

# How particular perceptive interactions could affect red wines' fresh fruity aroma?

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## ABSTRACT

A preparative HPLC method was applied to aromatic red wine extracts. 25 fractions with various flavors were thus obtained and several aromatic reconstitutions were produced by mixing some of these fractions. Discriminative tests revealed that the omission of some fractions from the mixture of fruity fractions or the addition of others affected the overall expression of fruity aroma. Sensory profile analyses identified significant differences among aromatic reconstitutions in terms of intensity of black-berry, as well as fresh-, and jammy-fruit descriptors. A fraction with a very low fruity note (fraction 17) had an additive effect on the fresh fruity aroma, while fractions with caramel and lactic notes (fractions 3 to 5) had a masking effect on this aroma. Further analysis revealed that ethyl 2-hydroxy-4-methylpentanoate was eluted in fraction 17, while diacetyl, acetoin, acetic acid, and  $\gamma$ -butyrolactone were eluted in fractions 3 to 5. Omissions tests established that ethyl 2-hydroxy-4-methylpentanoate was responsible for enhancing black-berry and fresh-fruit aroma and that a combination of diacetyl, acetoin, acetic acid, and  $\gamma$ -butyrolactone, at levels between 2 and 40% of their perception thresholds, had the same hypo-additive effect on the fresh fruity aroma as fractions 3 to 5.

## INTRODUCTION

Wines consist of highly complex mixtures of volatiles derived from grapes, fermentation processes, and aging. Is wine perception just a simple sum of these constituents? According to Francis et al. (2005) it is widely recognized that wine aroma is not the result of any single dominant compound, that confers to a specific aroma on a particular wine or wine type, but that, on the contrary, all wines owe their aromatic character to a multitude of volatiles. Perceptual interactions between multiple volatile components in combination remain difficult to predict in such a complex matrix as wine, where overall perception cannot be predicted

from the sum of perceptions of individual compounds. In addition, aroma of a reconstituted mixture only from pure odorants, considered as key compounds, differs substantially from the original wine (Barbe et al., 2008). Recent examples in simple but also in more complex mixtures showed that, via particular perceptive interactions, certain compounds, even present at levels far below their olfactory perception threshold, played a role in the overall aroma (Pineau et al., 2009). The main goal of this work was to highlight and study significative particular perceptive interactions on wine fruity aroma expression using various aromatic reconstitutions.

## **MATERIALS AND METHODS**

**Samples.** Two red wines were used: a Vin de Pays d'Oc (1) (2010 vintage) and a Margaux appellation wine (2) (2000 vintage). Dilute alcohol solution was prepared using double-distilled ethanol and microfiltered water (12%, v/v).

**Aromatic reconstitution made from HPLC fractions (AR).** Sample preparation was as optimized by Lytra et al. (2012). A 500 mL wine sample was extracted successively using 80, 80, and 50 mL dichloromethane, with a separatory funnel for 10 min. The organic phases were collected, blended, dried over sodium sulfate, and concentrated under nitrogen flow (100 mL/min) to obtain 1.25 mL wine extract. Reversed-phase (RP) HPLC was performed on this raw extract using a Nova-Pak C18 column (300 × 3.9 mm i.d., 4 μm, 60 Å, Waters, Saint-Quentin, France), without a guard cartridge. The HPLC system consisted of an L-6200A pump (Merck-Hitachi, Germany). Chromatographic conditions were as optimized by Pineau et al. (2009). Twenty-five fractions with various aroma were obtained in dilute alcohol solution. For aromatic reconstitutions, fractions were retained and added individually or blended together to reproduce the initial concentrations in the original wines, adding double-distilled ethanol and microfiltered water to obtain an ethanol level of 12% (v/v).

**HPLC fractions extraction.** Each fraction was diluted in distilled water to obtain 12% ethanol, then re-extracted by the same method as the wine, but with 4, 2, and 2 mL dichloromethane (5 min stirring for each extraction). The organic phases were collected, dried over sodium sulfate, and concentrated under nitrogen flow to obtain 100 μL extract.

**GC–MS analysis of wine fractions.** Two μL samples of organic extract were injected in splitless-split mode (injector temperature: 250 °C, interface temperature: 280 °C, splitless time: 45 s, split flow: 50 mL/min) using an HP 6890N gas chromatograph (Hewlett–Packard, Wilmington, DE, USA) coupled to a mass spectrometer (HP 5973i). The column was a BP20 (SGE, Ringwood, Australia), 50 m x 0.22 mm i.d., film thickness: 0.25 μm. The oven was

programmed at 40 °C for the first minute, then increased at a rate of 3 °C/min up to a final isotherm at 250 °C for 30 minutes. The carrier gas was Helium N55 (Air Liquide, France) with a column head pressure of 8 psi. The MSD was used in full-scan mode (m/z 40–300, 3.09 scans/ sec). MS data were recorded and processed using Chemstation software equipped with NIST 2008 MS library (US National Institute of Standards and Technology, Gaithersburg, MD, USA). The compounds were then characterized by comparing their LRI and mass spectra with those of standards.

**Quantification of indentified compounds.** The enantiomers of ethyl 2-hydroxy-4-methylpentanoate (ethyl DL leucate) were assayed using chiral gas chromatography–mass spectrometry ( $\gamma$ -cyclodextrin). Chromatographic conditions and sample preparation were as described by Lytra et al. (2012a). Diacetyl was assayed using gas chromatography–mass spectrometry. Chromatographic conditions and sample preparation were as optimized by de Revel et al. (2000). Acetoin and  $\gamma$ -butyrolactone quantitation were determined using gas chromatography coupled to a flame ionization detector method, as described by de Revel et al. (1994). Finally, an enzymatic test kit (Boehringer Mannheim/R-Biopharm, Germany) based on the UV spectrometry (V-530 UV/Vis Spectrophotometer, JASCO, Japan) was used to quantitate acetic acid.

**Sensory analyses.** Wine samples, aromatic reconstitutions, and HPLC fractions were evaluated by descriptive testing methods. Fractions were selected and preliminary tests carried out prior to preparing the aromatic reconstitutions. Triangular tests were performed for various aromatic reconstitution samples (Table 1). Sensory profiles of aromatic reconstitutions for red-berry, black-berry, fresh-, and jammy-fruit aroma intensity were evaluated, using a 0-7 point structured scale, as shown in Table 2. The panel consisted of 17 judges and all panelists were research laboratory staff at ISVV, Bordeaux University, selected for their experience in assessing fruity aroma in red wines.

Statistical data were analyzed using R analysis of variance (ANOVA) software: the homogeneity of variance was tested using Levene's and the normality of residuals was tested using Shapiro-Wilk Test. All descriptors are mean centered per panelist and scaled to unit variance. The statistically significant level is fixed at 5% ( $p < 0.05$ ).

Sensory profiles were also evaluated by 18 panelists for two different matrices (dilute alcohol solution and HPLC fruity fractions), supplemented with diacetyl, acetoin, acetic acid, and  $\gamma$ -butyrolactone (individually or mixed) at the concentrations found in fractions 3 to 5 in wine (1) (Table 3). The subjects had to evaluate the intensity of overall aroma intensity, fresh- and jammy-fruit aroma in 6 samples, using a 100 mm scale. All panelists were research laboratory

staff at ISVV, Bordeaux University, selected for their experience in assessing fruity aroma in red wines.

Experimental data were reported on a graph based on two parameters [ $\sigma = f(\tau)$ ] introduced by Patte and Laffort (1979). Tau ( $\tau$ ) reflects the ratio of perceived intensity of the aromatic reconstitution made from HPLC fruity fractions alone, to the sum of perceived intensities of mixture's components, prior to mixing:  $\tau = \text{IAR (17-22)} / (\text{IAR (17-22)} + \text{IC})$ , where IAR (17-22) and IC are the perceived odor intensities of an aromatic reconstitution containing HPLC fruity fractions and a test compound (diacetyl, acetoin, acetic acid, and  $\gamma$ -butyrolactone, individually or mixed) prior to mixing. Sigma ( $\sigma$ ) reflects the ratio of perceived intensity of the mixture, to the sum of perceived intensities of its components, prior to mixing: the degree of overall intensity addition in the mixture:  $\sigma = \text{Imix} / (\text{IAR (17-22)} + \text{IC})$ , where Imix is the perceived odor intensity of the mixture. Tau and sigma were calculated for the intensity of overall, fresh, and jammy fruit aroma. The mean experimental results for the panel were presented using the synthetic representation  $\sigma = f(\tau)$ . The graph was divided into several parts, according to the interaction level (Figure 2a). The position of experimental points reflects the interaction level (Frijters, 1987). Cain and Drexler (1974) referred to mixture interactions in terms of the overall perceived intensity of a mixture compared to the intensities of each separate component. They indicated that the perceived strength of a mixture may be: (a) as strong as the sum of the perceived intensities of the unmixed components, exemplifying complete addition ( $\sigma=1$ ); (b) more intense than the sum of its components, exemplifying hyper-addition ( $\sigma>1$ ); or (c) less intense than the sum of its components, exemplifying hypo-addition ( $\sigma<1$ ). Moreover, Frijters (1987) distinguished three cases of hypo-addition: the terms 'partial addition', 'compromise', and 'subtraction' are used if the quality intensity of the mixture is greater, intermediate, or smaller than that of the single intensities.

For each sample, the significance of the observed perceptual interaction was statistically tested by calculating the 95% confidence interval on the mean intensity of the subjects (18) for both  $\sigma$  and  $\tau$ .

## RESULTS

**Olfactive description of wines and fractions obtained by HPLC.** Applying reversed-phase HPLC to a wine extract resulted in 25 fractions in dilute alcohol medium and the aromatic characteristics of each fraction were assessed by direct olfaction. Analyses performed by on red wines confirmed that fruity characteristics were conserved from wines to fractions, as already observed by Pineau et al. (2009).

**Fraction selection.** First results suggested the importance of fractions 17 to 22, which had intense fruity notes as described by Lytra et al. (2012b). The addition of fractions 3 to 5 to the fruity mixture (17 to 22) and the suppression of fraction 17 from the fruity mixture (17 to 22) had an aromatic impact.

**Impact of some fractions on overall aroma.** During tests performed using wine (1) (Table 1), over 80% of the judges distinguished between the total AR (1 to 25) and less exhaustive aromatic reconstitutions (AR (18 to 22) and AR (17 to 22)). This is understandable due to the considerable difference in composition between total AR and simpler mixtures. For wine (1), it was also observed that the absence of fruity fraction 17 from the total fruity AR (17 to 22) had no impact on overall fruity aroma expression (Table 1). Nevertheless, this was not the case for wine (2), as over 80% of the judges detected the omission of fraction 17 (Table 1). This phenomenon may be explained by a difference in the composition of the fraction 17 obtained from these two wines. Finally, the addition of fractions 3 to 5 to the AR (17 to 22) of wine (1) was significantly perceived, indicating that volatiles in these fractions may play an important role (Table 1).

As shown in Figure 1, significant differences were found among the intensities of black-berry, fresh-, and jammy-fruit descriptors in AR prepared from HPLC fractions of wine (1). In total AR (1 to 25), jammy-fruit intensities were significantly higher and fresh-fruit were significantly lower than in fruity AR (17 to 22). This result suggests that fractions without any fruity character may contribute to overall jammy- and fresh-fruit aroma, as attested by the total AR intensities of these descriptors in model wine solution. The same results were observed when AR (17 to 22) was spiked with fractions 3 to 5, confirming that fractions without any clear fruity character may contribute to overall jammy- and fresh-fruit aroma. The mean intensities of descriptors for AR (17 to 22) spiked with fractions 3 to 5 and total AR led to statistically identical average scores for fresh- and jammy-fruit intensity, highlighting the importance of fractions 3 to 5, which seem to have the same aromatic impact as than the rest of the non-fruity fractions taken together on the expression of fresh- and jammy-fruit notes. This modification of fresh-fruit aroma by fractions with caramel and lactic aroma (3 to 5) suggests that components in these fractions may "mask" the fresh-fruit notes. Significant differences were found between AR (18 to 22) and AR (17 to 22): when fraction 17 was present, the average intensities of black-berry and fresh-fruit aroma were significantly higher than in AR (18 to 22). These results indicated that fraction 17, which had little fruity character, contributed to overall black-berry and fresh-fruit aroma, suggesting that its constituents may act as an enhancer of these notes.

**Identification of compounds in HPLC fractions.** Fraction 17 presented light, fresh-fruit aroma and was relatively simple, from an analytical point of view. Ethyl 2-hydroxy-4-methylpentanoate, recently identified in red wines by Falcao et al. (2012) was almost the only compound eluted from this fraction. An evaluation of its organoleptic impact by Lytra et al. (2012a) revealed that this ester contributed to a synergistic effect, enhancing the perception of fruity character. Fractions 3, 4, and 5, with their caramel and lactic aroma were found to be highly complex, from an analytical standpoint. The main compounds isolated were: diacetyl, acetoin, acetic acid, and  $\gamma$ -butyrolactone. Even if the origin and organoleptic properties of these individual compounds have been described in some detail, there was no previous data concerning their impact on fruity aroma expression.

**"Additive" effect on the fresh fruity aroma.** As shown in Table 4, the addition of fraction 17 or 550  $\mu\text{g/L}$  R- and S- ethyl 2-hydroxy-4-methylpentanoate (R/S:95/5, m/m) (as observed in fraction 17) to AR of HPLC fruity fractions 18 to 22 of wine (2) resulted in identical average scores for red-berry intensity and lower average scores for jammy-fruit intensity. Significant differences were found for black-berry and fresh-fruit aroma descriptors between AR (18 to 22) and both AR (18 to 22) when supplemented with 550  $\mu\text{g/L}$  R- and S- ethyl 2-hydroxy-4-methylpentanoate (R/S:95/5, m/m) or with fraction 17. Average intensities for black-berry and fresh-fruit aroma were significantly higher than in AR (18 to 22), revealing that ethyl 2-hydroxy-4-methylpentanoate alone played the same aromatic role as fraction 17 in the expression of black-berry and fresh-fruit notes. These results are in agreement with previous observations by Lytra et al. (2012a), demonstrating that ethyl 2-hydroxy-4-methylpentanoate was the only ester eluted from fraction 17 (the others were eluted from fractions 18 to 22) and that this compound was an active contributor to the black-berry and fresh-fruit notes.

**"Masking" effect on the fresh fruity aroma.** The results of perception of individual compounds demonstrated that the addition of A, Ac and GBL (at concentrations found in fractions 3 to 5 of wine (1)) to dilute alcohol solution did not affect the intensity of any descriptors, confirming triangular test results. A, Ac and GBL may, therefore, be present at subthreshold concentrations (Table 1). On the contrary, the presence of D caused a significant increase in overall intensity, confirming triangular test results (Table 1). In order to evaluate the impact of diacetyl, acetoin, acetic acid and  $\gamma$ -butyrolactone, the experimental data were reported on a graph based on two parameters [ $\sigma = f(\tau)$ ] introduced by Patte and Laffort (1979). As shown in Figure 2a, analyses of fresh-fruit aroma intensity revealed a compromise level of hypo-addition for both mixtures: AR (17-22) + D + A + Ac + GBL and AR (17-22) +

3 to 5. A decrease in fresh fruity intensity was observed when D + A + Ac + GBL or fractions 3 to 5 were added to AR (17 to 22), indicating that these mixture had the same effect. The omission of one of the four compounds from the total mixture (AR (17-22) + D + A + Ac + GBL) resulted in the same hypo-addition effect on fresh-fruit aroma intensity (Figure 2b). A compromise level ( $\sigma$  value $<1$ ) was observed for all mixtures. Interestingly, the simultaneous presence of A, Ac and GBL in AR (17-22), at subthreshold concentrations generally considered to have no impact on overall sensory perception, resulted in a marked attenuation of fresh-fruit notes, showing that these compounds have a considerable impact on the expression of fresh-fruit aroma. As shown in Figure 2c, the omission of two of the four compounds from the total mixture produced the same hypo-addition effect on fresh-fruit aroma intensity except AR (17 - 22) + A + GBL, where the hypo-addition was not significant as compared to hyper-addition and complete addition. It was observed that mixtures containing D had lower sigma ( $\sigma$ ) values (Figure 2c), highlighting the importance of D in attenuating fresh-fruit aroma intensity, and also the impact of A, Ac and GBL, as adding them in pairs to AR (17-22) markedly attenuated the fresh-fruit aroma. Our findings revealed that all 16 mixtures produced the same result, demonstrating the impact of each of these compounds on fresh-fruit aroma intensity. Diacetyl had the greatest direct impact, but acetoin, acetic acid, and  $\gamma$ -butyrolactone also contributed indirectly to the decrease in fresh-fruit aroma intensity (Lytra et al., 2012b). The impact of acetoin, acetic acid, and  $\gamma$ -butyrolactone was demonstrated conclusively, even at subthreshold concentrations. These findings highlighted the existence of new remarkable perceptual interactions impacting fresh-fruit aroma perception, indicating that constituents of the fractions with caramel and lactic aroma (3 to 5) had a "masking" effect on the fresh-fruit notes.

## DISCUSSION

These findings highlighted the direct role of diacetyl, as well as new perceptual interactions, including the indirect impact of acetoin, acetic acid, and  $\gamma$ -butyrolactone at concentrations representing about 2%, 12%, and 40% of their perception thresholds, respectively, on fruity aroma expression.

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**Table 1:** Triangular tests using various aromatic reconstitutions (AR)

Samples compared			Wines	
AR (1-25)		AR (17-22)	*	
AR (1-25)		AR (18-22)	*	(1)
AR (17-22)		AR (18-22)	-	
AR (17-22)		AR (17-22 + 3-5)	*	
AR (18-22)		AR (18-22) + 250 µg/L R- 2OH4MeC <sub>5</sub> C <sub>2</sub>	-	(1)
AR (17-22)		RA (18-22)	*	(2)
AR (18-22)		AR (18-22) + 550 µg/L R- and S- 2OH4MeC <sub>5</sub> C <sub>2</sub> (R/S:95/5, m/m)	*	
AR (17-22)		AR (17-22) + 4 mg/L D + 3,2 mg/L A + 25 mg/L Ac + 8,5 mg/L GBL	*	(1)
MS		MS + 4 mg/L D	*	
MS		MS + 3,2 mg/L A	-	-
MS		MS + 25 mg/L Ac	-	
MS		MS + 8,5 mg/L GBL	-	

AR, aromatic reconstitutions using HPLC fractions were supplemented with: D, diacetyl; A, acetoin; Ac, acetic acid; GBL,  $\gamma$ -butyrolactone; D, A, Ac, and GBL at concentrations found in fractions 3 to 5 of wine (1); 2OH4MeC<sub>5</sub>C<sub>2</sub>, ethyl 2-hydroxy-4-methylpentanoate at concentrations found in fraction 17 of the corresponding wine. MS, model wine solution (dilute alcohol solution); \*, 0.1% significant level; -, no significant difference.

**Table 2:** Aromatic reconstitutions (AR) compared by performing sensory profiles

Samples compared			Wines
AR (17-22)		AR (18-22)	
AR (17-22)		AR (1-25)	(1)
AR (17-22)		AR (17-22 + <b>3-5</b> )	
AR (17-22)	AR (18-22)	AR (18-22) + 550 µg/L R- and S- 2OH4MeC <sub>5</sub> C <sub>2</sub> (R/S:95/5, m/m)	(2)

AR, aromatic reconstitution made from HPLC fractions supplemented with: 2OH4MeC<sub>5</sub>C<sub>2</sub>, ethyl 2-hydroxy-4-methylpentanoate at concentrations found in fraction 17 of the corresponding wine.

**Table 3:** Compounds and fractions added to two different matrices (dilute alcohol solution and HPLC fruity fractions) for sensory profile evaluation

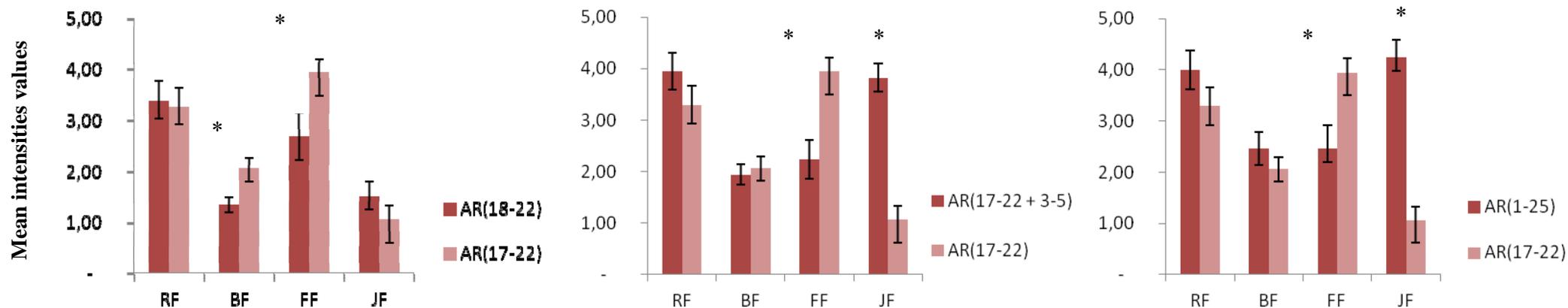
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + D + A + Ac + GBL</b>	<b>MS + F 3 to 5</b>	<b>AR (17-22) + D + A + Ac + GBL</b>	<b>AR (17-22) + F 3 to 5</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + D + Ac + GBL</b>	<b>MS + A + Ac + GBL</b>	<b>AR (17-22) + D + Ac + GBL</b>	<b>AR (17-22) + A + Ac + GBL</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + D + A + Ac</b>	<b>MS + D + A + GBL</b>	<b>AR (17-22) + D + A + Ac</b>	<b>AR (17-22) + D + A + GBL</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + A + GBL</b>	<b>MS + D + GBL</b>	<b>AR (17-22) + A + GBL</b>	<b>AR (17-22) + D + GBL</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + GBL + Ac</b>	<b>MS + A + Ac</b>	<b>AR (17-22) + GBL + Ac</b>	<b>AR (17-22) + A + Ac</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + D + A</b>	<b>MS + D + Ac</b>	<b>AR (17-22) + D + A</b>	<b>AR (17-22) + D + Ac</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + Ac</b>	<b>MS + GBL</b>	<b>AR (17-22) + Ac</b>	<b>AR (17-22) + GBL</b>
<b>MS</b>	<b>AR (17-22)</b>	<b>MS + D</b>	<b>MS + A</b>	<b>AR (17-22) + D</b>	<b>AR (17-22) + A</b>

MS, model wine solution (dilute alcohol solution); F, fractions; AR (17-22), aromatic reconstitution (AR) made from HPLC fruity fractions (17–22); supplemented with D, diacetyl; A, acetoin; Ac, acetic acid; GBL,  $\gamma$ -butyrolactone. D, A, Ac and/or GBL, at concentrations found in fractions 3 to 5.

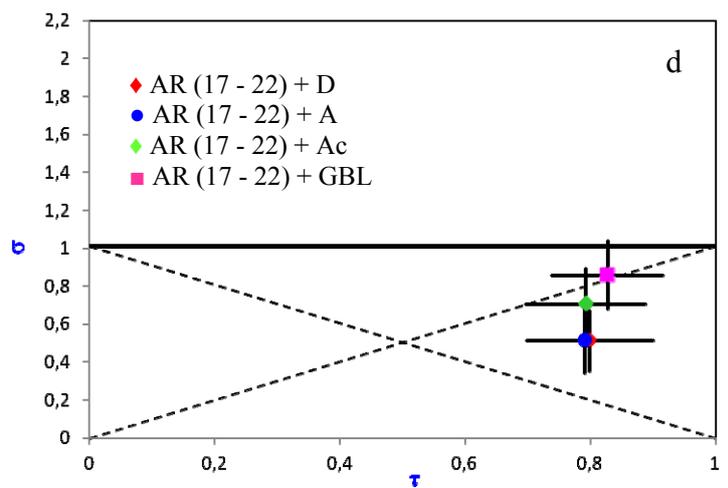
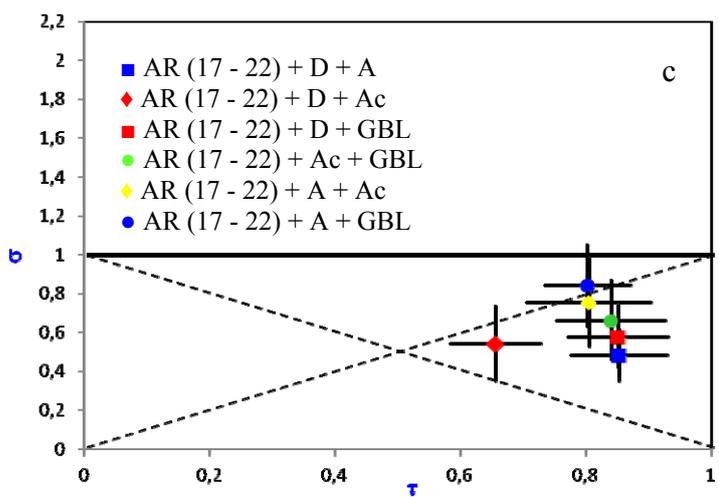
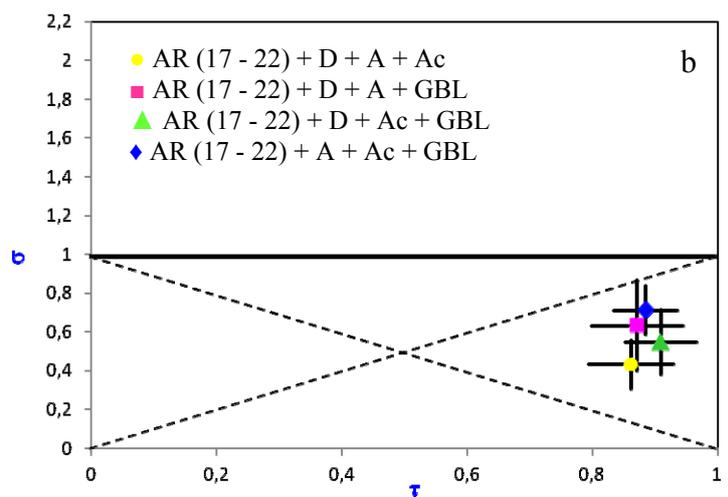
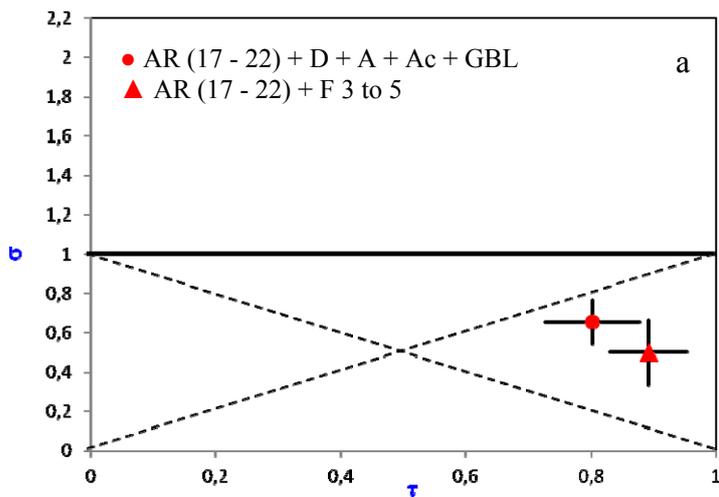
**Table 4:** Mean intensities of aromatic descriptors of AR made from HPLC fractions of wine (2) in dilute alcohol solution

Descriptors	Samples*		
	AR (18- 22)	AR (17- 22)	AR (18 to 22) + R- and S- 550µg/L ethyl 2-hydroxy-4-methylpentanoate (R/S:95/5, m/m)
Red-berry fruit	3.59 <b>a</b>	3.00 <b>a</b>	3.06 <b>a</b>
Black-berry fruit	1.76 <b>a</b>	2.76 <b>b</b>	2.94 <b>b</b>
Fresh fruit	2.64 <b>a</b>	3.64 <b>b</b>	3.82 <b>b</b>
Jammy fruit	3.23 <b>b</b>	2.35 <b>a</b>	2.41 <b>a</b>

\*p<0.05; values with different letters within each row are significantly different; AR, aromatic reconstitution; supplemented with ethyl 2-hydroxy-4-methylpentanoate at concentrations found in fraction 17 of wine (2).



**Figure 1:** Mean intensities of aromatic descriptors of AR prepared from HPLC fractions of wine (1) in dilute alcohol solution. \* $p < 0.05$ ; AR, aromatic reconstitution; RF, red-berry fruit; BF, black-berry fruit; FF, fresh fruit; JF, jammy fruit; Error bars indicate standard error deviation.



**Figure 2:** Impact of diacetyl (D), acetoin (A), acetic acid (Ac), and  $\gamma$ -butyrolactone (GBL) addition, individually or mixed, on fresh-fruit aroma. Symbols represent  $\sigma$  and  $\tau$  mean values; Error bars indicate the 95% confidence interval on the mean for both  $\tau$  and  $\sigma$ ; F, fractions; AR (17-22), aromatic reconstitution (AR) made from HPLC fruity fractions (17–22).