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Distribution and Organoleptic Impact of Ethyl 2-Hydroxy-4-methylpentanoate Enantiomers in Wine

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ABSTRACT: The enantiomers of ethyl 2-hydroxy-4-methylpentanoate (ethyl DL-leucate) were assayed in several wines using chiral gas chromatography (γ-cyclodextrin). Analyses of 55 commercial wines from various vintages and origins revealed different distributions. Generally, white wines presented only the *R* form, whereas red wines contained both enantiomers, in various ratios according to aging. The highest levels of the *S* form were found in the oldest samples. The *R*/*S* average enantiomeric ratio of this compound in red wine was approximately 95:5 with an average total concentration of ∼400 μg/L. The olfactory threshold of *R*-ethyl 2-hydroxy-4-methylpentanoate (126 μg/L) in hydroalcoholic solution was almost twice that of the *S* form (55 μg/L). The olfactory threshold of a mixture of *R*- and *S*-ethyl 2-hydroxy-4-ethylpentanoate (95:5, m/m) in hydroalcoholic solution was 51 μg/L, suggesting that both enantiomeric forms contribute to perception of this compound in wine, resulting in a synergistic effect. Both enantiomers have quite similar aromatic nuances. Sensory analysis was employed to demonstrate a synergistic effect of this ethyl ester on the perception of fruity aromas in wine: in hydroalcoholic solution supplemented with *R*- or *S*-ethyl 2-hydroxy-4-methylpentanoate or a mixture of the *R* and *S* forms (95:5, m/m) at their average concentrations in red wines, fruity character was perceived at concentrations 2.2, 4.5, and 2.5 times lower, respectively, than in hydroalcoholic solution alone. Sensory profiles of aromatic reconstitutions, using HPLC fruity fractions, highlighted the contribution of this compound to blackberry fruit and fresh fruit descriptors.

KEYWORDS: *ethyl 2-hydroxy-4-methylpentanoate, enantiomers, chiral GC, red wine, fruity aroma, aroma enhancer, perceptive interactions, synergistic effect*

INTRODUCTION

Ethyl 2-hydroxy-4-methylpentanoate (**1**) (ethyl DL-leucate) is a compound used in flavor chemistry. Luccarelli et al.[1] described its blueberry and valerian oil aroma and indicated that this compound was, alone, useful at levels of about 1−100 ppm to elaborate blueberry, tropical fruit, cashew, lime, and valerian oil flavors. In addition, these authors demonstrated that **1**, mixed with C4−C10 alkanoic acids, enhanced natural, ripe, tropical-fruit flavors in food.

1 has been identified in fresh fruits, such as cashew apple.[2,3] In distillates, it was initially identified in grape brandies by Schreier et al. in 1979[4] and, more recently, in freshly distilled Calvados and Cognac by Ledauphin et al.[5]

1 was first characterized in dry white wines by Câmpeanu et al.;[6] concentrations in wines made from the indigenous cultivar Feteasca Regala varied from 30 to 90 μg/L. Assays of this compound in Chardonnay wines from Changli County in China revealed concentrations of around 40 μg/L.[7] This ethyl ester was also found by Campo et al.[8] in aged Madeira wines and some types of sherry. More recently, **1** was also identified in dry red wines, at an average concentration of about 400 μg/L, by Falcão et al.,[9] who were the first to assess its organoleptic impact, suggesting that this compound contributed to fresh blackberry aromas but had a limited direct impact on overall red wine flavor.

Although **1** clearly has one asymmetrical carbon atom in position 2, indicating the possibility of two different enantiomers, previous works did not investigate this further. As the olfactory threshold and descriptors of an odoriferous compound may differ according to the stereoisomer considered,[10−14] it was important to separate the two enantiomers of **1** to obtain an accurate assessment of its organoleptic impact.

This paper reports the separation, distribution, and levels of enantiomers of **1** in wines from various vintages and origins and evaluates the organoleptic impact of this compound in red wines. For this last point, olfactory thresholds were determined and perceptive interactions were studied.

MATERIALS AND METHODS

Reagents. Dichloromethane (Pestipur quality, Carlo Erba, SDS, Italy) and absolute ethanol (analytical grade, 99.97%, Scharlau Chemie S.A, Barcelona, Spain) were distilled before use. Sodium sulfate (99%) was provided by Scharlau Chemie S.A. Microfiltered water was obtained using a Milli-Q Plus water system (resistivity = 18.2 MΩ cm, Millipore, Saint-Quentin-en-Yvelines, France). LiChrolut EN resin was obtained from Merck (Darmstadt, Germany). Standard compounds were obtained from commercial sources as follows: ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl pentanoate, ethyl 3-methylbutanoate, ethyl hexanoate, methyl octanoate, ethyl octanoate, ethyl 2-phenylacetate, propyl acetate, 2-methylpropyl acetate, butyl acetate, hexyl acetate, 2-phenylethyl acetate from Sigma-Aldrich, Saint-Quentin-Fallavier, France; methyl

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hexanoate, ethyl (*E*)-hex-2-enoate, from Alfa Aesar A Johnson Mattey Company, Bischheim, France; 3-methylbutyl acetate from VWR-Prolabo, Fontenay-sous-bois, France. Ethyl 2-hydroxy-4-methylpentanoate (ethyl DL-leucate) (>98%) was purchased from TCI Europe (Zwijndrecht, Belgium) and *R*-ethyl 2-hydroxy-4-methylpentanoate (>98.7%) and *S*-ethyl 2-hydroxy-4-methylpentanoate (>98.7%) were synthesized by Hangzhou Imaginechem Co., Ltd (Hangzhou, China). (Figure 1).

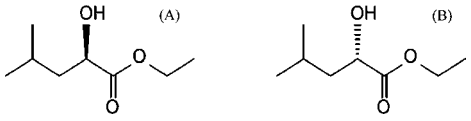

Figure 1. (A) *R*-(D)-Ethyl 2-hydroxy-4-methylpentanoate (CAS Registry No. 60856-83-9). (B) *S*-(L)-Ethyl 2-hydroxy-4-methylpentanoate (CAS Registry No. 60856-85-1).

Samples. **1** was assayed in wines from several vintages and origins: 42 red wines (vintages 1981−2010) and 13 white wines (vintages 1989−2008). Wine samples from the 2010 vintage were collected and analyzed 3 months after alcoholic fermentation. Two red wines were used for sensory analyses: a Vin de Pays d'Oc (2010 vintage) and a Margaux (2000 vintage). Hydroalcoholic solution was prepared with double-distilled ethanol and microfiltered water (12%, v/v). Dearomatized red wine was prepared according to the method of Pineau et al.,[15] by evaporating red wine (Bordeaux region) to obtain two-thirds of its original volume using a Rotavapor (Laborota 4010 digital Rotary Evaporator, Heidolph, Germany) with a 20 °C bath temperature. The liquid was then mixed with double-distilled ethanol and microfiltered water to reproduce the alcohol concentration and volume of the original wine. Dearomatization was then optimized using a method developed by Campo et al.[16] Dearomatized red wine (1000 mL) was supplemented with 5 g LiChrolut EN resin (40−120 μm) and it was stirred for 12 h. The solution was filtered and the protocol was repeated to eliminate all **1** traces. The resulting dearomatized wine had a very low-intensity neutral aroma.

Aromatic Reconstitution. A 500 mL wine sample was extracted successively using 80, 80, and 50 mL of dichloromethane, with magnetic stirring (700 rpm), for 15 min each, and separated in 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 of wine extract. Reversed-phase (RP) HPLC was performed on this raw extract using a Nova-PakHRC18 column (300 × 3.9 mm i.d., 4 μm, 60 Å, Waters, Saint-Quentin, France), without a guard cartridge. The HPLC system consists of an L-6200A pump (Merck-Hitachi, Germany). Chromatographic conditions were those optimized by Pineau et al.,[15] as follows: flow rate, 0.5 mL/min; injection volume, 250 μL of wine extract; program gradient, phase A, water, phase B, ethanol; 0−2 min, 100% A, linearly programmed until 100% B at minute 50. The effluent was collected in 1 mL fractions. Twenty-five fractions with various aromas were obtained. The 25 fractions in dilute alcohol solution were then directly evaluated by three trained assessors. 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).

Ethyl 2-Hydroxy-4-methylpentanoate Enantiomer Quantification in Wine Samples. A 50 mL wine sample was spiked with 100 μg/L octan-3-ol as an internal standard. It was then extracted using 4, 2, and 2 mL of dichloromethane, with magnetic stirring (700 rpm), for 5 min each and separated in a separatory funnel for 5 min. The organic phases were blended, dried over sodium sulfate, and concentrated under nitrogen flow (100 mL/min) to obtain 250 μL of wine extract. **1** was assayed using an HP 6890 gas chromatograph (Hewlett-Packard, Wilmington, DE) coupled to a mass spectrometer (MSD 5973i, Agilent Technologies Inc., Santa Clara, CA). The system was equipped with an ODO-I olfactometry port from SGE (Ringwood, Australia), for simultaneous sniffing and MS scanning (division 1:1). Two microliters of organic extract was injected in splitless−split mode (injector temperature, 180 °C; interface temperature, 200 °C; splitless time, 0.75 min; split flow, 48.3 mL/min). The column was a Chiraldex Gamma-TA (Astec, Whippany, NJ), 50 m × 0.25 mm i.d., film thickness = 0.12 μm. The oven was programmed at 40 °C for the first minute, raised to 100 °C at 4 °C/min, then programmed at a rate of 1 °C/min to 150 °C, and finally raised at 4 °C/min to a final isotherm at 170 °C, maintained for 5 min. The carrier gas was helium Alphagaz 2 (Air Liquide, France) with a column head pressure of 50 psi. The mass spectrometer was operated in electron impact mode at 70 eV with selected ion monitoring (SIM) mode, selecting the following ions: *m*/*z* 56, 59, 69, 76, 83, 87, 104, and 177. After enantiomeric synthesis by an external collaborator, the *R*-**1** and *S*-**1** were injected separately to identify its LRI, and the peaks of the reference products were compared with those naturally present in wine. In addition, GC-O analyses were carried out for both *R*-**1** and *S*-**1** to determine the possibility of the presence of any odiferous impurities in the reference compounds and to ascertain that odor properties come from the compound considered. Any olfactive impurity was detected by the three judges who performed this analysis.

Ester and Acetate Analyses in HPLC Fractions. Chromatographic conditions and sample preparation were as optimized by Antalick et al.[17] The fiber (Supelco, Bellefonte, PA) was coated with 100 μm stationary phase polydimethylsiloxane film (PDMS-100). A 10 mL sample was placed in a 20 mL headspace vial, 3.5 g of sodium chloride was added, and the vial was tightly sealed with a PTFE-lined cap. The solution was homogenized in a vortex shaker and then loaded onto a Gerstel (Mülheim an der Ruhr, Germany) autosampling device. The program consisted of swirling the vial at 500 rpm at 40 °C for 2 min, then inserting the fiber into the headspace at 40 °C for 30 min as the solution was swirled again, then transferring the fiber to the injector for desorption at 250 °C for 15 min. Gas chromatography analyses were carried out on an HP 5890 GC system coupled to an HP 5972 quadrupole mass spectrometer (Hewlett-Packard), equipped with a Gerstel MPS2 autosampler. Injections were in splitless mode for 0.75 min, using a 2 mm i.d. nondeactivated direct linear (temperature of the injector, 250 °C; temperature of the interface, 280 °C) and a BP21 capillary column (50 m × 0.32 mm, film thickness = 0.25 μm, SGE). The oven temperature was programmed at 40 °C for 5 min, then raised to 220 °C at 3 °C/min, and held at that temperature for 30 min. The carrier gas was helium Alphagaz 2 (Air Liquide) with a column-head pressure of 8 psi. The mass spectrometer was operated in electron ionization mode at 70 eV with full scan mode (*m*/*z* 40−300 mass range). Esters were characterized by comparing their linear retention indices and mass spectra with those of standards. Linear retention indices were determined from the retention time of linear alkanes. They were obtained by injecting a solution of alkanes with carbon chains of 7−23 atoms in the same conditions as the extract for analysis.

Sensory Analyses. *General Conditions.* Sensory analyses were performed as described by Martin and de Revel.[18] Samples were evaluated at controlled room temperature (20 °C), in individual booths, using covered, black AFNOR (Association Française des Normes) glasses,[19] containing about 50 mL of liquid, coded with three-digit random numbers. Sessions lasted approximately 10 min.

Sensory Panels. Panel 1 consisted of 15 judges, 7 males and 8 females of 30.5 ± 7.2 (mean ± SD) years of age. Panel 2 consisted of 19 judges, 8 males and 11 females of 30.7 ± 5.1 (mean ± SD) years of age. All panelists belong to the research laboratory staff at ISVV, Bordeaux University. The judges were selected for their experience in assessing fruity aromas in red wines. They attended three sessions of 5 min per week, for 4 weeks. Fresh berry-fruit standards were presented (blueberry, blackberry, blackcurrant, strawberry, cherry, and raspberry). Commercial jams, made from the same fruits, were presented directly as "jammy fruit" standards.

Olfactory Thresholds. *Experiment 1: Olfactory Thresholds of R- 1, S- 1, and a Mixture of R- and S- 1 (95:5, m/m) in Two Different Matrices (Hydroalcoholic Solution/Dearomatized Red*

Wine). Olfactory thresholds were determined by panel 1 in two sessions, using two different matrices, in a three-alternative, forced-choice presentation (3-AFC).[20] The first session consisted of 10 forced-choice tests in hydroalcoholic solution. Each test contained one positive sample supplemented with increasing concentrations of *R*-**1** (25, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12800 μg/L). The second session consisted of the same 10 tests performed using dearomatized red wine. The order of presentation of the positive sample was identical in both matrices, to obtain comparable results and avoid order effects. The olfactory thresholds of *S*-**1** (2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 μg/L) and a mixture of *R*- and *S*-**1** (95:5, m/m) (total concentrations of 26, 53, 105, 210, 421, 842, 1684, 3367, 6735, 13469 μg/L) were also determined in both matrices under the same conditions.

Experiment 2: Olfactory Thresholds of Fruity HPLC Fractions (18−22) in Four Different Matrices. Olfactory thresholds were determined by panel 2 in two sessions, using four different matrices, in a 3-AFC.[20] The first session consisted of 10 forced-choice tests in hydroalcoholic solution. Each test contained one positive sample supplemented with increasing volumes of fruity HPLC fractions (18−22), corresponding to an initial wine volume of 0.3, 0.6, 1.3, 2.5, 10, 20, 40, 80, or 160 mL, diluted in 50 mL of hydroalcoholic solution. The second session consisted of the same 10 tests, using hydroalcoholic solution supplemented with 400 μg/L of *R*-**1**. For each compound, the order of presentation of the positive sample was identical in both matrices, to obtain comparable results and avoid order effects. The "olfactory threshold" of these fruity HPLC fractions (18−22) was also determined in hydroalcoholic solution supplemented with 20 μg/L *S*-**1** and in hydroalcoholic solution supplemented with 420 μg/L of a mixture of *R*- and *S*-**1** (95:5, m/m), under the same conditions.

*Experiment 3: Olfactory Thresholds of R- **1**, S- **1**, and a Mixture of R- and S- **1** (95:5, m/m) in Two Different Matrices (Hydroalcoholic Solution/HPLC Fruity Fractions).* Olfactory thresholds were determined by panel 2 in two sessions, using two different matrices, in a 3-AFC.[20] The first session consisted of 10 forced-choice tests in hydroalcoholic solution, each containing one positive sample supplemented with increasing concentrations of *R*-**1** (25, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12800 μg/L). The second session consisted of the same 10 tests, using a reconstituted aromatic matrix containing HPLC fruity fraction 18−22 blended together in hydroalcoholic solution to reproduce the initial concentrations in the original wines. The order of presentation of the positive sample was identical in both matrices, to obtain comparable results and avoid order effects. The olfactory thresholds of *S*-**1** (2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 μg/L) and a mixture of *R*- and *S*-**1** (95:5, m/m) (total concentrations of 26, 53, 105, 210, 421, 842, 1684, 3367, 6735, 13469 μg/L), were also determined in both matrices under the same conditions.

Data Analysis. The results of all 3-AFC tests were statistically interpreted. The olfactory threshold corresponds to the minimum concentration below which 50% of the assessors statistically failed to detect the single sample. This statistical value was determined using an adaptation of the ASTM-E1432 method.[21] The concentration/response function is a psychometric function and fits a sigmoid curve ($y = 1/(1 + e^{(-\lambda x)})$). Detection probability was corrected using the chance factor ($P = (3*p - 1)/2$, where p = proportion of correct responses for each concentration and P = proportion corrected by the chance effect, 1/3 for 3-AFC). Sigma Plot 8 (SYSTAT) software was used for graphic resolution and ANOVA transform for nonlinear regression.[22]

The impact of the *R* and *S* enantiomeric mixture (95:5, m/m) in different matrices (hydroalcoholic solution and dearomatized red wine), determined by panel 1, was evaluated using an additive model,[23] as developed by Miyazawa et al.[24] Mixture interaction patterns were compared using a simple additive response model. Response addition equals the probability of detecting one or both of the components: $p(AB) = p(A) + p(B) - p(A)p(B)$, where $p(AB)$ represents the probability of detecting the mixture, $p(A)$ represents the probability of detecting component A, and $p(B)$ represents the probability of detecting component B. If detection performance for the mixture falls below the sum of probabilities, some degree of suppression has occurred relative to statistical independence. A performance above the sum of probabilities indicates that some form of mutual enhancement or synergy has occurred. Moreover, if detection performance matches the response addition, no mixture interaction has occurred. The psychometric functions were compared.

Olfactory Descriptors of Each Enantiomer. Descriptive analyses of the **1** enantiomers were carried out by panel 1 in hydroalcoholic solution, using the following concentrations: *R*-**1**, 800 μg/L; and **S-1**, 150 μg/L. Judges were asked to choose a maximum of three descriptors from the following list: rose, currant, banana, blueberry, caramel, violet, dried rose, black currant bud, green pepper, vanilla, strawberry, black currant, black olive, licorice−anise, mushroom, cherry, clove, prune, butter, strawberry jam, blackberry jam, tobacco, blackberry, apple, raspberry, and fresh oak wood. The last 10 descriptors on the list were also presented as physical samples.

Sensory Profiles. Red-berry, black-berry, fresh, and jammy fruit aroma intensities were evaluated by panel 1 using a 0−7 point structured scale, where 0 = no odor is perceived and 7 = high intensity is perceived. Two samples of aromatic reconstitutions in hydroalcoholic solution were presented. The first consisted of HPLC fruity fractions (18−22) and the second contained the same HPLC fruity fractions supplemented with 550 μg/L of a mixture of *R*- and *S*-**1** (95:5, m/m). Statistical data were analyzed using Statistica V.7 analysis of variance (ANOVA) software (Statsoft Inc., Tulsa, OK). Duncan's tests were used for comparison when ANOVA ($p < 0.05$) on sensory analysis results revealed significant differences.

RESULTS AND DISCUSSION

Ethyl 2-Hydroxy-4-methylpentanoate Enantiomers Distribution and Concentrations. The optical isomers of **1** were separated by chiral GC analysis on a γ-cyclodextrin phase. Commercial ethyl DL-leucate **1** was a 50:50 racemic mixture. The linear retention indices of *R*-**1** and *S*-**1** were 1278 and 1284, respectively, on a chiral γ-cyclodextrin column. As expected, in a solution such as wine, no enantiomeric interconversion was observed, considering the chemical structure as well as the potential reactivity of **1**.

As observed in previous studies,[9] in dry wines of the same age, **1** levels are generally higher in red than in white wines (maximum concentration in red wines = 660 μg/L, Margaux, 2005). Generally, white wines contained only the *R* form, whereas aged red wines presented both enantiomeric forms in various ratios, according to age. Young red wines of 2010 year contained only the *R* form. Table 1 shows the impact of aging on the *R*/*S* ratio in red wine with an *R*/*S* average enantiomeric ratio of approximately 95:5. The maximum enantiomeric *R* form was found in all 2010 vintage red wines with an *R*/*S* enantiomeric ratio of 100:0. The maximum enantiomeric *S* form was found in a Margaux wine (1990) with an *R*/*S* enantiomeric ratio of 85:15. In red wines, the highest *S*-**1** levels were found in the oldest samples (Table 1) (maximum concentration in red wines = 62 μg/L, Haut-Médoc, 1982). However, one white wine had particularly high **1** levels compared to red wines (Pessac-Leognan, 1989, 827 μg/L) and another one contained the *S* form with an enantiomeric ratio *R*/*S* of 97:3 (Bordeaux, 1994).

Generally, ethyl esters and acetates are produced by the yeast metabolism during alcoholic fermentation,[25] a phase when red- and black-berry fruit aromas are produced.[26] However, the origins of **1** are clearly far more complex than expected, with a different pathway for each enantiomer, and their elucidation will require specific investigation.

Table 1. Concentrations of Ethyl 2-Hydroxy-4-methylpentanoate (1) Enantiomers

vintage	no. of wines analyzed	av concn (μg/L)[a]			av ratio of R/S[a]
		RS-**1**	*R*-**1**	*S*-**1**	
		Red Wines			
1980–1990	10	408 ± 118	371 ± 113	32 ± 16	91:9 ± 5
1991–2000	10	449 ± 125	431 ± 122	18 ± 13	96:4 ± 3
2001–2009	14	361 ± 119	354 ± 114	7 ± 6	98:2 ± 1
2010	8	135 ± 47	135 ± 47	0 ± 0	100:0 ± 0
		White Wines			
1980–2000	8	342 ± 236	341 ± 237	2 ± 2	99:1 ± 1
2001–2010	5	182 ± 121	182 ± 121	0 ± 0	100:0 ± 0

[a] ± standard deviation over the average concentration.

Direct Organoleptic Impact of Ethyl 2-Hydroxy-4-methylpentanoate on Quantitative Odor Perception. The olfactory threshold of *R*-**1** in hydroalcoholic solution, determined by panel 1 (Eexperiment 1) was 126 μg/L, almost twice that of the *S* form (55 μg/L). These results clearly demonstrated that the thresholds were strongly dependent on the stereochemistry of the odorant. The olfactory threshold of a mixture of *R*- and *S*-**1** (95:5, m/m) was 51 μg/L, indicating that both enantiomeric forms contributed to its perception in wine and confirming the direct impact of this ester on aroma perception. Furthermore, the olfactory thresholds of *R*-, *S*-, and of a mixture of *R*- and *S*-**1** (95:5, m/m) in dearomatized red wine, determined by panel 1, were 431, 176, and 73 μg/L, respectively, revealing a clear matrix effect. These olfactory threshold results contradict the Olsson model,[27] which rarely corresponds to the sensory reality in wine. Generally, at a supraliminal concentration,fruity hplcand irrespective of perceptual interactions, the lower the perception threshold of a compound, the more aromatic impact it has. Olfactory threshold results obtained by panel 1 in both matrices suggest an hyperadditive effect in a binary mixture, where the mixture is perceived as more intensely aromatic than the sum of the two compounds.[28] An additive model was used to confirm the synergistic impact of the *R*- and *S*-**1** enantiomeric mixture (95:5, m/m) in different matrices (hydroalcoholic solution and dearomatized red wine). This method was applied to a particular binary model mixture, consisting of two enantiomers. After the detection curve for the *S*-enantiomeric form had been modeled, the response probabilities $p(L)$ for the range of concentrations of the mixture used were calculated in two different matrices. For both matrices, hydroalcoholic solution and dearomatized red wine, the measured probability of detecting the mixture was higher than the calculated probability (Figure 2), revealing a perceptual synergistic effect between the two enantiomeric forms in this binary mixture.

Direct Organoleptic Impact of Ethyl 2-Hydroxy-4-methylpentanoate on Qualitative Odor Perception. Despite the quantitative difference between the perception thresholds of the two enantiomers, their aromatic nuances were quite similar. At concentrations perceived by the whole panel, *R*- and *S*-**1** were mainly defined by descriptors such as blackberry fruit (14/15 for *R*- **1** and 12/15 for *S*-**1**). More specifically, fresh blackberry and blackberry jam obtained the highest ratings (8/15 for both *R*-**1** and *S*-**1**).

Distribution of Aromatic Compounds during HPLC Factionation. Applying HPLC to a wine extract resulted in 25 fractions. By sensory analysis, fractions 17–22 were selected for their intense fruitiness by Pineau et al.[15] Table 2 shows the esters and acetates with fruity notes in fractions 17–22, determined using headspace solid-phase microextraction. **1** was the only ester eluted in fraction 17. Thus, it was easy to obtain a pool of fruity wine esters without **1**, in order to investigate its indirect impact, by partial aromatic reconstitution of HPLC fruity fractions 18–22.

Indirect Organoleptic Impact of Ethyl 2-Hydroxy-4-methylpentanoate on Quantitative Odor Perception. *Experiment 2.* The "olfactory threshold of fruity HPLC fractions (18–22)" was calculated in four different matrices. The value was 1.8 ml in hydroalcoholic solution and 0.8 ml, 0.4 ml, and 0.7 ml (diluted in 50 ml of different matrices) in hydroalcoholic solution supplemented with 400 μg/L *R*-**1**, 20 μg/L of *S*-**1**, and 420 μg/L of a mixture of *R*- and *S*-**1** (95:5, m/m), respectively. As shown in Figure 3, comparing the olfactory threshold in hydroalcoholic solution matrix supplemented with *R*-**1**, *S*-**1**, and a mixture of *R*- and *S*-**1** revealed that the "olfactory threshold of fruity HPLC fractions (18–22)" was

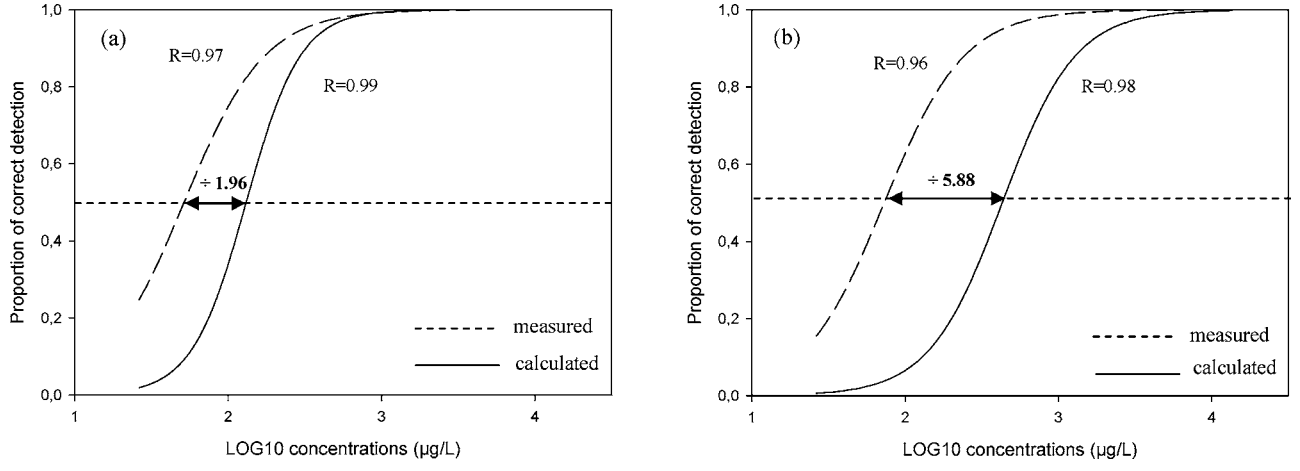

Figure 2. Comparison between probability of measured detection and calculated detection of the *R*- and *S*-enantiomeric mixture (95:5, m/m) in (a) hydroalcoholic solution and (b) dearomatized red wine.

Table 2. Distribution of Esters and Acetates with Fruity Notes in HPLC Fractions (F)[a]

compound	F16	F17	F18	F19	F20	F21	F22
Esters							
ethyl propanoate	–	–	x	–	–	–	–
ethyl 2-methylpropanoate	–	–	x	x	–	–	–
ethyl butanoate	–	–	x	–	–	–	–
ethyl 2-methylbutanoate	–	–	–	x	x	–	–
ethyl pentanoate	–	–	–	–	x	–	–
ethyl 3-methylbutanoate	–	–	–	x	x	x	–
methyl hexanoate	–	–	–	–	x	–	–
ethyl hexanoate	–	–	x	x	x	x	x
ethyl (*E*)-hex-2-enoate	–	–	–	–	–	x	–
ethyl 2-hydroxy-4-methylpentanoate	x	x	–	–	–	–	–
methyl octanoate	–	–	–	–	–	–	x
ethyl octanoate	–	–	–	–	–	–	x
ethyl 2-phenylacetate	–	–	–	x	x	–	–
Acetates							
propyl acetate	–	–	x	–	–	–	–
2-methylpropyl acetate	–	–	x	–	–	–	–
butyl acetate	–	–	–	x	x	–	–
3-methylbutyl acetate	–	–	–	x	x	x	–
hexyl acetate	–	–	–	–	–	–	x
2-phenylethyl acetate	–	–	–	x	x	–	–

[a] "x" indicates the presence of listed compounds eluted in the various fractions; "–" incidates the absence of listed compounds.

(a) 2.2, (b) 4.5, and (c) 2.5 times higher than that of hydroalcoholic solution supplemented with (a) 400 μg/L of *R*-**1**, (b) 20 μg/L of *S*-**1**, and (c) 420 μg/L of a mixture of *R*- and *S*-**1** (95:5, m/m), respectively. These results, obtained using the average *R*-**1**, *S*-**1**, and mixed *R*- and *S*-**1** concentrations found in red wines, demonstrated that **1** had a synergistic effect on the perception of fruity aromas in wine. These results are in agreement with those recently presented by Falcão et al.,[9] who found that the omission of **1** was clearly perceived and that simultaneous omission of **1** and ethyl butanoate was perceived even more clearly, suggesting perceptive interactions between **1** and another ethyl ester.

In the literature, the behavior of other compounds has been also studied by comparing two detection thresholds. Romano et al.[29] demonstrated that the addition of isobutyric and isovaleric acids to wine resulted in a remarkable increase in the olfactory threshold for ethylphenols.

Even if "synergy" was strictly defined by Berglund et al.[30] as describing quantitative effects in heterogeneous binary mixtures and "hyperaddition", according to Cain and Drexler,[28] for quantitative effects in homogeneous binary mixtures, both terms are actually used in the literature for odor intensification, even in more complex mixtures.

Recent evidence established the additive effect of low-impact odorants on fruity wine aroma. Pineau et al.[15] were the first to demonstrate that very small variations in certain ethyl esters (as little as 1.3% of the olfactory threshold of ethyl 2-methylpropanoate, for example) were perceived in complex mixtures in dearomatized red wine.

Experiment 3. The olfactory threshold of *R*-**1**, *S*-**1**, and the mixture of *R*- and *S*-**1** (95:5, m/m) was calculated in two different matrices. In hydroalcoholic solution, the olfactory thresholds of *R*-**1**, *S*-**1**, and the mixture of *R*- and *S*-**1** (95:5, m/m) were 167, 70, and 90 μg/L, respectively. In an aromatic reconstitution of HPLC fruity fractions, the olfactory thresholds of *R*- **1**, *S*- **1**, and the mixture of *R*- and *S*- **1** (95:5, m/m) were 1576, 688, and 460 μg/L respectively. As shown in Figure 4, a comparison of the olfactory thresholds of each compound in both matrices revealed that the olfactory thresholds of (a′) *R*- **1**, (b′) *S*- **1**, and (c′) the mixture of *R*- and S- **1** (95:5, m/m) were (a′) 9.4, (b′) 9.7, and (c′) 5.1 times higher in an aromatic reconstitution of HPLC fruity fractions than in hydroalcoholic solution. These results revealed that a more complex matrix had a masking effect on **1** perception.

Results concerning the masking effects of matrix complexity on **1** perception are in agreement with those reported by Pineau et al.,[31] highlighting the very low β-damascenone olfactory threshold in dilute alcohol solution as compared to red wine. Earlier research revealed that olfactory thresholds in complex matrices were higher than those determined in simpler solutions.[32−35]

A decrease in intensity is the most frequent effect of odor mixtures.[36−38] Some bibliographical data strictly differentiate "hypoaddition" for quantitative effects on odor intensity in homogeneous binary mixtures,[28] "antagonism" for quantitative effects on odor intensity in heterogeneous binary mixtures, and "masking" for qualitative effects (concerning odor quality). Nevertheless, actually, these three terms are generally used to describe attenuated intensity in odor mixtures.

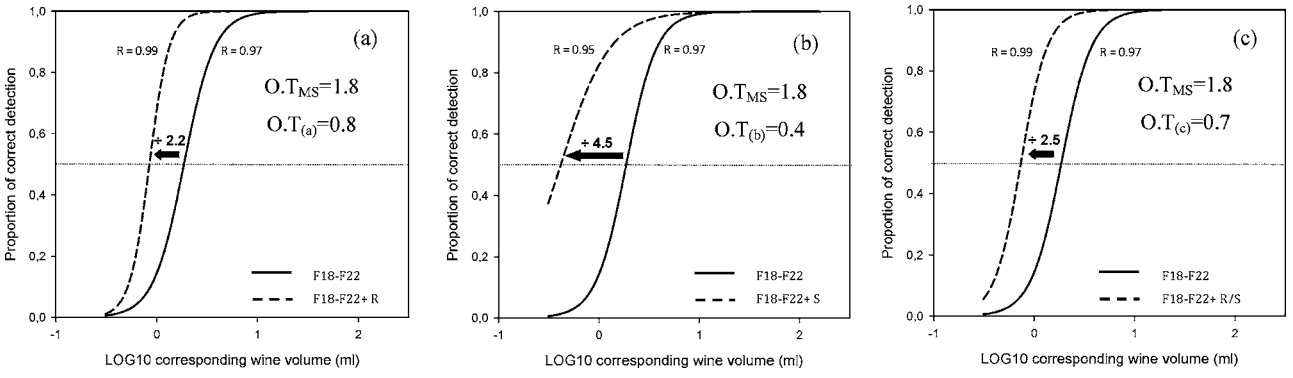

Figure 3. Comparison of the perception threshold of fruity HPLC fractions (18−22) in hydroalcoholic solution (MS = wine model solution) with the value in hydroalcoholic solution supplemented with (a) 400 μg/L *R*-ethyl 2-hydroxy-4-methylpentanoate, (b) 20 μg/L *S*-ethyl 2-hydroxy-4-methylpentanoate, and (c) 420 μg/L of a mixture of *R*- and *S*-ethyl 2-hydroxy-4-methylpentanoate (95:5, m/m). O.T = olfactory thresholds of fruity HPLC fractions (18−22) expressed in wine volume (mL) diluted in 50 mL of different matrices.

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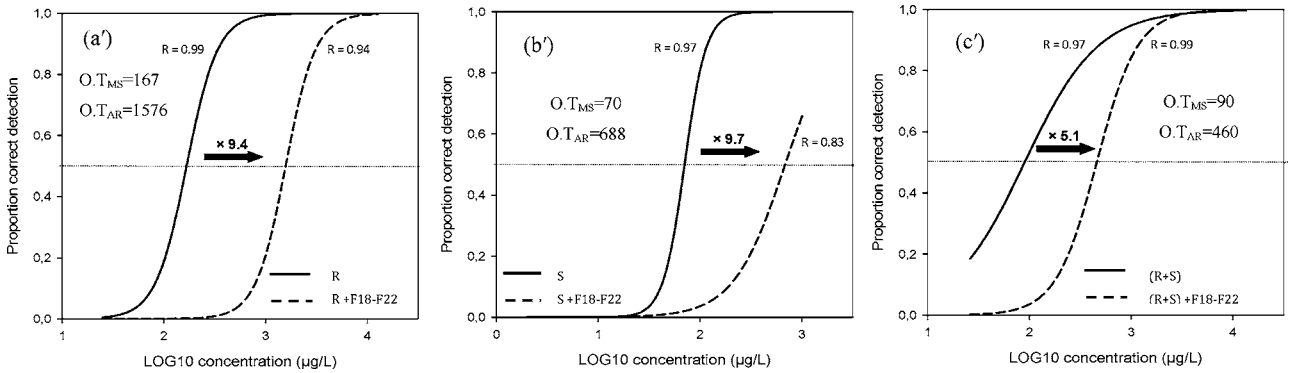

Figure 4. Comparison between perception thresholds of (a′) *R*-ethyl 2-hydroxy-4-methylpentanoate, (b′) *S*-ethyl 2-hydroxy-4-methylpentanoate, and (c′) a mixture of *R*- and *S*-ethyl 2-hydroxy-4-methylpentanoate (95:5, m/m) in hydroalcoholic solution (MS = wine model solution) and an aromatic reconstitution (AR) made from HPLC fruity fractions (18−22). O.T = olfactory thresholds (μg/L) of ethyl 2-hydroxy-4-methylpentanoate enantiomers in different matrices.

Organoleptic Impact of Ethyl 2-Hydroxy-4-methylpentanoate on Qualitative Odor Perception. Significant differences were found between the two samples for the red-berry, black-berry, fresh, and jammy fruit aroma descriptors, on a 0−7 point scale of intensity (Table 3).

Table 3. Mean Intensities of Four Aromatic Descriptors of Aromatic Reconstitutions (AR) in Hydroalcoholic Solution

	samples[a]	
descriptor	AR fractions 18−22	AR fractions 18−22 + 550 μg/L mixture of *R*- and *S*-ethyl 2-hydroxy-4-methylpentanoate (95:5, m/m)
red-berry fruit	3.59 a	3.06 a
black-berry fruit	1.76 a	2.94 b
fresh fruit	2.64 a	3.82 b
jammy fruit	3.23 b	2.41 a

[a]ANOVA fraction effect on aromatic intensity; values with different letters within each row are significantly different (Duncan'stest, $p \leq 0.05$).

The panel effect was not significant ($p > 0.05$), confirming that the judges' evaluation of fruity nuances was homogeneous. The average scores for red-berry fruit intensity were identical after addition of the ester, whereas jammy fruit intensity was significantly lower. The average scores for black-berry and fresh fruit aromas were significantly higher for the aromatic reconstitution of HPLC fruity fractions (18−22) supplemented with **1**. These results confirmed the sensory importance of ethyl 2-hydroxy-4-methylpentanoate, suggesting that it is an active contributor to the black-berry and fresh fruit nuances in the wine studied.

Taken together, these data revealed that ethyl 2-hydroxy-4-methylpentanoate was generally present at levels slightly above its perception threshold in model solution or dearomatized red wine. In a more complex matrix, from an olfactive and chemical point of view, such as an aromatic reconstitution from fruity fractions, the perception threshold was higher but still quite close to the ethyl 2-hydroxy-4-methylpentanoate content of wine. These facts clearly indicate that ethyl 2-hydroxy-4-methylpentanoate does not play a direct role as a key compound in red wine aroma. In contrast, our findings highlighted the indirect contribution of ethyl 2-hydroxy-4-methylpentanoate to wine aroma, showing that this ester contributed to a synergistic effect, enhancing the perception of fruity character. Finally, it was clearly demonstrated that this compound acts as a natural enhancer for black-berry and fresh fruit notes in red wine.

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