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Grape mistelles are much better than grape C18-extracts to study grape aromatic potential

Belén González-Martínez, Arancha de-la-Fuente-Blanco, Cristina Peña, Vicente Ferreira *

Laboratorio de Análisis del Aroma y Enología (LAAE), Department of Analytical Chemistry, Universidad de Zaragoza, Instituto Agroalimentario de Aragón (IA2) (UNIZAR-CITA), Associate Unit to Instituto de las Ciencias de la Vid y el Vino (ICVV) (UR-CSIC-GR), c/ Pedro Cerbuna 12, 50009, Zaragoza, Spain

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ABSTRACT

The aromatic compositions of hydrolysates containing grape aroma precursors obtained by two different procedures were compared. The first procedure used simply diluted mistelle, while the second employed a reconstituted C18 extract of the original mistelle containing both aromatic precursors and polyphenols. Hydrolysis was carried out under strict anoxic conditions at 75 °C and pH 3.5 for varying times (0–190 h). Varietal aroma compounds were analysed by GC-SCD (volatile sulphur compounds, VSCs), GC-MS (minor volatile compounds) and HPLC-MS (polyfunctional mercaptans, PFM's). Odorants were additionally controlled by GC-O. Aromas and aroma precursors lost during sample extraction were also investigated. Results revealed that polar precursors of relevant varietal aroma compounds, including DMS, limonene, dihydromyrcenol, geraniol, linalool, β-ionone, γ-nonalactone and vinylphenols are lost during C18 extraction, which demonstrates that traditional aroma precursor research has missed a significant fraction of precursors. On the other hand, hydrolysed mistelles accumulated high levels of H_2S and DMS, low levels of MH due to the presence of sugars, and developed detectable levels of amino acid related odorants, such as phenylacetaldehyde and 2-acetylpyrazine. While this explains why mistelles are not the best choice for sensory evaluation, also demonstrates that they are much better for the chemical assessment of grape aromatic potential.

1. Introduction

Many fruits and vegetal products accumulate specific flavour precursors (FPs), which are non-volatile odourless molecules, that by different spontaneous and/or enzymatically catalysed chemical processes are transformed into aroma molecules (De Rosso et al., 2022; Liang et al., 2022; Sarry & Günata, 2004). FPs can influence the flavour characteristics of these products in different ways. On one hand, a little fraction of FPs may release aroma in fast hydrolytical processes catalysed by saliva enzymes during degustation, therefore contributing to the actual flavour of the product (Ferreira & López, 2019). On the other hand, FPs can release aroma molecules during the food processing (Diez-Simon et al., 2019). In the particular case of wine, FPs are responsible for key grape-derived aromas, some of which accumulate only after some aging time and play an essential role in defining the wine's sensory properties and varietal characteristics (Ferreira & López, 2019; Parker et al., 2017).

Most FPs in wine are under the form of glycosides (Liu et al., 2017). However, other precursor structures have been also described as non-glycosidic polyols for some terpenols (Cebrián-Tarancón et al., 2021), and cysteinyl-derivatives for polyfunctional mercaptans (Cordente et al., 2015; Subileau et al., 2008). Furthermore, the complexity of the FP fraction is very high, because in many cases, different combination of monosaccharides composes the glycosidic part, and in some cases, there are also different aglycones able to form the same aroma molecule (De Rosso et al., 2022). Such complexity explains why the FP fraction is very often studied by analysing the aroma molecules formed after the hydrolysis, rather than targeting by HPLC-MS the different precursor molecules.

To date, almost all studies on FPs have been carried out on extracts obtained from the grape musts by solid-phase extraction (SPE) using non-polar sorbents, such as C18 or divinylbenzene polymers (Ibarz et al., 2006; Williams et al., 1982). This strategy makes it possible to concentrate FPs and, depending on the solvents used, to obtain them more or less separated from polyphenols, which can interfere with glycosidases or in HPLC-MS analysis. Nevertheless, recent reviews point out that the

* Corresponding author.
E-mail addresses: b.gonzalez@unizar.es (B. González-Martínez), arandlfb@unizar.es (A. de-la-Fuente-Blanco), cpena@unizar.es (C. Peña), vferre@unizar.es (V. Ferreira).

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odour properties of the different hydrolysates from neutral grapes obtained in earlier works were relatively weak and not clearly related to wine aroma (Campo et al., 2008; Francis et al., 1992; Loscos et al., 2009).

A recent procedure (Alegre, Arias-Pérez, et al., 2020) uses mistelles as starting material and carries out the extraction of FPs on C18 sorbents in conditions in which FPs are co-extracted with polyphenols. This is achieved by using de-alcoholised mistelle, low loading conditions and elution with ethanol to obtain the polyphenolic and aromatic fractions (PAFs). When PAFs are diluted and acidified to mimic wine, and are further incubated in anoxic conditions, develop strong grape-variety dependent aroma characteristics reminding of fruits and flowers, suggesting that the procedure can effectively assess grape varietal aroma. However, although this method avoids the presence of sugars, amino acids and ions that could form highly reactive systems in which powerful aroma molecules may be generated, it is labour intensive, time consuming and expensive, precluding its use in field studies.

Therefore, the aim of the present work is to explore the possibility of using simply diluted and pH-adjusted grape mistelles as the hydrolysis media to avoid this tedious sample preparation. Furthermore, considering that the presence of sugars or amino acids could alter the hydrolysis rates of FPs and induce the formation of artefactual odorants by Maillard processes, the addition of these compounds to the PAF was studied. Both aspects, together with the losses of aromas and FPs during the preparation of the PAFs will be evaluated in order to provide a clearer view about grape aroma potential and its measurement. To the best of our knowledge, grape mistelles have not yet been employed for this purpose, making this approach a novel and unexplored strategy in the field.

2. Materials and methods

2.1. Reagents, standards, and solvents

Tartaric acid, sodium hydrogen carbonate, ascorbic acid, sodium chloride, D-glucose and D-fructose were supplied by Panreac (Barcelona, Spain). Dichloromethane and methanol of Lichrosolv® quality and methanol of LC-MS quality were obtained from Fisher Scientific. Ethanol of Lichrosolv® quality and ammonium formate of LC-MS quality were acquired from Merck (Darmstadt, Germany). The chemical standards used in the analysis of minor compounds were supplied by Merck with a purity greater than 98 %, except for 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which was synthesized by Synchem UG & Co (Felsberg, Germany) with a purity of 80 %. The resin used to prepare the SPE cartridges was ISOLUTE® ENV + supplied by Biotage (Uppsala, Sweden) and the C18 sorbent used to extract the phenolic aromatic fraction was acquired from Waters (Ireland).

Dimethyl sulphide (DMS) was obtained from Merck. Ebselen, dimethyl disulphide (DMDS), ethyl methyl sulphide (EMS), ethylenediaminetetraacetic acid (EDTA), hexyl butyrate, L-cysteine and L-gluthatione reduced were supplied by Sigma-Aldrich (MO, USA). For the preparation of hydrogen sulphide (H_2S), methanethiol (MeSH), and ethanethiol (EtSH) used as chemical standards, sodium sulphide (Na_2S), sodium methanethiolate (CH_3SNa), and sodium ethanethiolate (CH_3CH_2Sna) (also from Sigma-Aldrich) were dissolved in water at pH 9.6. Potassium metabisulfite and a commercial standard mixture of alkanes at 1000 μg mL^{-1} C7-C30 were supplied by Supelco (PA, USA). Diethyl ether was supplied by VWR Chemicals. Nitrogen, hydrogen, helium, and air were supplied by Air Liquide. Pure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA). The deuterated chemical standards used in the polyfunctional mercaptans method were acquired from EPTES (Vevey, Switzerland).

2.2. Sample preparation and treatment

A flowchart outlining the experimental process of this study can be found in Supplementary Material Figure I.

2.2.1. Mistelles

Three kilograms of Garnacha grapes from the D.O. Campo de Borja with average Brix value of 30 were destemmed and introduced in a large beaker, spiked with 50 mg kg^{-1} of potassium metabisulfite, and gently crushed by hand. Subsequently, 190 mL kg^{-1} of pure ethanol were added. The resulting mixture was distributed in three 1.5 L PET bottles, which were squeezed to displace the remaining air, and were left to macerate for 2 weeks at 8 °C with daily manual agitation. After maceration, supernatant liquids were recovered, mixed with the liquid obtained by pressing the solid parts, and left to settle for one additional week at 8 °C. After this, the liquid (mistelle) was centrifuged (Allegra X-22R (Beckman Coulter, Germany)) at 4500 rpm and 4 °C for 10 min and bottled in 1 L PET bottles which were stored at 4 °C.

2.2.2. PAF extraction

The C18 extraction of the phenolic and aromatic fraction (PAF) from the mistelle was carried out following the procedure developed by Alegre, Arias-Pérez, et al. (2020). According to this procedure, the mistelle obtained from 1 kg of grapes (670 mL) was partially de-alcoholised using a rotary evaporator to a final volume of 366 mL. The resulting de-alcoholised mistelle was then percolated through a 10 g Sep Pack C18 cartridge. Sugars, amino acids, and ions were removed by washing with Milli-Q water at pH 3.5. The polyphenolic and aroma precursor fractions were subsequently recovered by elution with 100 mL of absolute ethanol.

2.2.3. Sample adjustment

The mistelle was diluted and pH adjusted so that its final alcohol content was 13.3 % (v/v) and pH 3.5. For that, 352 mL of the initial mistelle (28.4 % v/v) were diluted with tartaric water (5 g L^{-1} and pH 3.5) and pH adjusted with NaOH or HCl 1M. In order to ensure equivalent content in grape material, reconstituted PAF was prepared by mixing 52.5 mL of PAF with 47.5 mL of ethanol and bringing the mixture to 750 mL with tartaric water (5 g L^{-1} and pH 3.5).

2.2.4. Hydrolysis

One litre volumes of diluted mistelle and of reconstituted PAF were introduced in the anoxic glove chamber (GP(Concept) from Jacomex, France) with an oxygen content below 1 mg L^{-1}. The volumes were each distributed in twenty-seven 20 mL vials and thirty-six 10 mL screw capped vials. Once sealed, the vials were grouped in packs of 7 (3 × 20 mL plus 4 × 10 mL) and each pack was vacuum sealed with two heat-sealed plastic bags (Coimbra Pack S.L., Spain), containing AnaeroGen™ activated charcoal (Thermo Scientific, USA). Each pack is an independent sampling point (1 × 20 mL vial for sensory evaluation, 2 × 20 mL for GC-MS analysis, 2 × 10 mL for GC-SCD analysis and 2 × 10 mL for HPLC-MS analysis).

Anaerobically bagged samples were stored in an oven (JP Selecta, Barcelona, Spain) at 75 °C for 0, 8, 24, 48, 96, and 190 h for reconstituted PAF, while in diluted mistelle, additional time points of 36, 72, and 120 h were added. After the hydrolysis period, samples were stored in a refrigerator at 5 °C until their use.

2.2.5. Study of losses of aroma and aroma precursors during PAF preparation

This experiment was carried out with the same mistelle prepared in 2.2.1, but it took place 12 months later. In this case, two fractions were collected during the preparation of PAF. Firstly, the 263 mL of condensed vapours containing 44.8 % ethanol produced in the de-alcoholisation of 670 mL of mistelle. Secondly, the 398 mL of the mostly aqueous (2 % ethanol) percolate obtained in the SPE extraction of the de-alcoholised mistelle. The distillate and percolate were reconstituted to 13.3 % (v/v) in ethanol, 5 g L^{-1} of tartaric acid, and adjusted to pH 3.5. The percolate was then diluted to 670 mL, and both the

mistelle and diluted percolate were hydrolysed for 48 h as described in section 2.2.4. Minor compounds and BR-VSCs were quantified as described in sections 2.3.1 and 2.3.2, respectively, in the hydrolysed percolate (at 48 h of hydrolysis) and in the mistelle (at both 0 and 48 h of hydrolysis). For the distillate, only minor compounds were analysed after 0 h of hydrolysis. The comparison of volatile compounds in the distillate and the mistelle at 0 h of hydrolysis allowed for the estimation of losses during de-alcoholisation. Meanwhile, the comparison of volatile compounds in the percolate and the mistelle after 48 h of hydrolysis allowed for the estimation of losses during extraction.

2.2.6. Effects of sugars and cysteine/glutathione on hydrolysis rates

One additional 500 mL reconstituted PAF sample from the original mistelle was spiked with different concentrations of sugars and of cysteine and glutathione. The sample contained 125 g L^{-1} of glucose, 125 g L^{-1} of fructose, 20 mg L^{-1} of reduced glutathione and 10 mg L^{-1} of cysteine. This sample was distributed in different vials, packed under anoxic conditions, and subjected to hydrolysis at 75 °C during 0, 8, 24, 36, 48, 72 and 96 h. This sample was compared with the original reconstituted PAF. Minor compounds and PFM's were analysed as described later.

Additionally, two 10 mL reconstituted PAF samples from the original mistelle were spiked with different concentrations of sugars and of cysteine and glutathione. The first sample had 125 g L^{-1} of glucose and 125 g L^{-1} of fructose, while the second one contained 20 mg L^{-1} of reduced glutathione and 10 mg L^{-1} of cysteine. These samples were packed under anoxic conditions, subjected to 96 h of hydrolysis and PFM's were analysed as described later.

2.3. Chromatographic methods

2.3.1. Minor compounds

Minor compounds were analysed as described by Pérez et al. (2022). According to this methodology, 15 mL of sample, spiked with 100 μL of the internal standards (2-octanol, 3-octanone, and 3,4-dimethylphenol, all at 5 mg L^{-1}), were percolated through a previously conditioned 1 mL SPE cartridge packed with 70 mg of ISOLUTE® ENV + resins. Interferences were removed with 1.5 mL of a 30 % water-methanol (v/v), 1 % $NaHCO_3$ (w/v) solution, the resins were then dried, and the analytes eluted with 0.8 mL of dichloromethane containing 5 % (v/v) methanol. The extract was analysed using a GC-MS (QP2010 Shimadzu, Japan). Calibration was performed by response factors (RFs) calculated by analysing a synthetic wine solution containing known concentrations of the analytes.

2.3.2. Brine releasable – volatile sulphur compounds

BR-VSCs includes free and metal-complexed VSCs and were analysed as described by Ontañón et al. (2019). For this purpose, 1.2 mL of sample and 10.8 mL of brine were transferred to a screw capped headspace 20 mL vial within the anoxic chamber, spiked with 40 μL of a standard solution (2 mg L^{-1} ethylmethylsulfide in ethanol) and analysed by GC-SCD. Calibration plots were built by analysing, following the procedure, model wines spiked with known amount of sulphur compounds.

2.3.3. Polyfunctional mercaptans

Polyfunctional mercaptans (PFM's) were analysed as described by Vichi et al. (2015). The method consisted of a single-step derivatization/extraction procedure of 10 mL of sample, spiked with 20 μl of the deuterated standards solution (0.25 mg L^{-1} 3-mercaptohexyl-d_5-acetate, 0.05 mg L^{-1} benzyl mercaptan-d_5, 0.1 mg L^{-1} 2-furfurylthiol-d_2, 0.75 mg L^{-1} 3-mercapto-1-hexanol-d_5 and 0.15 mg L^{-1} 4-methyl-4--mercapto-2-pentanone-d_{10}), followed by UHPLC-QqQ-MS analysis using Ebselen as a derivatization agent.

2.4. Sensory and olfactometric analysis

2.4.1. Sensory analysis

The panel consisted of five experienced panellists ranging in age from 21 to 59 years old. First, panellists were asked to indicate the descriptors that they consider better characterized each sample, as well as its overall odour intensity. Second, they were presented pairs of hydrolysed mistelle and hydrolysed reconstituted PAF -at equivalent hydrolysis times- and were required to identify the pair displaying maxima differences. In all cases, tempered samples were served in coded black glasses with a three-digit random number, covered with a Petri dish and with a different order for each judge.

2.4.2. Olfactometric analysis

Volatile compounds of 80 mL of hydrolysed selected samples were isolated and preconcentrated using the dynamic headspace technique coupled with solid-phase extraction (HS-SPE), following the method described by Escudero et al. (2014). The extracts were analysed with a Trace GC-FID (Thermoquest, Italy) with olfactometric port ODO-I from SGE (Ringwood, Australia). Sniffing was carried out by a panel of 6 judges (1 man and 5 women, aged between 21 and 38), who were members of the research group and had experience in olfactometric analysis. Modified frequencies percentages (% MF) were calculated using the frequency of citation and the intensity of each odour zone according to the expression proposed by Dravnieks (1985). Compounds were identified using odour descriptors, retention indices, data from scientific literature and MS spectra when available.

3. Results and discussion

Quantitative data for aroma molecules formed during the anoxic incubation at 75 °C of diluted mistelle and reconstituted PAF in model wine can be seen in the plots given in Figs. 1–3. These figures also included odour thresholds (San-Juan et al., 2012) where available. The released aroma compounds analysed included volatile sulphur compounds (H_2S and DMS), polyfunctional mercaptans (4-methyl-4--mercapto-2-pentanone, MP; 3-mercapto-1-hexanol, MH; and 2-furfurylthiol, FFT), seven terpenes (β-citronellol, dihydromyrcenol, geraniol, linalool, limonene, α-terpineol and linalool oxide), five norisoprenoids (β-damascenone, β-ionone, TDN, vitispiranes and Riesling acetal), six volatile phenols (guaiacol, syringol, vanillin, methoxyeugenol, 4-vinylphenol and 4-vinylguaiacol) and two lactones (γ-nonalactone and massoia lactone). Fig. 4 compiles the plots showing the relative amounts of aroma compounds lost during the different stages of PAF preparation. It compares the levels found in mistelle with those in the distillate and percolate at different hydrolysis times (0 and 48 h). Fig. 5 summarises how the presence of sugars and amino acids, as in mistelle, can influence the hydrolysis rates of the different precursors present in PAF. The complete quantitative data can be found in supplementary material (Tables I–VI). All these results will be discussed in the next sections.

3.1. Volatile sulphur compounds

Hydrolysed mistelles contained significant levels of H_2S and DMS, as can be seen in Fig. 1A and B. The accumulation of H_2S in mistelles is surprising, as this compound and its precursors are majorly formed during fermentation. Results presented here indicate, however, that compounds naturally present in the must release relatively large levels of this compound in the accelerated aging conditions used in the study. H_2S could be formed by the metal catalysed degradation of cysteine contained in the mistelle (Ferreira et al., 2018), or, alternatively, by the metal catalysed chemical reduction of residual elemental sulphur present in the must. Both origins are consistent with the low levels developed in the PAF extract, since amino acids and metals are poorly extracted during the PAF preparation (Alegre, Arias-Pérez, et al., 2020).

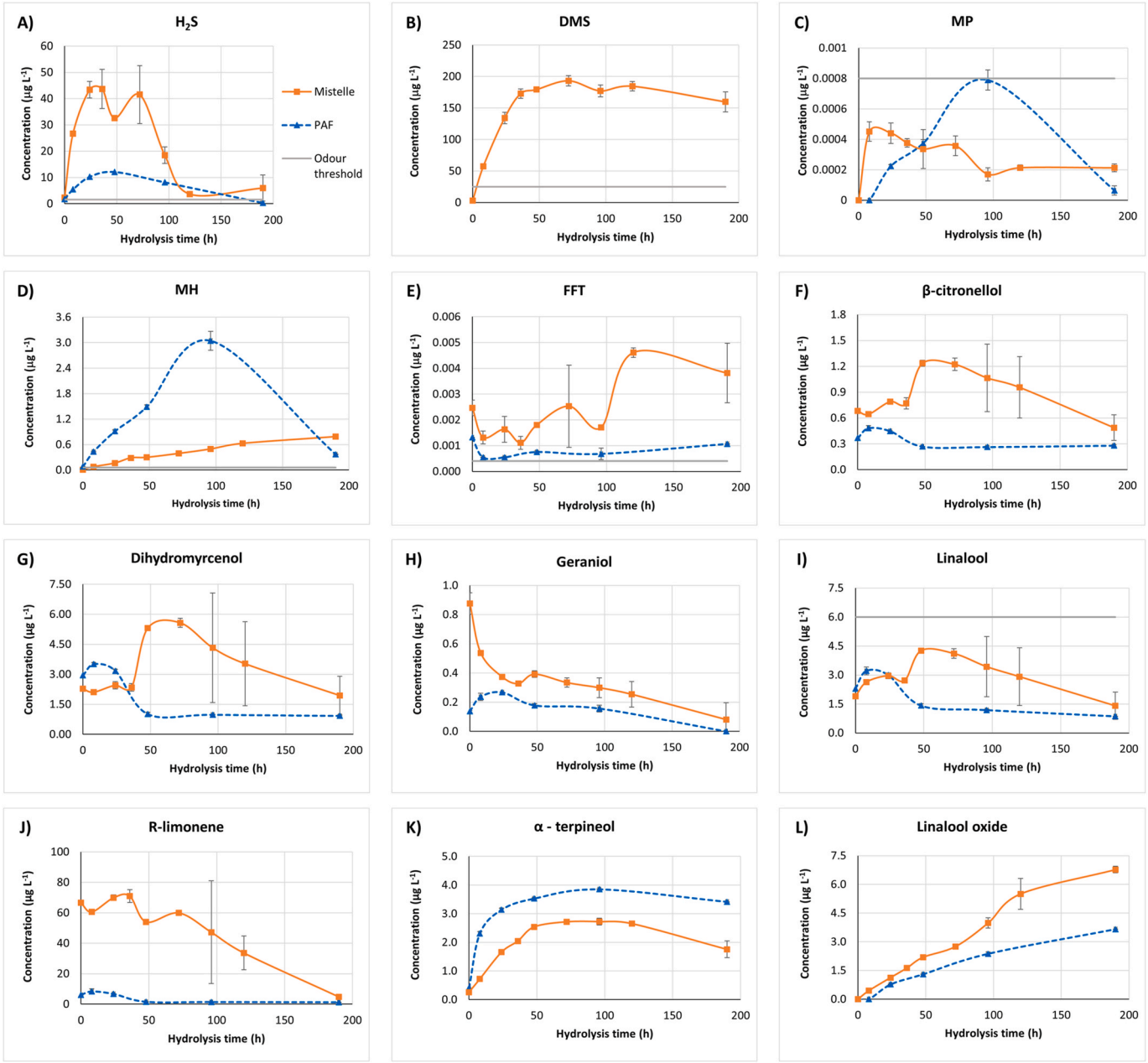

Fig. 1. Average concentrations at various hydrolysis times of selected aroma compounds in mistelle and PAF: A) H_2S, B) DMS, C) MP, D) MH, E) FFT, F) β-citronellol, G) dihydromyrcenol, H) geraniol, I) linalool, J) R-limonene, K) α-terpineol, and L) linalool oxide. Error bars are standard deviations.

However, the fact that the hydrolysis of the eluate recovered during the extraction of the PAF did not produce any H_2S (supplementary material, Table III), would favour the hypothesis of the chemical reduction of elemental sulphur, which should not be present in the eluate.

Dimethyl sulphide (DMS) was exclusively found in hydrolysed mistelle, as seen in Fig. 1B. Its formation followed a strongly increasing linear trend in the first 36 h, reaching then a plateau followed by a slight decrease in the last sampling point. The plot suggests that the precursors of DMS are completely hydrolysed in a couple of days at this temperature (75 °C), in contrast to observations made at 50 °C, where the plateau was not reached even after 7 weeks (Vela et al., 2017). The absence of this compound in the reconstituted PAF (Fig. 1B) has to be attributed to the loss of its precursors during sample preparation, and in fact, the analysis of the hydrolysed percolate (Fig. 4A) reveals that DMS precursors were not extracted by C18. This is not surprising, since the most important DMS precursor in grapes is S-methyl methionine (Segurel et al., 2005), which at the studied pH is positively charged and, therefore, cannot be retained in a non-polar sorbent.

From a practical point of view, these observations confirm what has already been seen in previous studies: that PAF is not suitable for assessing the DMS potential of grapes (Alegre, Arias-Pérez, et al., 2020), which can be studied in mistelles. On the other hand, the presence of significant amounts of DMS and H_2S in mistelle hydrolysates will have a great sensory impact, as will be discussed later.

3.2. *Polyfunctional mercaptans*

Three polyfunctional mercaptans could be quantified in the hydrolysed samples: FFT, MH, and MP (Fig. 1C–E). MH and MP derive from cysteinyl or glutathionyl precursors present in grape (Roland et al.,

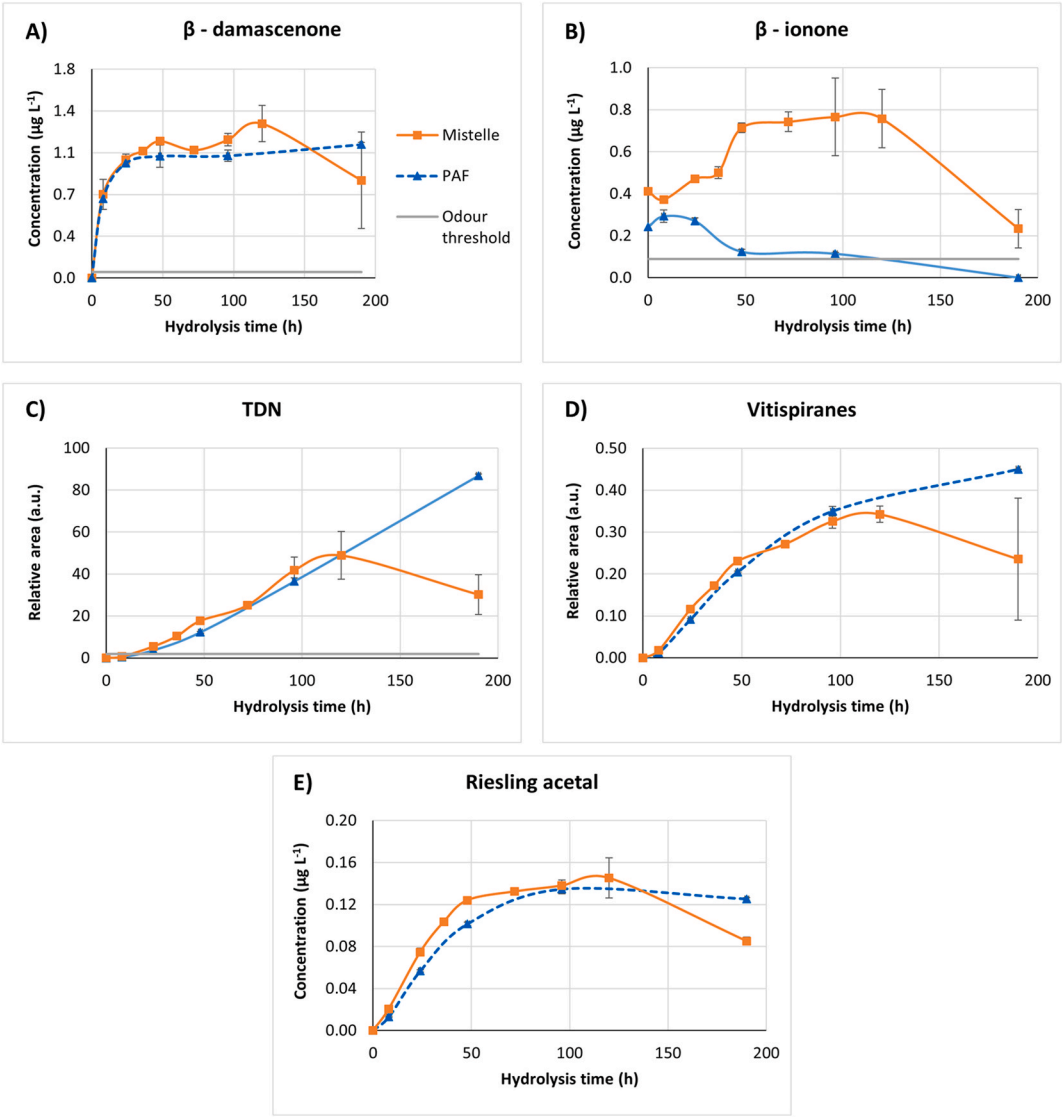

Fig. 2. Average concentrations at various hydrolysis times of selected aroma compounds in mistelle and PAF: A) β-damascenone, B) β-ionone, C) TDN, D) vitispiranes, and E) Riesling acetal. Error bars are standard deviations.

2011; Tominaga et al., 1998), while FFT is most likely an artifact formed by the reaction of H_2S with furfural (Blanchard et al., 2001). As can be seen in Fig. 1C and D, the evolutions in PAFs are similar for MP and MH, whose concentrations continuously increase, become maxima at 96 h of hydrolysis and then strongly drop. This increasing pattern of MH had already been observed by Sánchez-Acevedo et al. (2024). However, only MH reaches relatively high concentrations, up to 3.0 µg L^{-1} in the hydrolysed PAF, while levels of MP barely reached the odour threshold at 0.0008 µg L^{-1}, in accordance with previous observations about the varietal character of Garnacha (Sánchez-Acevedo et al., 2024). The patterns of release in mistelle are completely different. For MH, the release is much slower than for PAF, with no decrease observed. Moreover, the levels are much smaller than those of PAF, except at the end. In the case of MP, maximum levels are observed at the first sampling point, followed by a steady decrease, and with a maximum smaller than that observed for PAF. In any case, levels of this compound are too close to the limits of detection to be further considered.

The accumulation curve of MH in mistelle suggests that the hydrolysis of its precursors is being slowed down by some of the mistelle components not present in PAF. Therefore, the effects associated with the addition of sugars and cysteine or glutathione were tested in an independent experiment (see section 2.2.6). These results are shown in Fig. 5A, and, combined with those in Supplementary Material Table VI, confirm that the presence of reducing sugars reduces by more than a factor 3 the hydrolysis rate of MH precursors (relative areas of 1.660 a.u. in the original PAF *vs* 0.527 a.u in the added PAF after 96 h of hydrolysis). This suggests that, for assessing the levels of PFM's precursors in grapes, reconstituted PAFs are better than mistelles.

Incidentally, the highest levels of MH found in PAF, where levels of H_2S were very low, suggest that in the present case, the route of formation of MH from the reaction between H_2S and 3-hexenal (Schneider et al., 2006) should be of minor importance.

3.3. Terpenes

Fig. 1F–L shows the accumulation of terpenes during different hydrolysis times. As can be observed, the levels of terpenes released are consistently higher in mistelle in all cases except α-terpineol, strongly suggesting that some FPs and/or aroma molecules were lost during the preparation of the PAF. This is confirmed by the plots shown in Fig. 4. Losses by evaporation of the free aroma already present in the mistelle during distillation of the mistelle ranged from 20 % (geraniol) to 74 % (β-citronellol), as shown in the bars at 0 h of hydrolysis. Furthermore, hydrolysis of the percolate revealed the presence of FPs not extracted by

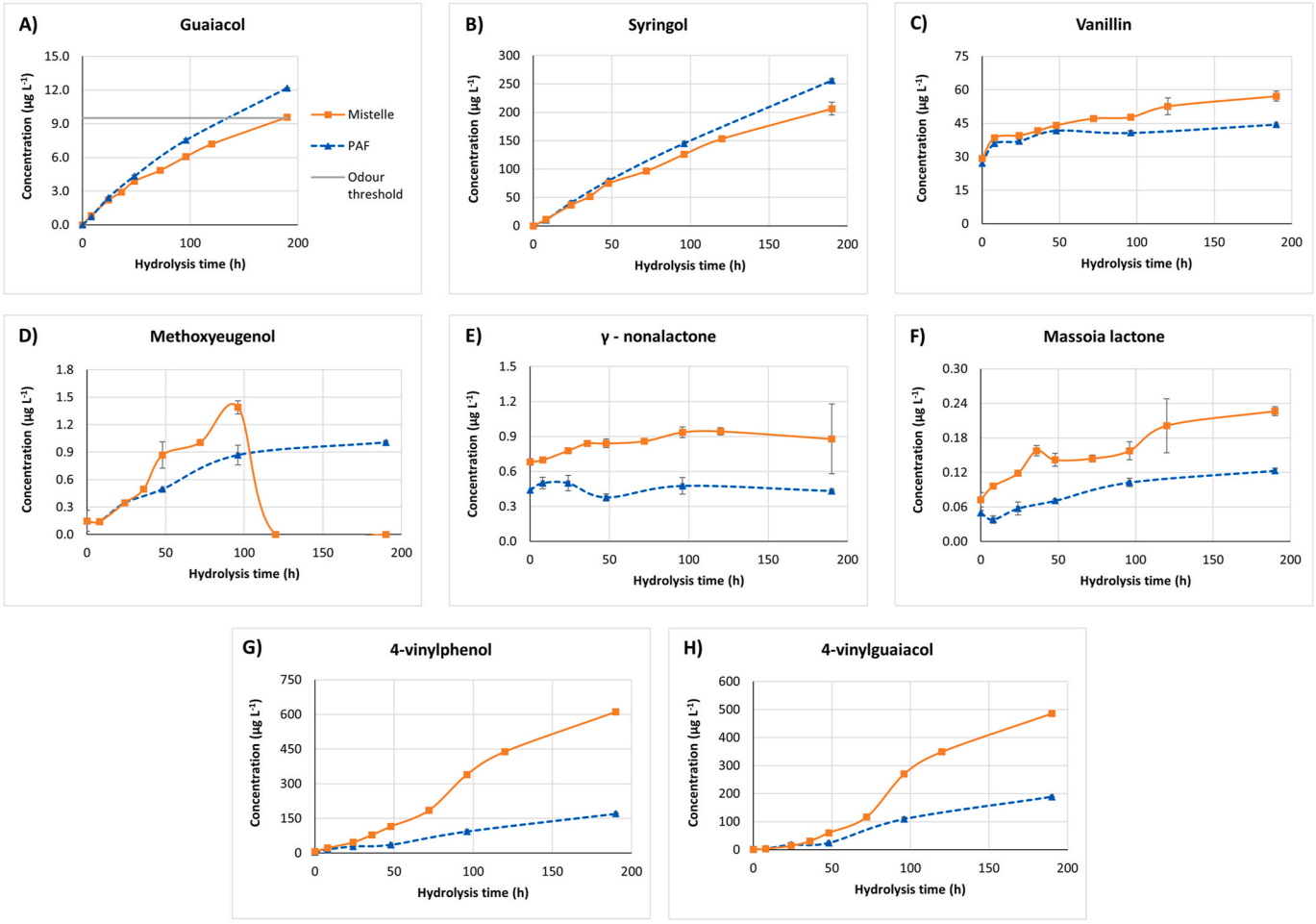

Fig. 3. Average concentrations at various hydrolysis times of selected aroma compounds in mistelle and PAF: A) guaiacol, B) syringol, C) vanillin, D) methoxyeugenol, E) γ-nonalactone, F) massoia lactone, G) 4-vinylphenol, and H) 4-vinylguaiacol. Error bars are standard deviations.

C18 of all terpenes except linalool oxide, as can be seen in the bars at 48 h of hydrolysis in Fig. 4. Levels of FP lost ranged from 8 % of α-terpineol to an amazing 118 % in the case of limonene, with most compounds in levels between 35 % (β-citronellol) to 52 % (dihydromyrcenol). Some of those highly polar FPs are, probably, di or trisaccharides of polyols, that dehydrate rapidly (Hjelmeland et al., 2015). Precursors of this type have been reported for β-citronellol, dihydromyrcenol, geraniol, and linalool (Michlmayr et al., 2012), but to the best of our knowledge, have not been reported for limonene.

A second relevant observation is that all terpenes, except a-terpineol and linalool oxide, show clear maxima in their plots, revealing the coexistence of reactive processes in which the aroma molecule is consumed. This is not surprising as these aroma molecules are quite unstable at acidic pH. α-terpineol and linalool oxide, on the other hand, are more stable molecules and are formed in whole or in part, from the degradation of other terpenols rather than from specific precursors, although glycosidic precursors of α-terpineol have been described (Ni et al., 2021).

A third conspicuous observation is that for the five terpenes with specific FPs, the maxima observed in the mistelle, in all cases observed after 48–72 h of hydrolysis, do not coincide with the maxima observed in the PAF, observed after 12–24 h of hydrolysis. In the case of linalool, two maxima are observed in the mistelle; the smallest of which is aligned with that observed in PAF, while the second and most intense is absent in this matrix. In the case of limonene, there are also two maxima in mistelle, and neither of them is aligned with the one in PAF. In order to assess whether the lack of alignment corresponds to the absence of the polar FPs in PAF, or to a delay in the hydrolysis induced by the presence of sugars in the mistelles, the hydrolysis of PAF was repeated in the presence of sugar (Fig. 5), which had no significant effect on the position of the maxima, confirming that the cause of the discrepancy between the hydrolysis curves is the loss of precursors during PAF preparation. This, in turn, shows that the assessment of terpenic potential should be based used on mistelles or musts, rather than extracts, as has traditionally been the case.

3.4. Norisoprenoids

Fig. 2 shows the accumulation curves of the five norisoprenoids consistently found in the present study. As can be seen, apart from the last sampling point, the evolutions in mistelle and PAF are quite parallel, except for β-ionone, which suggests that, with this exception, the FPs of the norisoprenoids were well retained in the PAF extraction. The analysis of the hydrolysed percolate confirms the presence of only β-ionone (Fig. 4I), suggesting that around 56 % of its FPs were not extracted by C18. To the best of our knowledge, the existence of polar FPs for this compound has not been previously indicated (Mendes-Pinto, 2009).

On the other hand, the formation of TDN, vitispiranes, and Riesling acetal takes a relatively long time, especially compared to β-damascenone and β-ionone. This may be due to the complexity of their formation. With the exception of Riesling acetal, these aroma molecules lack hydroxyl groups and cannot accumulate directly as glycosides. Instead, there is a complex pool of non-volatile glycosidic forms (Winterhalter & Schreier, 1994), which release the corresponding aroma

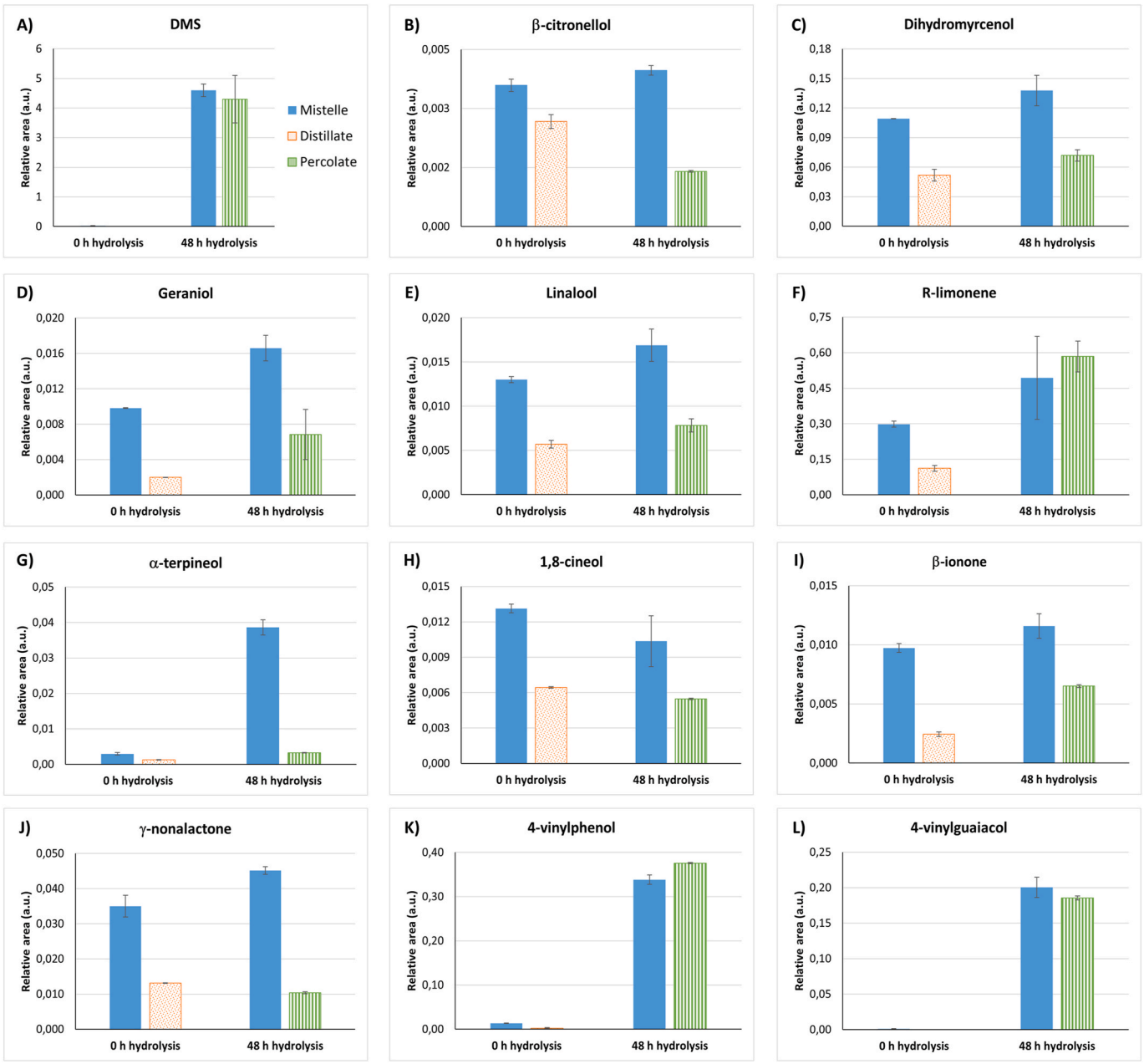

Fig. 4. Relative areas of: A) DMS, B) β-citronellol, C) dihydromyrcenol, D) geraniol, E) linalool, F) R-limonene, G) α-terpineol, H) 1,8-cineol, I) β-ionone, J) γ-nonalactone, K) 4-vinylphenol, and L) 4-vinylguaiacol lost during the distillation (distillate) and C18 extraction (percolate) stages of PAF preparation compared with their respective amounts in mistelle hydrolysates. Error bars are standard deviations.

compounds through a series of intricate reactions such as hydrolysis, dehydration, and other chemical rearrangements. Furthermore, norisoprenoids, with the exception of β-ionone, are relatively stable at wine pH, which explains why their hydrolysis plots mostly show increasing trends (TDN, vitispiranes and Riesling acetal) or clear plateaus (β-damascenone). However, the significant decreases observed in mistelles but not in PAFs, at the final sampling point were unexpected, except possibly for β-damascenone, which can react with sulphur dioxide (SO_2) (Daniel et al., 2008). The explanation for these decreases may be due to the precipitation observed at the last sampling point. It seems plausible that TDN, β-damascenone, β-ionone and vitispiranes, which are highly hydrophobic, with Log P 4.9, 4.0, 3.85 and 3.8, respectively, were partly retained in the precipitate due to non-polar interactions in addition to π-stacking processes (Charlton et al., 2002). The decrease in Riesling acetal, which is relatively polar (Log P 1.4), may be due to the fact that this compound can be further transformed into TDN (Daniel et al., 2009).

These observations demonstrate that both methodologies (reconstituted PAF or mistelle) are suitable for the quantification of norisoprenoids, provided that very long hydrolysis times are not used for mistelles.

3.5. Phenols and lactones

Fig. 3 shows the release profiles of the lactones and phenols at different hydrolysis times. As can be seen, all of them increase with time, in line with the presence of FPs of all these aroma compounds in the grapes. A similar pattern was observed by Sánchez-Acevedo et al. (2024) for volatile phenols in different Garnacha PAF 75 °C hydrolysates.

However, the release behaviour varies depending on the specific

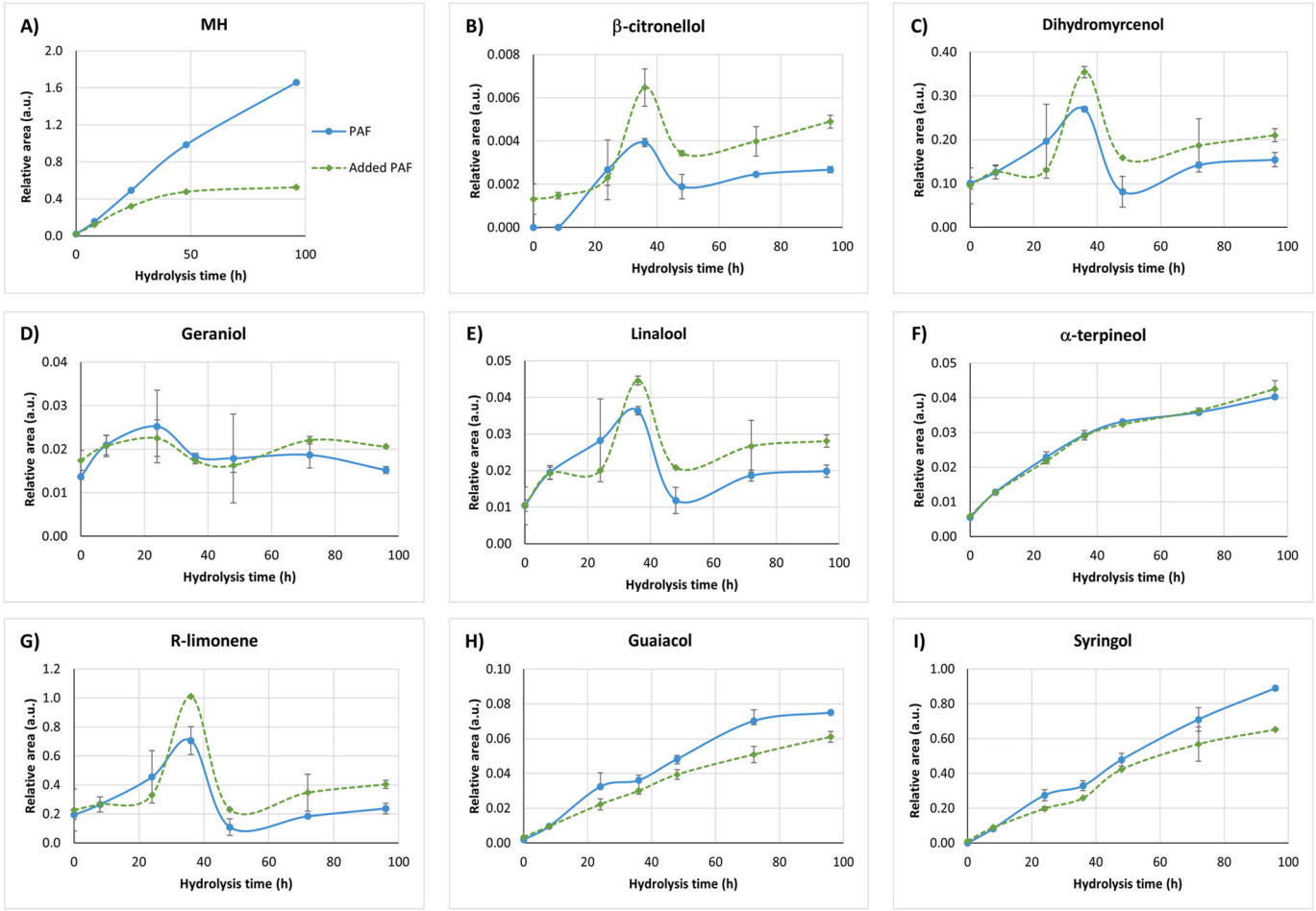

Fig. 5. Average relative areas of PAF and PAF added with sugars, cysteine and reduced glutathione at various hydrolysis times for the following compounds: A) MH, B) β-citronellol, C) dihydromircenol, D) geraniol, E) linalool, F) a-terpineol, G) R-limonene, H) guaiacol, and I) syringol. Error bars are standard deviations.

phenol. Firstly, guaiacol and syringol show relatively parallel curves in mistelle and PAF along the initial sampling points. Thereafter, the levels released in mistelles become slightly, but progressively, lower than those found in PAFs. This similarity in behaviour may be explained by the similar chemical and glycosidic precursor structures of both compounds (Hayasaka et al., 2010; Hjelmeland & Ebeler, 2015; Tikunov et al., 2013). The smaller release observed in mistelles should be attributed to the reduced hydrolysis rates induced by the presence of reducing sugars for these two compounds in mistelle, as shown for sugar-supplemented PAF in Fig. 5H and I. This was previously observed by Alegre, Arias-Pérez, et al. (2020) after 7 weeks at 45 °C. Secondly, the levels of aroma found in the hydrolysate of mistelle for the remaining compounds are significantly and, in some cases, remarkably much higher than those found in the PAFs. Vanillin and methoxyeugenol follow similar trends in the first 100 h, but levels of the latter compound in mistelle, drop sharply after this time, suggesting that it has selectively coprecipitated with some polyphenols, as already observed for some norisoprenoids in Fig. 2. The higher levels of vanillin and methoxyeugenol in the mistelle hydrolysate do not have a clear explanation, as the effects of sugar on their hydrolysis are inconsistent with the observations. Furthermore, both aroma compounds were absent or present at low levels (5.6 % in the case of vanillin) in the hydrolysed percolate (Supplementary Material Table III). Thirdly, the lower content of lactones in PAF compared to mistelle can be easily explained by two factors: their co-evaporation with the distillate and the poor extraction of their FPs, as shown by the presence of γ-nonalactone in the hydrolysed percolate (Fig. 4J). Finally, in the case of vinylphenols, the much large amount observed in the mistelles is clearly due to the poor extraction of a relevant part of their FPs, as these compounds are major compounds in the hydrolysed percolate which rendered 89.1 and 92.5 % of the amounts of 4-vinylphenol and 4-vinylguaiacol found in the mistelle, respectively (Fig. 4K and L).

These observations demonstrate that mistelles are more suitable than reconstituted PAFs for the quantification of volatile phenols. However, it is important to note that the presence of sugars slightly delays the hydrolysis of the precursors of guaiacol and syringol.

3.6. Sensory and olfactometric analysis

According to sensory analysis, hydrolysed mistelles exhibited aromas of truffle, asparagus, caramel or honey, in clear contrast to those of PAFs, which displayed mostly green and fruity aromas. Quantitative results seen in previous paragraphs support part of these differences, since truffle and asparagus are often associated to DMS, which, together with H_2S, was found at higher levels in mistelles. The smaller levels of MH in mistelles could also be related to a reduced perception of green and fruity attributes. The existence of caramel or honey descriptors could be related to the presence of specific odorants formed by Maillard reactions. In order to check this possibility, a semiquantitative GC-O experiment comparing two pairs of PAF and mistelle hydrolysates (at 48 and 96 h) was carried out. Results are summarized in Table 1. The modified frequencies percentages (%) obtained should be closely related to the olfactory importance of the odorants in the vapor phases of the samples (de-la-Fuente-Blanco & Ferreira, 2020).

As can be seen, 32 odour zones were detected in the four samples, with 29 odorants identified (13 lipid derivatives, 8 phenols and

Table 1
Summary of the GC-O of the mistelle and PAF hydrolysates during 48 and 190 h. Retention indices on polar capillary column (RI on DB-Wax), olfactory description, chemical identity and modified frequencies expressed as percentage (% MF) are shown.

RI	Odour description	Chemical identity	PAF 48 h	Mistelle 48h	PAF 96 h	Mistelle 96 h
957	Solvent, alcohol, liquor, Nenuco, fruit, red fruit, sweet	Ethyl isobutyrate[b]	–	35	26	55
1121	Strawberry, raspberry, fruit, sweet, solvent, rancid, bitter	n.i. 1121[c]	–	–	40	59
1223	Soil, humidity, geranium, green, metallic, unpleasant	1-hepten-3-one[a]	55	7	29	25
1258	Black fruit, floral, violets, dried herbs, geranium, green, dry	Ethyl hexanoate[a] and E−2-heptenal[b]	10	12	33	55
1306	Green, floral, sweet, fruit, orange, tangerine, citrusy, Fairy, air freshener	Octanal[a]	14	7	59	30
1313	Fruity but solvent, old, damp, rotten, buttery, vegetal	2-octanone[b]	–	–	57	–
1360	Toasted cereal, toasted, biscuit, cleaning product, floor cleaner	1-hexanol[a]	–	–	35	7
1376	Dairy, food, chicken stock, dried herbs, dry, clean clothes, cologne, detergent.	Z-3-hexen-1-ol[a]	*30*	29	*57*	*48*
1402	Green, floral, dried herbs, metallic, motor oil, wet	Nonanal[a]	19	7	33	29
1425	Mushroom, metallic, fungi, wet, grease	1-nonen-3-one[b]	60	29	65	19
1527	Food, meat stew, paper, fruit, vegetable, green, closed, chlorine, smoked	Z-2-nonenal[b]	19	63	19	50
1557	Alcohol, toasted cereal, bitter almond, dry, oil, butter, acidic, dairy, chlorine	1-octanol[a] and E−2-nonenal[b]	36	55	38	57
1613	Spiced, caramel, floral, muscatel, spicy	Z-2-decenal[b]	7	48	36	14
1659	Roasted, burnt, coffee, sewage water, caramel, toffee	2-acetylpyrazine[b]	17	45	10	22
1679	Fruit, plum, compote, floral	Phenylacetaldehyde[b]	–	12	–	31
1699	Cheese, feet, unpleasant, cooked meat, sausage	3-methylbutyric acid[b]	17	22	33	29
1715	Bleach, vomit, rancid, acidic, greasy, strong cheese, sweat, acid, garbage, fetid, moisture, spicy	Pentanoic acid[b]	50	69	57	47
1774	Rancid, closed, dusty, dry	E−2-undecenal[b]	13	38	19	26
1847	Ripe fruit, compote, blackberry, strawberry, pineapple, plum, peach, grape, raspberry	β-damascenone[a]	75	64	65	73
1871	Green, flowers, infusion, tea, sweet, compote, spicy, straw, dry leaves, spicy	3-mercaptohexanol[b]	–	44	45	39
1896	Mint, dry, sweet, nuts, bacon, smoky, floral, green, spicy	Benzyl alcohol[a]	43	61	59	59
1966	Honey, freshness, smoky, leather, phenolic	n.i. 1966[c]	38	–	–	–
1971	Floral, green, toasted, burnt, animalic, musky, smoky, bacon, dry	n.i. 1971[c]	–	–	22	36
1981	Smoky, dry, toasted, synthetic, plastic, cooked vegetables	n.i. 1981[c]	7	10	–	31
2126	Barn, musk, animal stable, manure, garbage	p/m-cresol[b]	70	57	66	80
2135	Phenolic, synthetic, gorgonzola, medicinal	4-propylguaiacol[b]	12	4	37	–
2157	Dry, chlorinated, leather, unpleasant, garbage, rancid, phenolic, rubber/plastic, burnt hair, medicine, spicy, caramel	2-phenoxyethanol[b]	–	47	50	53
2161	Roasted corn, sweet ("cinnamate-type"), fruity	Ethyl cinnamate[b]	48	–	22	–
2273	Sweet, floral, geranium, spices, cooked meat, bacon, smoky, medicine, burnt plastic	2,6-dimetoxyphenol[b]	43	36	59	47
2309	Smoky, bacon, phenolic, cresol, toasted, roast, liquorice	n.i. 2309[c]	19	–	37	37
2391	Floral, sweet, clove, smoky, damp	E-isoeugenol[b]	38	7	17	31
2698	Old, closed wardrobe, cinnamon, vanilla	Acetovanillone[b]	–	–	33	–

[a] Identified by the odour descriptor, retention index on DB-WAX column and GC-MS.
[b] Identified by the odour descriptor and retention index on DB-WAX column.
[c] n.i. Not identified compound.

benzenoids, 2 esters, 2 acids, 1 pyrazine, 1 norisoprenoid, 1 Strecker aldehyde and 1 polyfunctional mercaptan), in agreement with previous reports (Alegre, Arias-Pérez, et al., 2020; Alegre, Sáenz-Navajas, et al., 2020). The presence of lipid derivatives is common to all grape hydrolysates and seems to constitute a common green-vegetal background of unclear origin as previously reported (Alegre, Sáenz-Navajas, et al., 2020).

Differences between hydrolysates from mistelle or PAFs are rather qualitative and seem to be related to the profile of "green" odorants linked to the oxidation of grape fatty acids. For instance, mistelles have higher intensities of Z-2-nonenal and 1-octenol, whereas PAF hydrolysates have higher levels of 1-hepten-3-one, octanal and 1-nonen-3-one. Furthermore, ethyl cinnamate was detected only in PAF, while phenylacetaldehyde was detected only in mistelles and 2-acetylpyrazine was also found in mistelles with higher GC-O scores. Phenylacetaldehyde contributes to honey aromas and is derived from the Strecker degradation of phenylalanine (Bueno et al., 2018), suggesting that this reaction may be active in the mistelles. On the other hand, 2-acetylpyrazine is a potent Maillard reaction product with a low odour threshold (0,062 μg L^{-1}) (Bösl et al., 2021).

In summary, results suggest that the sensory deviations noted in hydrolysed mistelles may simply be the consequence of the higher levels of DMS and H_2S, the lower levels of 3 MH and the likely higher presence of phenylacetaldehyde and 2-acetylpyrazine.

4. Conclusions

The study showed that polar FPs of relevant varietal aroma compounds, including DMS, limonene, dihydromyrcenol, geraniol, linalool, β-ionone, γ-nonalactone and vinylphenols are lost during the extraction of the PAF fraction. This implies that the use of non-polar extraction techniques (C18) for the evaluation of grape aroma potential introduces a bias, and therefore the direct use of mistelle is much more appropriate. On the other hand, the presence of sugars in the hydrolysis medium has as its main effect a significant reduction in the formation of 3 MH, making it difficult to evaluate this molecule directly in mistelles. Finally, the GC-O profiles of hydrolysed mistelles are qualitatively not very different from those of PAF hydrolysates, although some odorants produced by the reaction between sugars and amino acids, such as phenylacetaldehyde or 2-acetylpyrazine, systematically scored higher in mistelles. This, together with the strong accumulation of DMS and H_2S in mistelles and the smaller levels of MH, may explain why the sensory evaluation of grape aroma potential using mistelles can be problematic. However, it should be the method of choice for the chemical assessment of grape aromatic potential.

CRediT authorship contribution statement

Belén González-Martínez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Arancha de-la-Fuente-Blanco:** Writing – review & editing, Writing – original

draft, Methodology, Investigation, Formal analysis. **Cristina Peña:** Methodology. **Vicente Ferreira:** Writing – original draft, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117650.

Data availability

Data will be made available on request.

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