar ones, such as those of DMS, more than 85% of the total phenolics and just traces of metal cations and of amino acids. PAFs reconstituted in model wine, aged in strict anoxia 7 weeks at 45ºC or 24h at 75ºC, develop strong aromas. At least 30 different odorants including lipid derivatives, volatile phenols, vanillins, norisoprenoids, terpenes, bencenoids and 3-mercaptohexanol were identified by GC-Olfactometry and GC-MS. Methodological aspects of the extraction, hydrolysis and analysis are optimized and discussed.

Keywords: glycosidic precursors, polyphenols, grape quality, grape aroma, wine, aging, hydrolysis

1. Introduction

In neutral grapes, most aroma compounds are present as non-volatile precursors, such as glycosidic precursors, polyolic forms, cysteinylated and glutathionylated precursors or dimethyl sulfide (DMS) precursors. From a quantitative point of view, the glycosidic fraction is the most important and was the first discovered (Gunata, Bitteur, Brillouet, Bayonove, & Cordonnier, 1988; Williams, Strauss, Wilson, & Massy-Westropp, 1982a). Some relevant wine aroma compounds belonging to the groups of terpenols, such as linalool and geraniol, nor-isoprenoids, such as β-damascenone, β-ionone and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), benzenoids, such as ethyl cinnamate, volatile phenols, such as guaiacol, eugenol, 4-vinylphenol and 4-vinylguaiacol or vanillins, such as vanillin and acetovanillone, derive from different glycosidic precursors already present in the grape (Hjelmeland & Ebeler, 2015). These precursors are formed by a glycone (sugar moiety) linked to an aglycone. The complexity of the fraction arises because the aglycone in some cases is the aroma compound, but in many other instances, notably the case of nor-isoprenoids (Waterhouse, Sacks, & Jeffery, 2016) is a related molecule which only after different spontaneous but slow chemical rearrangements will form the aroma compound. Overall, more than 100 different aglycones broadly classified in norisoprenoids, shikimates (bencenoids, volatile phenols and vanillins) and terpenoids have been identified (Schneider, Razungles, Augier, & Baumes, 2001; Williams, Sefton, & Wilson, 1989; Williams, Strauss, Wilson, & Massy-Westropp, 1982b; Winterhalter, 1992). Furthermore, aglycones are linked to β-D-glucose forming monosaccharides, trisaccharides (Hjelmeland, Zweigenbaum, & Ebeler, 2015), or most frequently, disaccharides of four major types (6-O-β-d-glucosyl-β-d-glucopyranosides, 6-O-β-d-apiofuranosyl-β-d-glucopyranosides, 6-O-α-l-rhamnopyranosyl-β-d-glucopyranosides, and 6-O-α-l-arabinofuranosyl-β-d-glucopyranosides), as recently reviewed (Hjelmeland & Ebeler, 2015; Liu, Zhu, Ullah, & Tao, 2017). This makes that the final number of precursor molecules is too high for being directly monitored. Only in the case of terpenols there are some recent studies trying to assess the existence of quantitative and qualitative differences between grape cultivars (Godshaw, Hjelmeland, Zweigenbaum, & Ebeler, 2019).

The release of the aglycone from the glycoside is produced during the winemaking process by the action of wine yeasts (Delfini et al., 2001; Fernández-González & Di Stefano, 2004; Hernández-Orte et al., 2008), by exogenous or endogenous glycosidases

(Gunata, Bayonove, Tapiero, & Cordonnier, 1990; Sánchez-Palomo, Díaz-Maroto Hidalgo, González-Viñas, & Pérez-Coello, 2005), or by slow acid hydrolysis (López, Ezpeleta, Sánchez, Cacho, & Ferreira, 2004; Skouroumounis & Sefton, 2000).

Although there is previous scientific evidence supporting the existence of a link between wine aromatic quality and content in aroma precursors in grape (Abbott, Coombe, & Williams, 1991; Francis, Sefton, & Williams, 1992), there remain many gaps in our knowledge. This is particularly true in the case of red wines, where some aroma nuances are formed slowly along the aging process from grape aroma precursors. For instance, recent reports have revealed the contribution to mint and fresh notes in aged Bordeaux wines of piperitone and of different lactones derived from menthofuran (Picard, de Revel, & Marchand, 2017; Picard, Franc, de Revel, & Marchand, 2018; Picard et al., 2016) and also the increase with time of tobacco aroma-related compounds derived from nor-isopresnoids in Valpolicella wines (Slaghenaufi & Ugliano, 2018). Therefore, new strategies able to assessthe aromas derived from grape aroma precursor fractions should be sought in order to study the effects of different of different agronomical or environmental conditions on the grape aroma potential, to assist in the chemical characterization of the precursors and, ultimately, to improve our understanding about the relationship between grape aroma composition and wine aroma properties.

The glycosides of aromatic aglycones are usually isolated on a chromatographic support of C18 type (García-Muñoz, Asproudi, Cabello, & Borsa, 2011) or by a polymeric adsorbent of styrene-divinylbenzene (Gunata, Bayonove, Baumes, & Cordonnier, 1985; Ibarz, Ferreira, Hernández-Orte, Loscos, & Cacho, 2006). Then, they are released by enzymatic or acid hydrolysis (Delfini et al., 2001; Loscos, Hernández-Orte, Cacho, & Ferreira, 2009) that can be carried out at different pHs and temperatures. While enzymatic hydrolysis is far more efficient in terms of breaking the glycosidic bond than acid hydrolysis (Liu et al., 2017), many relevant aroma molecules that are further formed by chemical rearrangement, or esterification such as β-damascenone, TDN or ethyl cinnamates (Waterhouse et al., 2016) are not even formed via enzymatic hydrolysis. This explains why the sensory properties of acid hydrolyzates obtained at mild temperatures (40-50 ºC) are more intense than those of enzymatic hydrolyzates, and in fact, that only acid hydrolyzates seem to have sensory relevance in wine aroma

(Francis et al., 1992; Sefton, Francis, & Williams, 1993). However, in order to speed the process, acid hydrolysis is usually carried out without particular antioxidant precautions and at high temperatures (Loscos et al., 2009), which implies an intense degradation of labile molecules, such as geraniol or linalool.

Other analytical strategies make use of the indirect evaluation of the sugar released after hydrolysis, for which a commercial trial has been even proposed (Salinas, de la Hoz, Zalacain, Lara, & Garde-Cerdán, 2012) which may be suitable for making comparisons between grapes from the same type. However, this strategy requires pre-calibration, provides limited information and its real usefulness still requires proper validation. Furthermore, strategies for the direct quantification of the aglycones based on direct HPLC-MS have been recently proposed (Flamini et al., 2014; Godshaw et al., 2019; Hjelmeland et al., 2015; Schievano et al., 2013) but due to the complexity of the number of aglycones and the difficulty in relating them to the aromas revealed, their use has not been extended.

The case of cysteinylated and glutathionylated precursors is completely different, since there is a limited number of well defined precursors (Darriet, Tominaga, Lavigne, Boidron, & Dubourdieu, 1993; Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Peyrot des Gachons, Tominaga, & Dubourdieu, 2002) which can be easily determined by direct HPLC-MS. Finally, DMS precursors are rarely determined, in spite of the relevant role that this molecule can play in the aroma of red wines (Picard et al., 2015; Segurel, Razungles, Riou, Salles, & Baumes, 2004).

Because of all the complex transformations suffered by grape aroma precursors, it can be argued that the best possible assessment of grape aroma potential will be obtained by hydrolyzing precursors under conditions as close as possible to those observed in real wine aging. For that, it is expected that best results will be obtained if the hydrolysis is carried out in a matrix as similar as possible to real wine regarding alcoholic content, presence of polyphenols, pH and acidity. It can be also anticipated that sugar and amino acids will have to be removed and that the process will have to take place under strict anoxic conditions. All these hypotheses are checked in the present paper, whose main goal is to develop a new strategy able to obtain an assessment of the aroma potential of winemaking grapes.

2. Materials and methods

2.1. Chemicals

HPLC quality Dichloromethane and LiChrosolv quality Methanol were purchased from Merck (Darmstadt, Germany), ACS quality absolute ethanol was purchased from Panreac (Barcelona, Spain) and pure water was obtained from a Milli-Q purification system (Millipore, USA).

LiChrolut EN resin cartridges were obtained from Merck (Darmstadt, Germany), while Sep Pak-C18 resins, prepacked in 10g cartridges were from Waters (Ireland), and Solid Purple-C18 resins, prepacked in 7g cartridges, were obtained from Análisis Vínicos (Tomelloso, Spain). A semiautomated solid phase extraction was carried out with a VAC ELUT 20 station supplied by Varian (Walnut, Creek, USA). Sodium chloride, L-tartaric acid, ammonium sulfate, and $NaHCO_3$ were supplied by Panreac (Barcelona, Spain).

2.2. Preparation of ethanolic musts (mistelles)

Ten kilograms of Tempranillo grapes from Dominio Pingus (D.O., Ribera del Duero) and 10 kg of Grenache grapes from Bodega Ramon Bilbao (D.O.Ca Rioja) were first destemmed and crushed in the presence of 15% (p/p) of ethanol and 5g/hL of potassium metabisulfite (Merck, Germany). After 7 days macerating at 13ºC, the mistelles was pressed, filtered (obtaining a total volume of 7 L) and stored at 5ºC in the dark.

2.3. Optimization of PAFs extraction

2.3.1. Optimization of cartridges and breakthrough volume

Mistelle was centrifuged at 4500 rpm, 10ºC for 20 min (Allegra X-22R Beckman Coulter). Then, three different mistelle preparations were used to obtain a higher volume of loaded sample: i) mistelle; ii) mistelle diluted to 50% with milli-Q water at pH 3.5 and iii) mistelle dealcoholized. For the dealcoholization, 750 mL of the mistelle were put into a rotatory evaporator system (Buchi R-215 equipped with a V-700 vacuum pump from Buchi, Flawil, Switzerland) hold at 23ºC and at pressure of 20 mbar for 3 hours, achieving a final volume around 410 mL containing just 2-3% (v/v) of ethanol as determined by distillation and measurement of density. For extraction, two types of high capacity cartridges were used, Sep Pak-C18 10g (from Waters, Ireland) and Solid Purple-C18 7g (from Análisis Vínicos, Tomelloso, Spain).

156 The 7 and 10g-C18 cartridges were first conditioned by passing through them 35 and
157 44mL of methanol (corresponding to 4 dead volumes of the cartridges of 7 and 10g)
158 followed by 35 and 44mL of milli-Q water with 2% ethanol, respectively.

159 Thereafter, the mistelle, diluted mistelle and dealcoholized mistelle were further passed
160 through the 7 and 10g-C18 cartridges. After letting the dead volume pass (7mL and
161 11mL in the case of the 7 and 10g cartridges, respectively), fractions were collected
162 every 5mL and were analyzed by means of the total polyphenol index (TPI).

163 *Spectrophotometric measurements.* TPI was determined as optical density at 280 nm
164 (OD 280) following the method described by Ribéreau-Gayon et al. (Ribéreau-Gayon,
165 Glories, Maujean, & Dubourdieu, 2006). The absorbance measurements were done
166 using a UV–vis spectrophotometer UV-1700 Pharma Spec from Shimadzu (Kyoto,
167 Japan).

168 *Determination of breakthrough volume (V_B).* The comparison of TPI in each of the
169 fractions obtained from the three types of mistelle with the TPI in each of the initial
170 mistelles (mistelle, diluted mistelle and dealcoholized mistelle) was used to determine
171 the breakthrough volume. A loss of TPI of less than 15% was considered to represent a
172 good V_B.

173 **2.3.2. Optimization of elution volume**

174 After a washing step with 88mL (corresponding to 8 dead volumes of the 10g cartridge)
175 of milli-Q water at pH 3.5, pure ethanol was passed through the cartridge. Fractions of
176 50mL of pure ethanol were taken and the presence of glycosidic precursors were
177 analyzed in each fraction. In addition, the anthocyanins and total tannins in these
178 fractions were also studied.

179 *Glycosidic precursors analysis.* The presence of glycosidic precursors in each fraction
180 was investigated with an indirect method based on the harsh acid hydrolysis of each
181 fraction, following the procedure described by Ibarz et al. (Ibarz et al., 2006), followed
182 by a sensory analysis of the released compounds.

183 *Total anthocyanins content.* The determination of anthocyanins content was carried out
184 following the method described by Ribéreau-Gayon et al. (Ribéreau-Gayon et al.,
185 2006).

Total tannins content. The determination of tannins content was carried out following the method described by Ribéreau-Gayon et (Ribéreau-Gayon et al., 2006).

2.4. Extraction of phenolic and aromatic fractions (PAFs)

750mL of mistelle were dealcoholized in a rotatory evaporator system. The resulting dealcoholized mistelle was passed through a 10g-C18 prepared cartridge previously conditioned with 44mL of methanol followed by 44mL of milli-Q water with 2% of ethanol. The cartridges were then washed with 88mL of milli-Q water pH 3.5 and dried by letting air pass through them. The polyphenolic and precursor fractions were recovered by elution with 100mL of ethanol.

2.5. Hydrolysis conditions

Samples. The preparation of the samples was based on the reconstitution of the PAF from Tempranillo and Grenache mistelles in synthetic wine. The PAF was added to a synthetic wine with 5g/L of tartaric acid at pH 3.5 and 13.3% of PAF (corresponding to 13.3% of ethanol) (rPAF). In addition, to study the effect of sugar, PAF was added to a synthetic wine that also contained sugar (100g/L of glucose and 100g/L of fructose) (rsPAFs). Besides, to study the aroma compounds lost during the extraction, mistelle was used and was adjusted at pH 3.5. These three kinds of samples were prepared in duplicate

Assay. The samples were placed in the anoxic chamber and divided into two 20mL-vials. The vials were hermetically closed and bagged with two certified oxygen permeability thermos-sealed plastic bags containing an activated charcoal with an oxygen-scavenger (AnaeroGen™ from Thermo Scientific Waltham, Massachusetts, United States) (Vela, Hernández-Orte, Franco-Luesma, & Ferreira, 2017). The bagged samples were then incubated under different conditions.

Accelerated hydrolysis at 45ºC. The samples were put in a stove at 45°C for 2, 4 and 7 weeks in the case of rPAFs and mistelles, and only for 7 weeks in the case of rsPAFs. In addition, two 20mL-vials of each kind of sample (rPAF, rsPAF and mistelle) were used as controls to test the effect of oxygen. The controls were closed hermetically but not bagged, then were incubated at 45ºC for 7 weeks.

215 *Accelerated hydrolysis at 75ºC*. The rPAF from the Tempranillo variety was incubated
216 at 75ºC. Two vials were taken out at different times until 72h (3, 8, 14, 24, 38, 48, 60
217 and 72h).

218 Thereafter, in all cases, the compounds released from glycosidic precursors were
219 analyzed by sensory analysis and gas chromatography-mass spectrometry (GC-MS).

220 **2.6. Sensory analysis of aroma released from accelerated hydrolysis**

221 Four different sensory tasks were carried out. The first one was carried out to determine
222 the elution volume. The second and third sensory tasks consisted of a descriptive task
223 for both samples obtained from the accelerated hydrolysis at 45ºC and at 75ºC. The
224 fourth sensory task consisted of a triangle test for rPAFs obtained at 75ºC.

225 Fifteen wine experts (45.5% men and 54.5% women from 26 to 63 years) from the
226 Laboratory for Analysis of Aroma and Enology (LAAE) took part in the study. They
227 were semi-trained assessors with experience in the sensory description of wine,
228 considered wine experts according to the specifications of Parr et al. (Parr, Heatherbell,
229 & White, 2002).

230 In all cases, one hour before the sensory tasks, samples were removed from the 5°C cold
231 room and 10mL were served at room temperature in dark approved wine glasses (ISO
232 NORM 3591, 1977) labeled with 3-digit random codes and covered by plastic Petri
233 dishes. Besides, for each panelist, samples were presented simultaneously in a different
234 random order.

235 *Elution volume determination*. This sensory analysis was carried out to determine the
236 presence/absence of the aroma compounds released from glycosidic precursors in each
237 of the collected fractions. The panelists were asked to smell each hydrolyzed fraction
238 and indicate "yes" if in the smelled fraction there was any aroma or "no" if there was no
239 aroma. They were then asked to indicate one to three free attributes to describe each
240 fraction. The descriptors cited by at least 20% of the panel were used.

241 *Descriptive tasks*. In both sessions (one for rPAFs hydrolyzed at 45ºC and the other for
242 rPAFs hydrolyzed at 75ºC), the panelists were asked to smell each sample and describe
243 them with 1 to 5 attributes. In addition, they were also asked to indicate the intensity of

each of the samples as “low, medium or high intensity”. Attributes mentioned by at least 20% of the panel were used.

Triangle test. In addition, rPAFs incubated at 75ºC during 14, 24, 38 and 48h were submitted to different triangle tests to identify the presence/absence of significant differences between the pairs: i) arPAF incubated for 14h and 24h; ii) arPAF incubated for 24h and 38h; and iii) arPAF incubated for 24h and 48h. Panelists performed tests in duplicate. In each triangle test, three glasses were presented to each panelist and, based on the orthonasal aroma, they were asked to select the different sample. To identify the presence of significant differences between the samples incubated during different times, the number of correct answers was compared with the tabulated values.

2.7. Aroma compounds quantification

2.7.1. Aroma released from glycosidic precursors

This analysis was carried out using the method proposed and validated by López et al., (Lopez, Aznar, Cacho, & Ferreira, 2002) with the following modifications: 65 mg of LiChrolut EN resins were placed in standard SPE cartridges, the cartridges were conditioned with 2mL of dichloromethane, 2mL of methanol and 2mL of water containing 12% of ethanol. Then, 15mL of sample with 100µL of ethanol solution of 3 internal standards (2-octanol, 3-octanone and 3,4-dimethylphenol) were passed through the cartridges (2mL/min), followed by a washing step using 1.5mL of a 30% water-methanol, 1% $NaHCO_3$ solution. The resins were then dried by letting air pass through them and finally eluted with 0.6mL of dichloromethane with 5% methanol. 2µL of the extract was injected in a QP2010 gas chromatograph equipped with a quadrupole mass spectrometer detector from Shimadzu (Japan) following the method described by Oliveira et al., (Oliveira & Ferreira, 2019).

2.7.2. Volatile sulfur compounds (VSCs)

To determine if the DMS precursor was extracted in the PAFs, an accelerated hydrolysis of rPAF and mistelle was carried out. The determination of DMS was conducted using the method described by Franco-Luesma et al., and Lopez et al., (Franco-Luesma & Ferreira, 2014; López, Lapeña, Cacho, & Ferreira, 2007).

2.7.3. Data analysis

One-way analysis of variance (ANOVA) followed by Duncan's post-hoc test were applied to establish the significant differences among the hydrolyzed samples. The analyses were carried out using SPSS (SPSS Inc., Chicago, IL) for Windows, version 19. Different letters express significant differences with a significance level of 95%.

Furthermore, principal component analysis (PCA) using XLSTAT software (version 2014.2.02) was carried out to illustrate the quantitative data obtained in the different accelerated hydrolysis.

2.8. Gas Chromatography-olfactometry (GC-O)

2.8.1. GC-O analysis

One microliter of the extracts of rPAF and mistelle from Tempranillo incubated in anoxic conditions during 7 weeks and of rPAF incubated at 75ºC for 24h was injected for GC-O analyses with a Trace GC gas chromatograph (ThermoQuest, Milan, Italy) equipped with a sniffing port ODO-I and a flame ionization detector (FID) supplied by SGE (Ringwood, Australia), as described by Escudero et al. (Escudero, San Juan, Franco-Luesma, Cacho, & Ferreira, 2014). The temperature program used was 40ºC for 5min, increased by 4ºC/min to 100ºC and then 6ºC/min to 220ºC, holding for 10min.

Sniffing was carried out by 4 trained judges (75% women and 25% men from 25 to 30 years) from the laboratory staff. The sniffers indicated the time, odor intensity and description when they detected an aroma. The measurement of the perceived odor intensity was based on a 7-point structured category scale: 0 = not detected; 1 = weak odor, 2 = clear odor; 3 = extremely strong odor with intermediate values.

The odorants identification was carried out by comparing their descriptors and chromatographic retention index in DB-Wax and DB5 columns with those of pure reference compounds.

2.8.2. Data analysis

The GC-O data were treated calculating the modified frequency percentage (%MF) from the formula given by Dravnieks (Dravnieks, 1985):

$$\%MF = \sqrt{\%F \; x \; \%I}$$

where F (%) is the aromatic attribute detection frequency expressed as a percentage and I (%) is the average intensity expressed as a percentage of the maximum intensity.

2.9. Amino acids quantification

Amino acids present in rPAF and mistelle were determined by HPLC with fluorescence detector according to the method reported by Hernández-Orte et al., (Hernandez-Orte, Ibarz, Cacho, & Ferreira, 2003).

2.10. Metal cations quantification

The most abundant and enologically relevant transition metals of musts and wines (Fe,Cu, Mn and Zn) were determined in the rPAF measuring the most abundant isotopes (^{56}Fe, ^{63}Cu, ^{55}Mn and ^{66}Zn) by inductively coupled plasma mass spectrometry using a procedure published by Grindlay et al., (Grindlay, Mora, de Loos-Vollebregt, & Vanhaecke, 2014).

3. Results and discussion

3.1. Solid Phase Extraction

Mistelles are *a priori* suitable matrixes for studying the aroma potential of grapes, since contain all the grape metabolites potentially extracted by physical processes during wine making, are relatively stable from the microbiological point of view, and do not contain all the volatiles produced by yeast. However, they have large amounts of glucose and fructose and significant levels of amino acids and of different metal cations. Since these chemical species form highly reactive systems in which powerful aroma molecules can be formed, such as Strecker aldehydes or Maillard-derived aroma compounds, they likely have to be removed before aroma development. Therefore, a first goal was to separate grape polyphenols and aroma precursors from sugar and amino acids. This was achieved by solid phase extraction on large capacity C18 sorbent beds.

The extraction abilities of different SPE beds or the effect of the different operations carried out on the mistelle on such extraction abilities were studied by plotting the corresponding breakthrough curves. As the most abundant group of grape secondary metabolites are phenols displaying some absorbance at 280nm, this parameter was selected to monitor the effluent. Given that some phenols are chemically more polar than most aroma precursors, it is expected that absorbance at 280nm gives quite a conservative assessment about the ability of the SPE bed to extract aroma precursors. Breakthrough curves were built by estimating at each loaded volume the fraction of absorbance not retained in the cartridge (Poole, 2003; Poole, Gunatilleka, & Sethuraman, 2000). Plots of this kind for three different mistelle preparations (diluted mistelle, dealcoholized mistelle and mistelle), and two C18 cartridges (Solid purple 7g and Sep Pak 10g) are summarized in Figure 1.

As can be seen in Figures 1a and 1b, in the case of untreated mistelles, the 15% breakthrough volume is as little as 7 or 10mL indicating, as expected, that the high presence of ethanol has a pernicious effect on the extraction ability of the bed. In fact, dilution had a very positive effect, as can be seen in Figures 1c and 1d, and breakthrough volumes for 1:1 dilutions were 62 and 91mL, equivalent to 31 and 45.5mL of the undiluted mistelle, more than 4x larger than the initial ones. The best results were obtained by previous dealcoholization of mistelle, as can be seen in Figures 1e and 1f. Breakthrough volumes of 132mL and 411mL, equivalent to 240 and 750mL

of the original mistelle, were obtained for the 7g and the 10g cartridges, respectively. These last conditions (dealcoholization of 750mL of mistelle and retention in a 10g Sep Pak-C18 cartridge) were retained as optimal, in spite of the fact that most polar precursors, such as those of DMS, could be lost, as will be latter discussed.

In order to optimize elution volume, a cartridge containing the grape extract, was eluted with five consecutive 50mL-volume fractions of ethanol. Each fraction was analyzed for total anthocyanins, total tannins and aroma precursors. These last were indirectly measured after aroma generation by harsh acid hydrolysis (pH 2.2, 100ºC, 1h) using a sensory panel. The results are shown in Table 1.

Regarding aroma precursors, only the first two fractions produced relevant levels of aromas, the first being more intense, fruity, syrupy and jammy and the second more terpenic (green, herbal). Only one of the judges was able to detect some unspecific aroma in the third fraction. Most anthocyanins (92.4%) were eluted in the first fraction, which also contained 79.1% of total tannins. The second fraction contained 5.64% and 12.5% of anthocyanins and total tannins, respectively. In light of these results, an elution volume of 100mL of ethanol was chosen.

The characterization of amino acids and transition metal cations present in this polyphenolic and aromatic fraction (PAF) reveals that these species are nearly completely lost during the sample treatment. No amino acids were found above the method detection limits in the PAF, and only very little amounts of Fe and Cu, less than 5% and 2% of the initial content of the mistelle, respectively, were found (Tables 1S and 2S). Two other relevant transition metal cations, Zn, and Mn were also completely lost. The very low levels of metals can in fact be positive, since these compounds, particularly Cu and Fe, are determinant for O_2 consumption (Bueno, Carrascón, & Ferreira, 2016; Carrascón, Bueno, Fernandez-Zurbano, & Ferreira, 2017), and seem to be also active catalysts for some reactions in which aroma compounds are formed or degraded (Bueno et al., 2018). The absence of amino acids in the PAF is also positive, since these compounds can form powerful aroma compounds, such as phenylacetaldehyde or methional (Bueno et al., 2018), but this implies that precursors for DMS will be most surely not present in the PAF.

3.2. Aroma development

3.2.1. Accelerated hydrolysis at 45ºC

Assuming that acid hydrolysis provides the best possible assessment of the grape aroma potential, different hydrolysis conditions were studied. In all cases, the PAFs were rediluted with water containing tartaric acid to 13% ethanol and pH 3.5 to form the reconstituted rPAFs. These rPAFs were first hydrolyzed at 45ºC under strict anoxic conditions at three different times (2, 4 and 7 weeks). In order to assess the relevance of the presence of sugar and also to assess the potential losses of some precursors, the original mistelle and a rPAF enriched in sugars (named rsPAF) were also processed. In order to assess the effect of strict anoxia on aroma development, one of the series was aged 7 weeks in the presence of a little chamber of air and without any special insulation. Aroma compounds released from glycosidic precursors were analyzed by sensory analysis and by GC-MS.

The results of the sensory analysis of the hydrolyzed samples (rPAF, rsPAF and mistelle) reveal that aroma development takes a long time, since intensity and aromatic complexity increased with time and, in fact, it was only after 7 weeks of anoxic aging that the samples developed complex and intense aromas, as summarized in the last lines of Tables 2 and 3. The most interesting aromas were developed in rPAFs after 7 weeks of anoxic aging at 45ºC. These samples were described by the panelists as containing fresh fruit, fruit in syrup, sweet, spicy and phenolic notes in the case of the rPAF from Tempranillo, and fruit in syrup, floral and tea notes in the case of rPAF from Grenache. Some of these notes, such as syrupy, sweet, spicy or tea, are typical from hydrolysates obtained from glycosidic precursors (Alegre et al., 2017; Fischer, 2007; Loscos, Hernández-Orte, Cacho, & Ferreira, 2007), but they were present at much higher intensity and were richer in fruity aromas.

The presence of sugar in the hydrolytic media had a surprising sensory effect since after 7 weeks of aging, strong kerosene notes were detected in the rsPAF samples. On the other hand, untreated mistelles developed some distinctive notes, such as tomato and truffle notes, which were attributed to the presence of DMS, further confirming that the precursors for this molecule are lost during the preparation of PAFs. However, untreated mistelles also developed strong and very sweet caramel-like and raisin-like aromas likely related to Strecker degradation and Maillard reaction that masked other varietal aromas. This suggests that untreated mistelles may be not adequate for

assessing varietal aroma, which, except for DMS, seems to best expressed in reconstituted PAFs.

The presence of oxygen in all cases was extremely detrimental to aroma development, since samples not stored under strict anoxic conditions developed typical oxygen-related wine off-odors (Chisholm, Guiher, & Zaczkiewicz, 1995; Escudero, Asensio, Cacho, & Ferreira, 2002; Lopes et al., 2009), suffered browning (Cheynier, Basire, & Rigaud, 1989; Fernandez-Zurbano et al., 1995; Ma & Waterhouse, 2018) and did not retain any of the typical aromas noted under anoxic conditions.

In order to get a better insight into the chemicals potentially responsible for these differences, samples were submitted to quantitative GC-MS of selected odorants (tables 2 and 3), and two of them (together with rPAF at 75ºC), were also subjected to semiquantitative GC-O (table 4) in order to screen for the potential presence of relevant odorants different to those targeted.

Samples sent to GC-O were the rPAF and mistelle incubated at 45ºC for 7 weeks from Tempranillo, and rPAF aged at 75ºC for 24h, which displayed the strongest sensory notes. Results revealed the presence of up to 32 different odorants at levels potentially relevant from the sensory point of view. Odorants present in the samples can be classified into several categories:

1. Lipid derivatives, that with 10 different odorants is the most numerous group and includes Z-3-hexenal, 1-octen-3-one, (Z)-1,5-octadien-3-one, Z-3-hexenol, E-2-octenal, Z-2-nonenal, E-2-nonenal, (E,Z)-2,6-nonadienal, (E,E)-2,4-nonadienal, γ-decalactone and massoia lactone.
2. Volatile phenols and vanillins, including guaiacol, cresols, eugenol, 2,6-dimethoxyphenol, E-isoeugenol and vanillin.
3. Nor-isoprenoids and terpenes, including linalool oxide (and/or dihydromyrcenol), linalool, TDN, β-damascenone and β-ionone.
4. Amino acid derivatives, including methional and sotolon.
5. Benzenoids and miscellaneous compounds, including β-phenylethanol, ethyl cinnamate, furaneol, 3-mercaptohexanol and three unidentified compounds.

As can be seen in Table 4, there is a close proximity between the olfactometric profiles of the samples, since most of the odorants were present at not very different