Ethyl 2-hydroxy-3-methylbutanoate enantiomers: quantitation and sensory evaluation in wine

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Abstract

Aim: To investigate chemical and sensory characteristics of ethyl 2-hydroxy-3-methylbutanoate in wines.

Methods and results: Ethyl 2-hydroxy-3-methylbutanoate has been recently identified as a potential marker of lactic acid bacteria esterase activity. Enantiomers of this fruity ester were quantitated in 99 wines from various vintages and French regions using chiral gas chromatography (γ-cyclodextrin phase). Analyses revealed the predominant presence of the R enantiomer in red and white wines, with a maximum R/S enantiomeric ratio of 94/6 in a 1993 red wine. Results also highlighted greater levels of the ester in red than white wines, depending on grape origin. The detection thresholds of the R- and S-form were estimated at 4 µg/L and 1.5 µg/L in water and 51 mg/L and 21 mg/L in red wine, respectively. Moreover, ranking tests made with levels found in wines did not show significant sensory differences.

Conclusion: The concentrations found in wines were considerably below the detection threshold, indicating no direct effect of these compounds on fruity aroma modulation. The absence of significant difference in sensory tests demonstrates that ethyl 2-hydroxy-3-methylbutanoate does not contribute significantly to the fruity aroma of red wine.

Significance and impact of the study: To our knowledge, no previous research had determined the enantiomeric distribution and the sensory characteristics of this compound in wine.

Keywords: ethyl 2-hydroxy-3-methylbutanoate, enantiomers, chiral GC, red wine, fruity aroma

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Introduction

Ethyl 2-hydroxy-3-methylbutanoate (E2H3MB) occurs widely in the aroma volatiles of fruits, beverages, and other foodstuffs. It has been identified in olive oil (De Angelis *et al.*, 2015) as well as in fruits such as pineapple (Elss *et al.*, 2005), blueberries (Hirvi and Honkanen, 1983), banana (Pino and Febles, 2013), and some Asian fruits (Wong and Siew, 1994).

E2H3MB has also been quantified in alcoholic beverages such as apple cider (Xu *et al.*, 2007), mango (Pino and Queris, 2011a) and guava (Pino and Queris, 2011b) wines, and several spirits (Cognac, Armagnac, Calvados, and apple brandies) (Schreier *et al.*, 1978; Ledauphin *et al.*, 2003; Fan and Qian, 2005). Its presence in several types of wines has also been confirmed, including Chardonnay (Cejudo-Bastante *et al.*, 2013), Bobal (Atienza *et al.*, 1998), Tempranillo (Alañón *et al.*, 2013), Fiano (Genovese *et al.*, 2007), and Madeira (Campo *et al.*, 2006). The concentrations of E2H3MB found in these various beverages varied from several dozen to a few hundred micrograms per liter.

Various organic extracts were analyzed by gas chromatography coupled with olfactometry to characterize its odor. In fruit studies, E2H3MB was described as "ripe pineapple-like" (Umano *et al.*, 1992) or "blueberry" (Hirvi and Honkanen, 1983). In other studies dealing with alcoholic beverages, such as hard cider, Chinese spirits, or Madeira, this compound was mainly described as "fruity", "floral", "jasmine", or "strawberry" (Fan and Qian, 2005; Campo *et al.*, 2006; Xu *et al.*, 2007). However, no detection threshold could be found in the literature.

E2H3MB has one stereogenic center in position 2, indicating the potential existence of two enantiomers. Nevertheless, no study related to E2H3MB enantiomers could be found, neither in fruit, nor alcoholic beverages nor other foodstuffs. Recently, Lytra *et al.* (2012) highlighted the major differences in the distribution and the organoleptic impact of ethyl 2-hydroxy-4-methylpentanoate (E2H4MP) enantiomers in red wine. To our knowledge, the enantiomeric distribution of E2H3MB, as well as its organoleptic impact, had never been investigated in wine. Moreover, the detection threshold and descriptors of an organic compound having a sensory impact can differ according to stereoisomer (Lytra *et al.*, 2012; Cretin *et al.*, 2015). Thus, it was important to separate the two enantiomers of E2H3MB to obtain a precise assessment of their organoleptic impact on fruity aromas in red wine.

The objective of this work was to separate and assay E2H3MB enantiomers, namely ethyl (2R)-2-hydroxy-3-methylbutanoate (R-E2H3MB) and ethyl (2S)-2-hydroxy-3-methylbutanoate (S-E2H3MB), in wines from different vintages and origins, and to evaluate their ability to modulate fruity aromas in red wines.

Materials and methods

1. Chemicals

The compound used as internal standard (octan-3-ol, 99%) was obtained from Sigma-Aldrich (Steinheim, Germany), dichloromethane (> 99%) from VWR Chemicals (Fontenay-sous-Bois, France), anhydrous sodium sulfate (99%) from Scharlau Chemie (Sentmenat, Spain), and ethanol (≥ 99.9%) from Merck (Darmstadt, Germany). R-E2H3MB (> 98%), S-E2H3MB (> 98%) (Figure 1), and R-E2H4MP (>98.7%) were synthesized by Hangzhou Imaginechem Co. (Hangzhou, China). Microfiltered water was obtained using a Milli-Q Plus water system (resistivity = 18.2 MΩ cm, Millipore, Saint-Quentin-en-Yvelines, France).

2. Gas chromatography - Olfactometry (GC-O) analysis of reference compounds

A panel of six trained judges was asked to perform GC-O analyses on the reference compounds. The aim was, first, to ensure that the compounds did not contain any odoriferous impurities and, second, to generate descriptors to define the racemic mixture and the enantiomers.

Olfactometry analyses were carried out using an HP-4890 gas chromatograph (Hewlett-Packard,

(a)

(b)

Figure 1. (a) R-ethyl 2-hydroxy-3-methylbutanoate (CAS registry number: 129025-85-0); (b) S-ethyl 2-hydroxy-3-methylbutanoate (CAS registry number: 63674-18-0).

Wilmington, DE, USA) equipped with a flame ionization detector (FID) and a sniffing port (ODO-I SGE, Ringwood, Australia), and connected by a flow splitter to the column exit. GC effluent was combined with humidified N_2 (Air Liquide, France) at the bottom of the glass-sniffing nose (SGE, Ringwood, Australia) to avoid nasal dehydration. A 1 μL sample of each pure odorant and the racemic mixture (50:50) was injected in splitless□split mode (injector temperature, 250 °C; splitless time, 30 s; split flow, 50 mL/min). The column was a BP21 (50 m x 0.32 mm, 0.25 μm film thickness, SGE, Courtaboeuf, France). The oven was programmed at 35 °C for the first minute and then increased at a rate of 4 °C/min to a final isotherm at 200 °C for 10 min. The carrier gas was hydrogen 5.5 (Linde, France) with a column head pressure of 15 psi.

3. Quantitation and separation of ethyl 2-hydroxy-3-methylbutanoate

E2H3MB was assayed in French wines from several vintages and origins: 60 red wines (3 – 34 years old; Bordeaux: 28, Burgundy: 16, Rhône Valley: 4, Loire Valley: 2, Languedoc: 10) and 39 white wines (1 – 11 years old; Bordeaux: 13, Burgundy: 12, Loire Valley: 9, Alsace: 5).

A 100 mL wine sample was spiked with 20 μL internal standard solution (octan-3-ol at 1.04 g/L in ethanol). The mixture was successively extracted with 8 mL, and twice with 4 mL dichloromethane. The organic phases were blended, dried over sodium sulfate, and concentrated under nitrogen flow (100 mL/min) to obtain 250 μL of wine extract.

Total ester concentrations were quantified using an Agilent 7890A gas chromatograph coupled to a mass spectrometer (MSD 5975C, Agilent Technologies Inc., Santa Clara, CA). One microliter organic extract was injected in splitless mode (injector temperature, 250 °C; splitless time, 0.75 min). The column was a BP21 capillary type (50 m x 0.32 mm, 0.25 μm film thickness, SGE). The oven was programmed at 40 °C for the first minute, increased to 220 °C at a rate of 3 °C/min, and then maintained at that temperature for 20 min. The mass spectrometer was operated in electron impact mode at 70 eV with selected ion monitoring (SIM) mode, selecting the following ions: *m/z* 55, 69, 73, 76, 83, 87, 101, and 104.

Enantiomers of both esters were assayed as described by Lytra *et al.* (2012) using an Agilent 6890N gas chromatograph coupled to a mass spectrometer (MSD 5973i, Agilent Technologies Inc., Santa Clara, CA). One microliter organic extract was injected in split mode (injector temperature, 200 °C; split flow,

15 mL/min). The column was a Chiraldex Gamma-TA (50 m x 0.25 mm, 0.12 μm film thickness, Astec, Whippany, NJ). The oven was programmed at 40 °C for the first minute, increased to 100 °C at a rate of 1 °C/min, and finally increased at a rate of 3 °C/min to a final isotherm at 180 °C for 5 min. The mass spectrometer was operated in electron impact mode at 70 eV with SIM mode, selecting the same ions as previously described.

Quantitations were performed with calibration curves built in red wine.

4. Sensory analyses

4.1. General conditions

Sensory analyses were performed as described by Martin and de Revel (1999). Samples (around 50 mL) were poured into black INAO wine glasses (ISO 3591, 1977), labeled with random three-digit codes, and covered with a Petri dish. Evaluations were performed in a dedicated room (NF EN ISO 8589, 2010) equipped with individual booths to prevent communication between assessors, using normal daylight and at room temperature.

4.2. Sensory panel

Panel 1 consisted of 22 judges, 8 men and 14 women, aged 30.9 ± 4.3 years (mean ± SD). Panel 2 consisted of 23 judges (also members of panel 1), 8 men and 15 women, aged 30 ± 7.1 years (mean ± SD). Panel 3 consisted of 20 judges (also members of panel 1), 10 men and 10 women, aged 29.8 ± 7.1 years (mean ± SD). All the panelists were either research laboratory staff members at ISVV (Bordeaux University) or from the Laffort Company, and had previous experience in wine sensory evaluation.

4.3. Detection threshold

Olfactory detection thresholds of R- and S-E2H3MB were determined by panel 1 in microfiltered water and by panel 2 in a red Bordeaux wine, vintage 2012, using a three-alternative, forced-choice presentation (3-AFC) (ISO 13301, 2002). Each session consisted of ten forced-choice ascending tests. Each test contained one positive sample, supplemented with increasing concentrations of the compound to be evaluated (Table 1). By way of comparison, the detection threshold of the R form of E2H4MP, a compound recently described as an enhancer of fruity aromas in red wine (Lytra *et al.*, 2012), was determined by panel 2 in the same red wine. Each test was performed twice.

Table 1. Samples subjected to detection threshold determination in different matrices.

Compounds	Concentrations
In water (µg/L)	
R-Ethyl 2-hydroxy-3-methylbutanoate	0.2/0.4/0.9/1.8/3.6/7.2/14.4/28.8/57.5/115
S-Ethyl 2-hydroxy-3-methylbutanoate	0.1/0.2/0.4/0.8/1.6/3.1/6.3/12.5/25/50
In red wine (mg/L)	
R-Ethyl 2-hydroxy-3-methylbutanoate	0.6/1.1/2.2/4.5/9/17.9/35.9/71.8/143.5/287
S-Ethyl 2-hydroxy-3-methylbutanoate	0.6/1.1/2.2/4.4/8.8/17.7/35.4/70.7/141.4/282.8
R-Ethyl 2-hydroxy-4-methylpentanoate	0.2/0.4/0.8/1.7/3.4/6.7/13.5/27/53.9/107.8

4.4. Sensory evaluation

A ranking test (NF ISO 8587, 2006) of a red Bordeaux wine, vintage 2012, was performed orthonasally by panel 3 to evaluate the influence of E2H3MB on fruity aroma perception. The judges were asked to sort the four samples according to their fruitiness, from least to most. Equal ranking was not allowed. The concentrations tested were in accordance with those found in the 60 red wines analyzed, as follows: 25, 50, 100, 200 and 400 µg/L.

4.5. Statistical analyses

The Mann-Whitney test was used to evaluate the difference in E2H3MB levels between red and white wines from various vintages, and from different regions. The statistically significant level was 5% (XLSTAT 2016.03.30882 software, $p < 0.05$).

Results obtained from sensory tests were statistically interpreted following the norms published by the International Organization for Standardization (ISO).

The olfactory threshold was defined as the concentration at which the probability of detection was 50%. This statistical value was determined using an adaptation of the ASTM-E1432 method. 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 ($P = (3p - 1)/2$, where p = the proportion of correct responses for each concentration and P = the proportion corrected by a chance factor of 1/3 for 3-AFC). Sigma Plot 13 (SYSTAT) software was used for graphic resolution and ANOVA transform for nonlinear regression (Tempere *et al.*, 2011). The significance of the observed difference between olfactory thresholds was statistically tested by calculating the 95% confidence interval on detection probabilities.

The second test consisted of a ranking with a previewed order; therefore the Page test was used.

For each judge, a value between 1 and 4 was attributed to each sample, depending on the response of the assessor (1 for least intense, 4 for most intense). The sums of the rankings were obtained for each sample, then the parameters L and L' were calculated using Page test specifications, and L' was compared to reported values in order to determine if the test results were significant or not for the factor concerned.

Results and discussion

1. Ethyl 2-hydroxy-3-methylbutanoate enantiomers distribution and concentrations

The enantiomers of E2H3MB were separated by chiral GC analysis on a γ-cyclodextrin phase. Analysis of 99 wines from various French regions and vintages revealed different distributions.

E2H3MB levels were generally higher in red than white wines of the same age ($p < 0.05$) (Table 2). The same observations had already been made for other ethyl-branched esters, such as ethyl 3-hydroxybutanoate or E2H4MP (Lytra *et al.*, 2012; Lytra *et al.*, 2015), suggesting that red grapes may contain higher E2H3MB precursor levels. Moreover, E2H3MB concentrations were mostly higher in older rather than younger wines (Figure 2). Maximum levels were 314 µg/L and 164 µg/L in red and white wines, respectively (both from the 2004 vintage), and minimum levels were 18 µg/L and 0 µg/L, respectively (2012 and 2014 vintages). These concentrations are in accordance with those already found in red (Atienza *et al.*, 1998; Campo *et al.*, 2006) and white wines (Pet'ka *et al.*, 2001; Cejudo-Bastante *et al.*, 2013). Wine aging seems to be associated with a gradual increase in E2H3MB concentrations over time (Figure 2). In light of these findings, it seems probable that only a few micrograms per liter of E2H3MB were produced by yeast and/or lactic acid bacteria metabolism during fermentation. These results are in accordance with

those of several studies which observed a tendency toward branched-ethyl ester formation during wine aging (Díaz-Maroto *et al.*, 2005; Antalick *et al.*, 2014; Bordiga *et al.*, 2014). The authors also reported that concentrations were higher in old red wines than in young red or white wines, suggesting that they might be formed by esterification of the corresponding acid. Interestingly, concentrations were also linked with the origin of wine. Samples from Burgundy contained a significantly higher amount of E2H3MB than those from Bordeaux (min: 65 µg/L, max: 314 µg/L for red Burgundy; min: 24 µg/L, max: 231 µg/L for red Bordeaux; p < 0.05). According to Díaz-Maroto *et al.* (2005), branched-chain fatty acid ethyl esters, such as E2H3MB, could also be formed by yeasts during alcoholic fermentation from the corresponding amino acid. These findings suggest that Burgundy grape varieties may contain a larger amount of precursors than Bordeaux grape varieties. Furthermore, technical parameters during the fermentation processes could also influence E2H3MB levels, as highlighted by several studies. In particular, the amount of amino acids added to a nitrogen-deficient must is known to strongly affect the biosynthesis of branched-ethyl esters (Hernández-Orte *et al.*, 2006).

Considering the enantiomeric distribution of E2H3MB, the R enantiomer was found almost exclusively in all wines studied. Young red and white wines contained only the R form, whereas very small levels of the S form were quantitated in aged red wines. The S enantiomer was mostly formed after several years of aging for red wines, whereas only 4 white wines contained the S form (Figure 2). The highest S-E2H3MB concentrations were found in the oldest samples, with a maximum found at 5.6 µg/L in

a 1981 red Bordeaux. However, particularly low R/S ratios were also found in old red wines (1986 and 1994: 99/1 and 100/0, respectively), showing a high heterogeneity in S-E2H3MB amounts. These results highlight the complexity of the origin of these two enantiomers, with probable different biosynthetic and chemical pathways. If the S form seems only to result from the esterification of the corresponding acid during aging, R-E2H3MB could also have fermentative and/or varietal origins. Indeed, it is well known that esters are produced by yeasts during alcoholic fermentation. Recent studies have also highlighted the ability of lactic acid bacteria strains to synthesize esters (Sumby *et al.*, 2013), particularly the R form of E2H3MB (Gammacurta *et al.*, 2018). The elucidation of these different pathways will clearly require more specific investigation.

2. Odor significance of ethyl 2-hydroxy-3-methylbutanoate

The E2H3MB enantiomers used in this work were olfatively pure, and no odoriferous impurities were detected by the six judges who performed the analysis. They described the racemic mixture with artificial aromas such as candy, with strawberry, pineapple, and kiwifruit notes. R-E2H3MB was defined as having a heavier fruity odor , whereas the S form was characterized by "red fruits", "pineapple" and "green apple" descriptors.

Considering that the concentrations of S-E2H3MB found in almost all the wine samples were very low or zero, especially in young wines, an analysis of the enantiomeric ratio was not required.

The detection threshold of R-E2H3MB determined by panel 1 in microfiltered water was 4 µg/L, almost

Table 2. Concentrations of ethyl 2-hydroxy-3-methylbutanoate enantiomers.

Aging time (year)	No. of wines analyzed	Average concentration (µg/L) ± standard deviation			
		RS	R	S	ratio R/S
Red wines					
1-5	14	70 ± 27	70 ± 28	0 ± 0	99/1 ± 1
6-10	23	111 ± 30	111 ± 30	1 ± 0	100/0 ± 0
11-15	12	148 ± 45	148 ± 45	1 ± 0	99/1 ± 0
16-20	6	111 ± 25	109 ± 26	1 ± 1	98/2 ± 1
> 21	5	142 ± 63	139 ± 62	3 ± 2	98/2 ± 2
White wines					
1-5	25	28 ± 13	28 ± 13	0 ± 0	100/0 ± 1
6-10	12	38 ± 16	37 ± 15	1 ± 1	99/1 ± 2
11-15	2	120 ± 87	118 ± 83	2 ± 4	99/1 ± 2

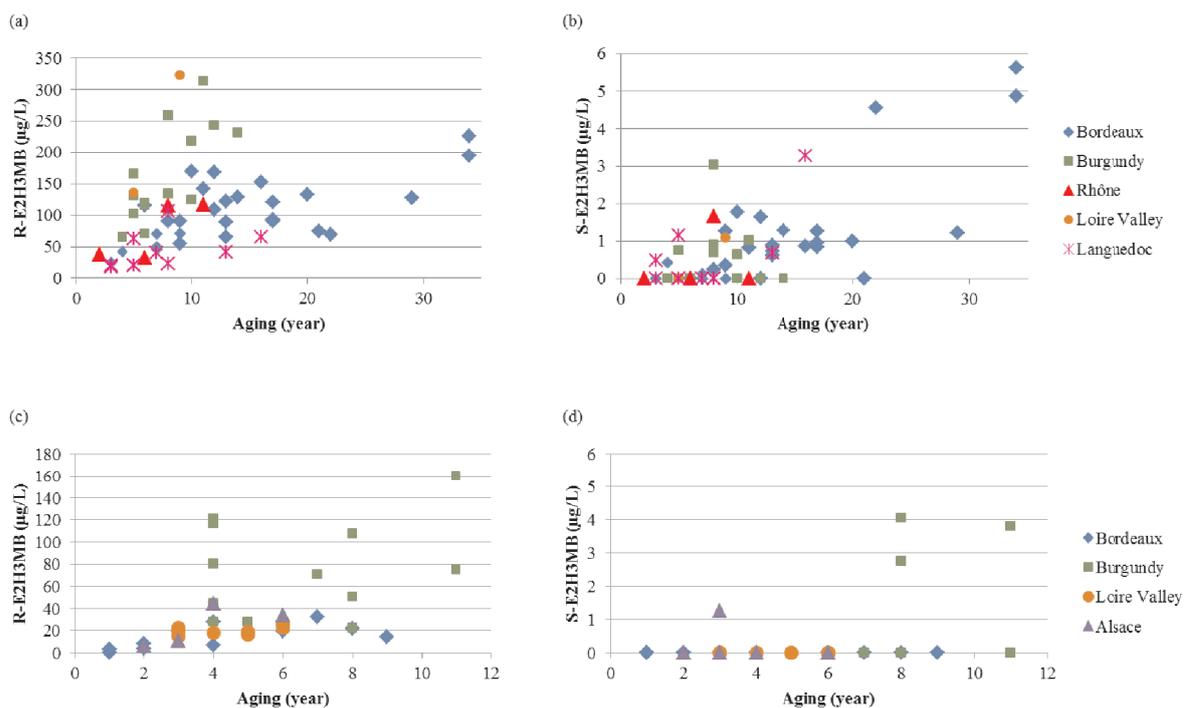**Figure 2. Concentrations of ethyl 2-hydroxy-3-methylbutanoate enantiomers (μg/L) in red (a, b) and white (c, d) wines from different French regions.**

twice that of the S form (1.5 μg/L) (Table 3). These results are in accordance with those obtained by Lytra *et al.* (2012) in a hydroalcoholic solution for another ester, E2H4MP, where the S form exhibited an olfactory threshold twice as high as the R form. These results confirm that the stereochemistry of the molecules has a strong influence on their perception, as already demonstrated in several studies (Pons *et al.*, 2008; Lytra *et al.*, 2012; Cretin *et al.*, 2015).

Detection thresholds were much higher in a young red Bordeaux (2012), found to be 51 mg/L and 21 mg/L for R- and S-E2H3MB, respectively, revealing a clear matrix effect. In order to compare with E2H4MP, which has a similar chemical structure and is known to be a strong enhancer of fruity aromas in wine model solution (Lytra *et al.*, 2012), its detection threshold was also determined in the same wine and estimated at 10 mg/L. As already observed for several esters (Etievant, 1991), this detection threshold was considerably higher than the concentrations found under the same conditions, confirming that these esters have no direct impact on the modulation of fruity aromas in red wine.

Recent studies of perceptive interactions have demonstrated the indirect potential role of a certain number of compounds, including esters. Indeed, Ferreira *et al.* (1998) suggested that the concentrations of compounds from the same chemical family could have a cumulative effect, resulting in the perception of their overall fruity character. Pineau *et al.* (2009) highlighted the importance of esters and acetates in red and black berry aromas, which was subsequently confirmed (Lytra *et al.*, 2012). They also demonstrated in de-aromatized wines that very small variations in ester levels can be perceived by panelists, even at concentrations far below their detection threshold.

Table 3. Olfactory thresholds in water and in a red wine.

Compound[b]	Olfactory threshold (μg/L)	
	In water	In red wine
R-E2H3MB	4	51 000
S-E2H3MB	1.5	21 000
R-E2H4MP	126[a]	10 000
S-E2H4MP	55[a]	N.Q.

[a] in hydroalcoholic solution (Lytra *et al.*, 2012)
[b] R-E2H3MB, R-ethyl 2-hydroxy-3-methylbutanoate; S-E2H3MB, S-ethyl 2-hydroxy-3-methylbutanoate; R-E2H4MP, R-ethyl 2-hydroxy-4-methylpentanoate; S-E2H4MP, S-ethyl 2-hydroxy-4-methylpentanoate
N.Q., not quantified

Based on these observations, a young red wine containing low levels of E2H3MB (25 µg/L for the R form, not detected for the S form) was spiked with varying concentrations of R-E2H3MB, covering the range found in the 60 red wines analyzed. Spiked samples were then orthonasally compared by a trained panel and ranked according to their fruitiness. The data presented in Table 4 show that judges were not able to distinguish modalities with different levels of R-E2H3MB, showing that this ester does not have a direct influence on fruity aroma modulation, at least at the concentrations tested in this study.

Conclusion

These data complement current knowledge of E2H3MB in wine. In particular, the relative abundance of its enantiomers revealed the preponderance of the R form in all wines studied, to varying degrees depending on the origin of the grapes and the vintage. Quantitation of both fruity enantiomers in 60 red wines and 39 white wines resulted in concentrations clearly below the sensory threshold estimated in a young red wine, demonstrating that this ester cannot have a direct impact on fruity aroma modulation. Ranking tests carried out with red wine samples spiked with increasing levels of R-E2H3MB did not show a significant difference in aroma. Thus, it is highly unlikely that this ester is involved in perceptive interactions influencing the fruity aroma of red wines.

Acknowledgments

Table 4. R-Ethyl 2-hydroxy-3-methylbutanoate impact on red wine fruity aromas.

Sum of rank							
V1	V2	V3	V4	V5	L	L'	Significance
53	60	57	73	57	921	**0.939**	ns

V1, V2, V3, V4 and V5 correspond to concentrations of 25, 50, 100, 200 and 400 µg/L, respectively.
L and L' were calculated as described in ISO 8587 (2006)

$$L = \sum_{i=1}^{p} iR_i \quad \text{and} \quad L' = \frac{12L - 3np(p+1)^2}{p(p+1)\sqrt{n(p-1)}}$$

with n: number of panelists and p: number of modalities
ns: not significant

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