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Formation Pathways of Ethyl Esters of Branched Short-Chain Fatty Acids during Wine Aging

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The particular behavior during wine aging of fermentative branched fatty acid ethyl esters, related to yeast nitrogen metabolism, compared that of their straight-chain analogues, related to yeast lipid metabolism, was first checked in 1−5 year aged Muscadet wines. Quantitative SIDA measurements showed that the levels of the former increased, whereas those of the latter decreased. Then, three hypothetical pathways suggested in the literature to explain these variations of branched esters were investigated. Two Muscadet and Sylvaner wines were spiked with levels of deuterated isobutanoic acid and its ethyl ester, similar to those of their natural analogues, then they were submitted to model aging. Quantitative SIDA measurements on the formation of these natural and labeled ethyl esters from the corresponding acids revealed that the behavior of the natural and labeled compounds were similar. The acid levels were much higher than the ester levels in the initial young wine, and a significant upward trend of their esterification ratios to those of the acid−ester equilibrium was observed with aging. Thus, this equilibrium proved to be the most effective in generating the branched fatty acid ethyl esters during wine aging. In contrast, the formation of these acids by Strecker-type degradation of wine amino acids in the conditions of the model aging or by hydrolysis of their glycoconjugates proved to be ineffective.

KEYWORDS: Wine; ethyl esters; branched short-chain fatty acids; stable isotope dilution assay

INTRODUCTION

Wine volatiles are originated in part from grape and in part from fermentation. The major fermentation aroma constituents are ethanol, fusel alcohols, and esters. These last compounds are essentially ethyl esters of organic acids, fusel alcohol acetates, and ethyl esters of fatty acids (*1*). These esters arise from alcoholysis of the corresponding acids activated as acyl-*S*-CoA by yeast (*2*, *3*).

Ethyl esters of straight-chain fatty acids with an even number of carbon atoms, such as ethyl hexanoate, ethyl octanoate, or ethyl decanoate, and fusel alcohol acetates, such as 2-methylbutyl, isopentyl, and 2-phenylethyl acetates, are considered important contributors to young wine aroma and exhibit floral and fruity odors (*4*, *5*). In contrast, ethyl acetate can give an unpleasant odor to the wine at concentrations higher than 100 mg/L (*6*).

However, it was generally admitted that the ethyl esters of branched short-chain fatty acids, such as ethyl isobutanoate, ethyl 2-methylbutanoate, and ethyl isopentanoate, were negligible contributors to wine aroma (*1*). It is only in the past decade that GC/olfactometry studies showed that these esters, derived from the amino acid metabolism of yeast, were also recognized as important odorants of wine (*7*−*11*). The variation of their levels during aging of wine is different to that of the ethyl esters of straight-chain fatty acids, derived from the lipid metabolism of yeast, and to that of the fusel alcohol acetates. The concentrations of the two last classes of esters generally decrease during wine aging. These variations are accelerated by high temperature and low pH and depend on the equilibrium between the esters and the corresponding acids, with an equilibrium constant close to 4, as demonstrated previously (*12*−*19*).

In contrast, the levels of the branched fatty acid ethyl esters are more or less stable or even can increase during the aging of wine (*10*, *20*), as well as of beer (*21*). Apart from their production by flor yeasts during the biological aging process of sherry wines (*20*), it is suggested in the literature that different chemical mechanisms might explain these variations. They could depend on the acid−ester equilibrium, if the esterification molar ratios of the branched fatty acids were lower in young wines than in aged wines at the equilibrium, as observed for the fixed acids (*1*). They could also be formed by the shifting of this acid−ester equilibrium, due to the formation of branched fatty acids. In nonoxidative aging, the branched fatty acids could be produced from the corresponding amino acids by Strecker degradation or from some amino acid derivatives formed during the fermentation, e.g., α-ketoacids (*10*). Similar hypothesis were

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Table 1. Levels of Some Fatty Acid Ethyl Esters[a] of Muscadet Wines of Different Vintages and Different Locations of the Muscadet Vineyard (Melon B. grapes)

wine[b]	age[c]	pH	2meC3	2meC4	3meC4	C4	C6	C8	C10
LIM 1999	1	3.39	95	4	6	141	824	862	408
MON 1999	1	3.22	31	3	1	100	755	728	357
DRA 1999	1	3.34	35	4	3	143	670	578	355
LOR 1999	1	3.33	91	3	3	178	884	981	511
mean level[a] 1999			63	3.5	3	140	783	787	408
molar level[d]			543	27	23	1207	5438	4576	2040
DRA 1996	4	3.25	361	7	30	162	602	506	153
MON 1995	5	3.17	328	21	135	80	424	218	150
mean level[a] 95–96			344.5	14	82.5	121	513	362	151.5
molar level[d]			2970	121	711	1043	4422	3121	1306

[a] Levels in μg/L; 2meC3: ethyl isobutanoate; 2meC4: ethyl 2-methylbutanoate; 3meC4: ethyl isopentanoate; C4: ethyl butanoate; C6: ethyl hexanoate; C8: ethyl octanoate; C10: ethyl decanoate. [b] See experimental part. [c] Natural aging at 12 °C in years before analysis. [d] Molar level in nmol/L.

also mentioned previously to explain the formation of phenylacetic acid in Spanish red wines (*22*). Technological parameters, such as high temperature and pH, lack of water, and dissolved oxygen and transition-metal contents, play an important role in the rate of formation of the Strecker aldehydes and acids, and consequently on aroma compounds formation (*23*, *24*). Conditions encountered during storage and aging of wine (aqueous medium, low temperature, and low pH) are not optimal for this reaction. However, in the last several years, it has been demonstrated that the Strecker reaction can occur under conditions close to those of wine (*25*, *26*). Finally, the branched fatty acid ethyl esters could occur through the slow hydrolysis at the wine pH of fatty acid glycosides, as fatty acids have been previously reported as aglycons of wine glycosides (*27*).

The purpose of the present work was first to check the particular behavior of the branched fatty acids and their ethyl esters during wine aging and then to investigate the hypothetical pathways suggested in the literature to explain these variations.

MATERIALS AND METHODS

Wines. Muscadet wines of the 1995, 1996, 1999, 2000 and 2002 vintages were produced using Vitilevure KD yeast (Martin-Vialatte, Epernay, France) at pilot scale (35 L) according to standard wine-making procedures by ITV France (Institut Technique du Vin, Unité de Nantes, France) from Melon B. grapes harvested in different locations of the Muscadet vineyard: La Limouzinière (LIM), Monnières (MON), Drain (DRA), and Le Loroux Bottereaux (LOR). After vinification, the wines were bottled with free sulfur dioxide levels ranging from 30 to 43 mg/L and stored at 12 °C. The Muscadet wines of the 1995, 1996, 1999 vintages (see pH in **Table 1**) were analyzed in 2000 to determine their levels in ethyl esters of branched- and straight-chain fatty acids. The 2000 Muscadet wine (40 mg/L of free SO_2 when bottled; pH 3.42) was used in the model aging experiment with [$^2H_3$]-leucine performed in 2001. The 2002 Muscadet wine (38 mg/L of free SO_2 when bottled; pH 2.98) and a 2002 Sylvaner wine (25 mg/L of free SO_2 when bottled; pH 3.38) were used in the model aging experiments carried out to investigate the equilibrium between the branched fatty acids and their ethyl esters. The 2002 Sylvaner wine was produced using the Levuline CHP yeast (Martin-Vialatte, Epernay, France) according to standard wine-making procedures by ITV France (Institut Technique du Vin, Unité de Colmar, France).

Chemicals. Sodium hydroxide, sodium sulfate, potassium hydroxide, acetic acid, and hydrochloric acid were all obtained from Merck (Darmstadt Germany). Diethyl ether, pentane, and dichloromethane were purchased from Riedel de Häen (St Quentin Fallavier, France), and ethanol (absolute) and methanol were from Carlo Erba (Rodano, Italy). Water was purified with a Milli-Q system from Millipore S.A. (Saint-Quentin Fallavier, France). The other chemicals were all purchased from Sigma-Aldrich (St Quentin Fallavier, France).

Labeled Chemicals. [$^2H_6$]-Ethanol (99.5% isotopic purity), [$^2H_6$]-acetone (99.9% isotopic purity), [$^2H_2$]-sulfuric acid (99.5% isotopic purity), deuterium oxide (99.9% isotopic purity), [$^2H_3$]-methyl [$^2H_5$]-methacrylate (99% isotopic purity), and L-[5,5,5-$^2H_3$]leucine (99% isotopic purity) were purchased from Aldrich (Saint-Quentin Fallavier, France).

[$^2H_5$]-Ethyl 2-methylbutanoate, [$^2H_5$]-ethyl isopentanoate, [$^2H_5$]-ethyl butanoate, [$^2H_5$]-ethyl hexanoate, [$^2H_5$]-ethyl octanoate, and [$^2H_5$]-ethyl decanoate were synthesized by esterification of the corresponding acids with [$^2H_6$]-ethanol as reported previously (*10*).

Synthesis of [$^2H_5$]-Ethyl Isobutanoate. Isobutanoic acid (632.7 mg, 7.2 mmol) and [$^2H_6$]-ethanol (158.1 mg, 3.1 mmol) were heated at 80 °C during 20 h using sulfuric acid (35 μL) as catalyst. The solution was cooled to room temperature, and then diluted with 30 mL of pentane, washed with two 5 mL portions of 1 N aqueous sodium hydroxide and with 5 mL water, dried over anhydrous sodium sulfate, and filtered. The solution of isobutanoic acid [$^2H_5$]-ethyl ester in pentane was kept at −20 °C. Its purity was checked by GC/EIMS (70 eV), *m*/*z* (%): 43 (100), 34 (47), 71 (38), 41 (28), 121 (16), 42 (16), 30 (11), 90 (9), 39 (9), 74 (6).

Synthesis of [$^2H_7$]-Isobutanoic Acid (Figure 1). A solution of isocyanomethyl *p*-tolyl sulfone (1035 mg, 5.3 mmol) in THF (2.7 mL) was added dropwise to a stirred suspension of potassium *tert*-butoxide (315 mg, 2.8 mmol) in THF (5.4 mL) at 7−10 °C. After cooling of the sample to −10 °C, a solution of [$^2H_6$]-acetone (212 μL, 2.8 mmol) in 2.7 mL of THF was added, and the resultant mixture was stirred for a further 15 min. Glacial acetic acid (150 mg, 2.5 mmol) was then added, and the solvent was removed by evaporation at 25 °C under vacuum. The residue was washed with 11.3 mL of water and extracted with 56.3 mL of methylene chloride. The organic phase was dried over sodium sulfate anhydrous, filtrated, and concentrated until dryness at 25 °C under vacuum to yield 787 mg (76%) of [$^2H_6$]-5,5-dimethyl-4-*p*-tolylsulfonyl-2-oxazoline. Its ^{1}H NMR spectrum was consistent with that of the natural compound, reported previously (*28*).

To obtain the [$^2H_6$]-1-formylamino-1-p-tolylsulfonyl-2-methyl-1-propene, a solution of the oxazoline obtained in the first step (253 mg, 1 mmol) in THF (1 mL) was added dropwise to a stirred suspension of potassium *tert*-butoxide (120 mg, 1 mmol) in THF (2 mL) at −10 °C. The resultant mixture was stirred for 15 min. Glacial acetic acid (60 mg, 1 mmol) was then added and the solvent was removed by evaporation at 25 °C under vacuum. The residue was washed with 2.5 mL of water and extracted with 5.3 mL of methylene chloride. The organic phase was dried over sodium sulfate anhydrous, filtrated, and concentrated until dryness at 25 °C under vacuum to yield 126 mg (49.8%) of [$^2H_6$]-1-formylamino-1-*p*-tolylsulfonyl-2-methyl-1-propene, which was used without further purification in the next step.

The hydrolysis of the crude [$^2H_6$]-1-formylamino-1-*p*-tolylsulfonyl-2-methyl-1-propene was carried out in 1 mL of a 2 N solution of deuterated sulfuric acid in deuterium oxide. The solution was then cooled, adjusted to pH 14 with aqueous sodium hydroxide 2 N, and washed twice with diethyl ether. The aqueous solution was then adjusted to pH 1 with sulfuric acid and extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulfate and filtered. The solution of [$^2H_7$]-isobutanoic acid in diethyl ether was kept at −20 °C.

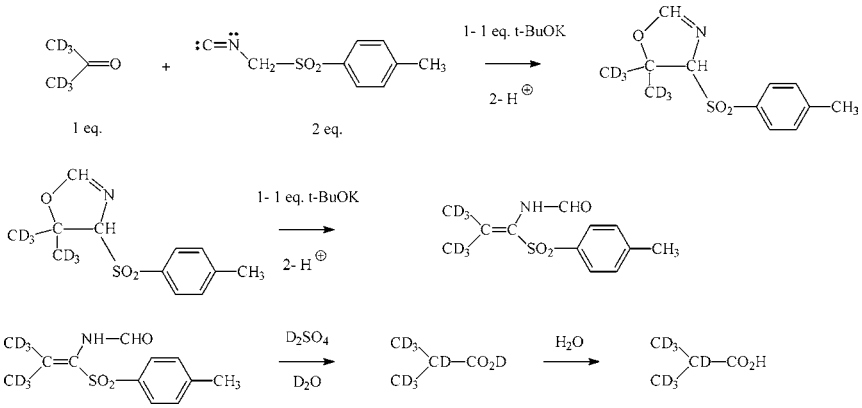

Figure 1. Synthesis of [$^2H_7$]-isobutanoic acid from [$^2H_6$]-acetone (*28*).

Table 2. Effect of Model Aging on the Levels (nmol/L) of Natural and [$^2H_5$]-Isobutanoic Acid and Their Ethyl Esters in a 2002 Muscadet Wine (pH 2.98) Initially Spiked with [$^2H_5$]-Isobutanoic Acid and Its Ethyl Ester

	before aging		after aging		aging effect		
	mean level[a]	%CV[a]	mean level[a]	%CV[a]	level variation[b]		ester deficiency[c]
ethyl [$^2H_5$]-isobutanoate	521.5	3.0	1190.9	6.3	669.4 ($p < 0.1\%$)	128.4%	–96.2 (ns)
[$^2H_5$]-isobutanoic acid	5387.1	6.2	4621.5	4.3	–765.6 ($p < 5\%$)	–14.2%	
[$^2H_5$]-ester/acid ratio	9.7%		25.8%				
ethyl isobutanoate	379.3	7.2	1031.0	1.2	651.7 ($p < 0.1\%$)	171.8%	–335.8 (ns)
isobutanoic acid	8883.0	5.7	7895.5	3.0	–987.5 ($p < 5\%$)	–11.1%	
ester/acid ratio	4.3%		13.1%				

[a] Mean levels (nmol/L) and variation coefficient (%CV) of the acids and esters in the samples analyzed ($n = 3$). [b] Difference between the levels after and before aging (nmol/L and %values) and level of significance of the Student test; ns= not significant. [c] Difference between the ester levels (nmol/L) observed and expected (acid loss totally esterified) after aging and level of significance of the Student test.

Table 3. Effect of Model Aging on the Levels (nmol/L) of Natural and [$^2H_5$]-Isobutanoic Acid and Their Ethyl Esters in a 2002 Sylvaner Wine (pH 3.38) Initially Spiked with [$^2H_5$]-Isobutanoic Acid and Its Ethyl Ester

	before aging		after aging		aging effect		
	mean level[a]	%CV[a]	mean level[a]	%CV[a]	level variation[b]		ester deficiency[c]
ethyl [$^2H_5$]-isobutanoate	1080.2	3.4	1461.2	6.0	381.0 ($p < 1\%$)	35.3%	–465.2 ($p < 5\%$)
[$^2H_5$]-isobutanoic acid	10862.4	1.7	10016.1	5.6	–846.2 (ns)	–7.8%	
[$^2H_5$]-ester/acid ratio	9.9%		14.6%				
ethyl isobutanoate	533.6	2.0	1050.0	5.0	516.4 ($p < 0.1\%$)	96.8%	–22.3 (ns)
isobutanoic acid	11609.1	1.6	11070.5	3.5	–538.6 (ns)	–4.6%	
ester/acid ratio	4.6%		9.5%				

[a] Mean levels (nmol/L) and variation coefficient (%CV) of the acids and esters in the samples analyzed ($n = 3$). [b] Difference between the levels after and before aging (nmol/L and %values) and level of significance of the Student test; ns= not significant. [c] Difference between the ester levels (nmol/L) observed and expected (acid loss totally esterified) after aging and level of significance of the Student test.

Its purity was checked by GC/EIMS (70 eV), *m*/*z* (%): 50 (100), 46 (50), 30 (33), 77 (27), 42 (12), 45 (11), 49 (10), 48 (8), 95 (7), 33 (6).

Synthesis of [$^2H_5$]-Isobutanoic Acid. [$^2H_3$]-Methyl [$^2H_5$]-methacrylate (486 mg, 4.5 mmol) in 6 mL of methanol was hydrogenated over 30 mg of palladium on carbon (5%) in a two necked flask at room temperature. When absorption of hydrogen ceased, the catalyst was separated out through filtration, and methanol was eliminated by concentration under vacuum at 40 °C from the filtrate. The residue (440 mg, 4 mmol) was subsequently heated at 60 °C for 1 h in a solution of 448 mg (8 mmol) of potassium hydroxide in 35 mL of methanol. Then, the solution was cooled to room temperature and concentrated to dryness under vacuum, and the residue was added with water (8 mL). The aqueous solution was washed with pentane, and then it was adjusted to pH 1 with 1 N hydrochloric acid and extracted with diethyl ether. The solution of [$^2H_5$]-isobutanoic acid in diethyl ether was dried over anhydrous sodium sulfate, filtered, and kept at −20 °C. Its purity was checked by GC/EIMS (70 eV), *m*/*z* (%): 45 (100), 46 (77), 47 (78), 48 (72), 44 (64), 49 (51), 43 (38), 75 (37), 76 (33), 42 (26).

Synthesis of Ethyl [$^2H_5$]-Isobutanoate Acid. [$^2H_5$]-Isobutanoic (111.6 mg, 1.2 mmol) and ethanol (55.2 mg, 1.2 mmol) were heated at 80 °C during 3 h using sulfuric acid (7 μL) as catalyst. The solution was cooled to room temperature, and then diluted with 30 mL of pentane, washed with two 5 mL portions of 1 N aqueous sodium hydroxide and with 5 mL water, dried over anhydrous sodium sulfate, and filtered. The solution of [$^2H_5$]-isobutanoic acid ethyl ester in pentane was kept at −20 °C. Its purity was checked by GC/EIMS (70 eV), *m*/*z* (%): 46 (100), 47 (94), 48 (83), 45 (85), 44 (54), 75 (53), 76 (43), 74 (39), 43 (33), 73 (28).

Model Aging of the Wines. A 2002 Muscadet wine and a 2002 Sylvaner wine (0.75 L of each wine) were spiked with the amounts of [$^2H_5$]-isobutanoic acid and ethyl [$^2H_5$]-isobutanoate mentioned under "before aging" in **Tables 2** and **3**. Each wine was divided in two portions of 0.6 and 0.15 L. The 0.6 L portions were aged in a laboratory oven at 45 °C during 3 weeks and then analyzed as described below, whereas the 0.15 L portions were analyzed at the starting time of the aging.

In addition, 0.5 L of a 2000 Muscadet wine were spiked with 40 mg of L-[5,5,5-$^2H_3$]leucine, aged as above, in a laboratory oven at 45 °C during 3 weeks, and then analyzed as described below.

Isolation of Volatiles from Wines. *General Procedure*. The wine was placed in a flask, and then the internal standards were added and equilibrated under stirring. Then the samples were extracted with

methylene chloride by stirring for 20 min under nitrogen. The emulsion was resolved by centrifugation at 8000 rpm and 4 °C for 20 min. The organic phases were blended, desiccated over anhydrous sodium sulfate, filtered, and concentrated on a Vigreux column. Finally, the concentrates were stored at −20 °C until analysis.

Quantitative Determination of Fatty Acid Ethyl Esters of Muscadet Wines of Different Vintages. The procedure used was reported previously to analyze these esters in Merlot wine (*10*). 13 μg of [$^2H_5$]-ethyl isobutanoate, 4.4 μg of [$^2H_5$]-ethyl 2-methylbutanoate, 5.5 μg of [$^2H_5$]-ethyl isopentanoate, 7.5 μg of [$^2H_5$]-ethyl butanoate, 92.6 μg of [$^2H_5$]-ethyl hexanoate, 33.9 μg of [$^2H_5$]-ethyl octanoate, and 22.4 μg of [$^2H_5$]-ethyl decanoate were added as internal standards to 250 mL of wine, which was extracted with 2 × 60 mL of methylene chloride. The final volume of the extract was about 4 mL.

Wines of the Aging Experiment with Deuterated Branched Fatty Acids and Ethyl Esters. A total of 24.6 μg of [$^2H_7$]-isobutanoic acid and 50.4 μg of [$^2H_5$]-ethyl isobutanoate were added as internal standards to 50 mL of the different portions of wine, which were extracted with 2 × 15 mL of methylene chloride. The final volume of the extract was about 1 mL. Each sample was analyzed in triplicate.

Wines of the Aging Experiment with Deuterated Leucine. 500 mL of wine were extracted with 2 × 100 mL of methylene chloride. The final volume of the extract was about 1 mL.

Isolation of Glycosides from Wines and Enzymatic Hydrolysis of the Glycosidic Extracts. The glycosides of 50 mL of a 2002 Muscadet and of a 2002 Sylvaner wines, diluted twice with Milli-Q water (Millipore Corp.), were extracted using C18 reversed phase cartridges, and the extracts were hydrolyzed using a hydrolase enzyme AR2000 (DSM, France), as reported previously for Melon B. juice (*29*). The aglycone extracts were kept at −20 °C until analyzed by GC/MS.

Gas Chromatography−Mass Spectrometry. A Hewlett-Packard 5890 series II gas chromatograph fitted with a DB-WAX capillary column (30 m × 0.25 mm, 0.5 μm, J&W Scientific Inc., USA) was used. The "on column" injector was heated from 30 to 250 °C at 180 °C/min. Helium (Linde gaz, Marseille, France) at a flow rate of 1.3 mL/min was used as the carrier gas. The oven temperature program was as follows: 25 °C, then increased at 70 °C/min to 50 °C (held for 3 min), then increased at 3 °C/min to 125 °C, then increased at 10 °C/min to 245 °C (held for 20 min). The GC instrument was coupled to a Hewlett-Packard 5989A mass spectrometer and a MS chemstation. The transfer line was heated at 250 °C. The electron impact (EI) energy was 70 eV, and the MS source and quadrupole temperatures were set at 250 and 120 °C, respectively. EIMS spectra were recorded in full scan mode in the range of 29 to 350 amu at 0.5 s intervals.

Calibration Curves. *Quantitative Determination of Fatty Acid Ethyl Esters of Muscadet Wines of Different Vintages*. Serial dilutions of each fatty acid ethyl ester, added with the same amount of the corresponding [$^2H_5$]-ethyl ester as internal standard, were made in methylene chloride with the following concentration ranges: 0.025−5.1 μg/mL of ethyl isobutanoate (1.3 μg/mL of [$^2H_5$]-ethyl isobutanoate), 0.042−2.68 μg/mL of ethyl 2-methylbutanoate (0.44 μg/mL of [$^2H_5$]-ethyl 2-methylbutanoate), 0.031−1.0 μg/mL of ethyl isopentanoate (0.55 μg/mL of [$^2H_5$]-ethyl isopentanoate), 0.14−4.31 μg/mL of ethyl butanoate (0.75 μg/mL of [$^2H_5$]-ethyl butanoate), 0.73−23.5 μg/mL of ethyl hexanoate (9.26 μg/mL of [$^2H_5$]-ethyl hexanoate), 0.08−24.0 μg/mL of ethyl octanoate (3.39 μg/mL of [$^2H_5$]-ethyl octanoate), and 0.20−19.6 μg/mL of ethyl decanoate (2.24 μg/mL of [$^2H_5$]-ethyl decanoate), respectively. The calibration curves were obtained from these solutions injected into the GC-MS system in the same chromatographic conditions as the wine extracts. The ions used as quantifiers for the natural ethyl esters were *m*/*z* 116 for ethyl isobutanoate, *m*/*z* 102 for ethyl 2-methylbutanoate, and *m*/*z* 88 for the other esters. The ions used as quantifiers for the [$^2H_5$]-ethyl esters were *m*/*z* 121 for [$^2H_5$]-ethyl isobutanoate, *m*/*z* 107 for [$^2H_5$]-ethyl 2-methylbutanoate, and *m*/*z* 93 for the other internal standards. For each compound and for the concentrations mentioned above, the peak area ratios (peak area of the ion used as quantifier ion for the natural ester/peak area of the ion used as quantifier ion for the corresponding [$^2H_5$]-ethyl ester) were plotted against the concentration ratios (micrograms/milliliter of each dilution of each natural ester/micrograms/milliliter of the corresponding [$^2H_5$]-ethyl ester). The standard curves were obtained by linear regression analysis.

Wines of the Aging Experiment with Deuterated Branched Fatty Acids and Ethyl Esters. Eight solutions of both natural compounds, ethyl isobutanoate and isobutanoic acid, and eight solutions of both spiked [$^2H_5$]-analogues, ethyl [$^2H_5$]-isobutanoate and [$^2H_5$]-isobutanoic acid, were made in methylene chloride with the following concentration ranges: 0.02−20.83 μg/mL of ethyl isobutanoate, 0.06−60.61 μg/mL of isobutanoic acid, 0.12−59.74 μg/mL of ethyl [$^2H_5$]-isobutanoate, and 0.77−48.20 μg/mL of [$^2H_5$]-isobutanoic acid, respectively. Then 1.35 μg/mL of [$^2H_5$]-ethyl isobutanoate and 3.00 μg/mL of [$^2H_7$]-isobutanoic acid were added to each solution as internal standards. The calibration curves were obtained from these solutions injected into the GC-MS system in the same chromatographic conditions as the wine extracts. For each compound, the peak area ratios (peak area of the ion *m*/*z* 71/peak area of the ion *m*/*z* 71; peak area of the ion *m*/*z* 73/peak area of the ion *m*/*z* 77; peak area of the ion *m*/*z* 76/peak area of the ion *m*/*z* 71; and peak area of the ion *m*/*z* 47/peak area of the ion *m*/*z* 50) were plotted against the concentration ratios (eight-point scale mentioned above: micrograms/milliliter of ethyl isobutanoate/1.35 μg/mL of [$^2H_5$]-ethyl isobutanoate; micrograms/milliliter of isobutanoic acid/3.00 μg/mL of [$^2H_7$]-isobutanoic acid; micrograms/milliliter of ethyl [$^2H_5$]-isobutanoate/1.35 μg/mL of [$^2H_5$]-ethyl isobutanoate and micrograms/milliliter of [$^2H_5$]-isobutanoic acid/3.00 μg/mL of [$^2H_7$]-isobutanoic acid, respectively). The standard curves were obtained by linear regression analysis.

RESULTS AND DISCUSSION

Levels of Fatty Acid Ethyl Esters during Aging of Muscadet Wines. To evaluate their variations during aging, the levels of the ethyl esters of straight-chain and branched fatty acids were determined in 1−5 year aged (vintages 1999, 1996, and 1995) experimental Muscadet wines (**Table 1**). These different wines were made, using the same wine-making procedure, from white Melon B. grapes grown in different locations of the Muscadet vineyard (northwest France). The quantitative determination of the straight-chain fatty acid ethyl esters is easy (relatively high levels in wine), but that of the branched esters is more difficult, due to their lower levels and volatility. Thus, these levels were determined using the stable isotope dilution assays previously reported for their quantification in Merlot wines (*10*), but dichloromethane was used as the solvent of extraction instead of pentane. [$^2H_5$]-ethyl esters of the fatty acids, obtained by esterification of the corresponding acids with [$^2H_5$]-ethanol, were used as internal standards (*10*).

The differences observed in these Muscadet wines of different vintages were consistent with those reported previously in the literature during wine aging (see introduction): the levels of the branched esters increased with age, whereas those of the straight-chain esters decreased. They were also consistent with the variations observed during the model aging of Merlot wines, which consisted in controlled heating, at 45 °C for 24 days (*10*), and was used previously as model aging for acid-catalyzed reactions occurring during wine aging (*30*). Thus, we chose the same model aging conditions, in the experiments undertaken afterward to elucidate the pathways explaining the variations of the branched esters during wine aging.

Formation of the Ethyl Esters of Branched Short-Chain Fatty Acids from Their Corresponding Acids in Model Aging Experiments. To study the equilibrium between the ethyl esters of branched short-chain fatty acids and their corresponding acids in wine, two one-year-old wines, a 2002 Muscadet and a 2002 Sylvaner wines, were spiked with a deuterated branched fatty acid and its ethyl ester, and submitted to the same model aging as above. Then, the levels of the spiked deuterated compounds were compared to those of the corresponding natural

compounds, before and after aging. Isobutanoic acid and its ethyl ester were chosen, as this ester was the most abundant of the branched fatty acid ethyl esters quantified in the Muscadet wines analyzed above (**Table 1**). To carry out these experiments, four deuterium labeled derivatives were necessary and were synthesized. [$^2H_5$]-Isobutanoic acid and ethyl [$^2H_5$]-isobutanoate were used to spike the wines, and [$^2H_7$]-isobutanoic acid and [$^2H_5$]-ethyl isobutanoate were used as internal standards to determine the exact amounts of the target compounds by means of stable isotope dilution assays.

Synthesis of the Deuterated Compounds. [$^2H_7$]-Isobutanoic acid was synthesized by conversion of [$^2H_6$]-acetone into the next higher carboxylic acid (**Figure 1**), using the procedure reported previously for the unlabeled compound (*28*). However, as the first step of the procedure reported did not convert totally the deuterated acetone to the propene adduct, it was slightly modified to isolate the [$^2H_6$]-5,5-dimethyl-4-*p*-tolylsulfonyl-2-oxazoline intermediate, as shown in **Figure 1**. Thus, [$^2H_6$]-acetone was first converted into [$^2H_6$]-5,5-dimethyl-4-*p*-tolylsulfonyl-2-oxazoline by reaction with α-metalated isocyanomethyl *p*-tolyl sulfone in strong basic conditions. The isolated oxazoline was then converted into [$^2H_6$]-1-formylamino-1-tosyl-2-methyl-1-propene in the same basic conditions. Finally, the hydrolysis of this adduct with a solution of deuterated sulfuric acid in deuterium oxide gave rise to [$^2H_7$]-isobutanoic acid, obtained as a pure isotopomer, as shown by GC-MS (molecular ion *m*/*z* 95).

[$^2H_5$]-Isobutanoic acid was obtained by hydrogenation of the double bond of commercial [$^2H_3$]-methyl [$^2H_5$]-methacrylate, followed by saponification of the deuterated methyl isobutyrate obtained. However, alkenes and even alkanes are well-known to exchange hydrogens during heterogeneous catalytic hydrogenation (*31*). Thus, as shown by GC-MS analysis, the partial exchange of deuterium by hydrogen atoms gave rise to a mixture of deuterated isobutanoic acid isotopomers, instead of [$^2H_5$]-isobutanoic acid, which was named, by convenience, [$^2H_5$]-isobutanoic acid throughout this text. As it contained only a trace of the natural isotopomer (molecular ion *m*/*z* 88 was less than 1% of the molecular ion cluster), it was used, with its ethyl ester, to spike the wines in the model aging experiment. Finally, [$^2H_5$]-isobutanoic acid ethyl ester and isobutanoic acid [$^2H_5$]-ethyl ester were obtained by acid-catalyzed esterification of the corresponding acids with ethanol and [$^2H_5$]-ethanol, respectively. The former was a mixture of isotopomers similar to the corresponding acid, whereas the latter was a pure isotopomer (molecular ion *m*/*z* 121).

SIDA Assays Using GC-MS. The quantification of the natural isobutanoic acid and its ethyl ester on one hand, and that of the deuterated analogues spiked in the wine, [$^2H_5$]-isobutanoic acid and its ethyl ester on the other hand, were achieved using [$^2H_7$]-isobutanoic acid and isobutanoic acid [$^2H_5$]-ethyl ester as internal standards. Extraction of these compounds from wine samples with methylene chloride and analysis of the extracts by GC/EIMS, used in full scan mode, gave sufficiently sensitive detection. In the GC conditions used, the three target esters appeared as two resolved peaks. [$^2H_5$]-Isobutanoic acid ethyl ester and isobutanoic acid [$^2H_5$]-ethyl ester were coeluted in the first eluted one and the natural ester was in the second eluted peak. The three target acids gave a first eluted peak with a shoulder, corresponding to the deuterated acids, and a second eluted peak, separated from the first one, corresponding to the natural acid. Reconstructed ion chromatograms (RIC) were used for the quantification of the different isotopomers of isobutanoic acid and its ethyl ester, and the requirements to choose the ions used were their relative ion abundance and their least interference from each other and from other wine volatile components.

Analysis of the Target Compounds in the Model Aging Experiments. **Tables 2** and **3** show the levels of the natural and labeled ethyl isobutanoate and their corresponding acids in the spiked Muscadet and Sylvaner wines, before and after aging at 45 °C for 3 weeks. In both wines, the natural acid levels were much higher than the corresponding ester levels in the initial young wine, leading to esterification molar ratios lower than 5%. As these ratios were expected to be higher when the acid−ethyl ester equilibrium was brought about, assuming values similar to those of straight-chain fatty acids (*18*, *32*), those chosen for the spiked deuterated compounds were doubled (∼10%). Despite this doubling, the model aging brought about significant increases ($p < 1\%$ and $p < 0.1\%$) in the molar concentrations of the deuterated ethyl isobutanoate, which were also observed for the natural ester, as expected according to the acid−ethyl ester equilibrium model. Simultaneously, decreases in the molar concentrations of their corresponding acids were observed, but they were significant ($p < 5\%$) in the Muscadet wine only. As a matter of fact, the relative variations in the acid molar levels (ranging from −4.6 to −14.2%) were much lower than that of the ethyl esters (ranging from +35.3 to +171.8%), due the much higher acid levels (about 10−20 times). Thus, despite similar fair coefficients of variation for the quantitative determination of the acids (1.6−6.2%) and ethyl esters (1.2−7.2%), the level of significance of the acid decreases in both wines (ns or $p < 5\%$) was much lower than that of the increases in the molar concentrations of the corresponding ethyl esters ($p < 1\%$ or $p < 0.1\%$). Furthermore, assuming the acid−ethyl ester equilibrium model, the absolute variations of the molar levels of each acid should be equal to that of the corresponding ethyl ester, but no ester in both wines reached these theoretical values. That was particularly evident in the cases of the deuterated acid−ester pair in Sylvaner wine (381 nmol vs 846.2 nmol) and of the natural acid−ester pair in Muscadet wine (651.7 nmol vs 987.5 nmol). These deficiencies in ethyl ester levels after model aging could be partly explained by the consumption of the acids by other constituents of wine than ethanol. However, that was not consistent with the high content in ethanol, by far the most abundant wine constituent reacting with the acids. In addition, the deficiencies observed for the natural and the labeled esters in both wines were different and in different value orders. Thus, these discrepancies could be attributed mainly to the difference in the magnitude orders of the ester and acid levels, as discussed above. That explained why these ester deficiencies were not statistically significant (except for the deuterated ester in the Sylvaner wine), not allowing rejection of the acid−ethyl ester equilibrium model.

Finally, assuming this model with first-order esterification and hydrolysis reactions and constant water and ethanol concentrations, the rate of the formation of the ethyl esters could be written:

$$de/dt = k_1(a_0 + e_0 - e)[\mathrm{EtOH}] - k_2 e[\mathrm{H_2O}]$$

which gave at the equilibrium ($de/dt = da/dt = 0$):

$$k_1(a_0 + e_0 - e_E)[\mathrm{EtOH}] = k_2 e_E[\mathrm{H_2O}]$$

where k_1 and k_2 are rate constants of the esterification and hydrolysis reactions, respectively, e, e_0, and e_E are the concentrations of the ester at t time, starting time and equilibrium, respectively, a and a_0 are the concentrations of the corresponding

acid at t time and starting time, respectively, and [EtOH] and [H_2O] are the concentrations of ethanol and water, respectively.

Assuming that the isotope effect was negligible (k_1 and k_2 equal for the natural and deuterated compounds), the same equation could be written for the natural and deuterated analogues, which led to the following equation:

$$e_E^H/e_E^D = (a_0 + e_0)^H/(a_0 + e_0)^D$$

where the H and D superscripts referred to the natural and deuterated compounds, respectively.

Hence, the ratio e_E^H/e_E^D should be equal to 1.57 in the Muscadet wine and 1.02 in the Sylvaner wine. These values were compared to those of the ratio e^H/e^D at the starting time (0.73 and 0.49) and at the end of the model aging time (0.87 and 0.72, respectively) in the corresponding wines, which showed that the equilibrium was not reached at the end of the model aging time. At this time, the esterification molar ratios were 13.1 and 25.8% for the natural and deuterated isobutanoic acid in the Muscadet wine, and 9.5 and 14.6% in the Sylvaner wine, respectively. The ester levels reached were lower than the levels of ethyl isobutanoate measured in the 1996 and 1995 Muscadet wines (natural aging of 4 and 5 years, respectively) (**Table 1**), but no comparison with the esterification ratios of these wines could be made, as the levels of isobutanoic acid were not determined in these wines. However, these esterification molar ratios were consistent with those measured at the equilibrium in Port wine (20% ethanol content V:V), for the straight-chain C-4, C-6, C-8, and C-10 fatty acid−ethyl ester couples, which ranged from 28 to 40% (*32*), that is, from ~18 to 26% for the ethanol content of the wines used in our experiments (~13% V:V).

Whatever these values are, as the labeled acid and ester were the only sources of labeled compounds in the wines used in the model experiments, the increase of their esterification ratios could be reasonably explained only under the assumption that the acid−ester equilibrium was the major process involved in the generation of branched ethyl esters during wine aging, although it was not possible to exclude the concomitant occurrence of other chemical processes shifting this equilibrium. Of course, the acid−ester equilibrium setting up would have afforded further insight into the balance between the branched acids and their corresponding ethyl esters. However, even if the 3-week aging period was shorter to reach the equilibrium, the model (wine medium, 45 °C) allowed promotion of variations of the levels of the branched acid and its ethyl ester in the same direction as those observed under natural aging (**Table 1**). In contrast, harder conditions for model aging, such as an increase of temperature above 45 °C, could favor reactions requiring higher energy, as Strecker degradation. Thus, this reaction, which could also occur during wine aging, was tested using the same model aging conditions.

Formation of Branched Short-Chain Fatty Acid by Strecker Degradation in Model Aging Experiment. The increase of ethyl esters of branched short-chain fatty acids during the model aging of the Muscadet and Sylvaner wines could be explained by the formation of these fatty acids from the corresponding amino acids by Strecker degradation. Indeed, the excess of acid formed in this process should shift the acid−ester equilibrium toward the ester formation. Similar hypothesis were also mentioned previously to explain the formation of phenylacetic acid in Spanish red wines (*22*) and that of ethyl esters of branched short-chain fatty acids during the model aging of Merlot wines (*10*).

To verify this hypothesis, the same model aging as above was performed on a Muscadet wine added with 40 mg/L of L-[5,5,5-$^2H_3$]leucine, which would give rise to [4,4,4-$^2H_3$]-isopentanoic acid by the Strecker degradation, and subsequently to its ethyl ester. The amount of deuterated L-leucine added to the wine was similar to the highest levels of natural L-leucine found previously in white wines, ranging from 21 to 43 mg/L (*33*). GC-MS detection of the wine volatiles, after the model aging, was performed with the method described above to detect the different isotopomers of isobutanoic acid and its ethyl ester. It revealed only natural isopentanoic acid and its ethyl ester, but not their trideuterated isotopomers, which would be expected in high amounts (theoretical amounts of 30.6 and 38.8 mg/L). Thus, in our conditions of medium and temperature, the excessive levels of deuterated leucine here added allowed us to conclude that the levels of isopentanoic acid ethyl ester produced during Muscadet wine aging cannot be explained by the Strecker degradation of leucine, but the formation of branched fatty acids from some amino acid derivatives formed by yeast, e.g., α-ketoacids, cannot be excluded.

Formation of Branched Short-Chain Fatty Acid from Grape Glycoconjugates. One of the sources of branched fatty acid ethyl esters during wine aging could be the release of isobutanoic acid through the slow hydrolysis at the wine pH of its glycosilated forms (*27*) and subsequently the esterification in its corresponding ethyl ester. To elucidate the importance of this route, the glycosides of a 2002 Muscadet and of a 2002 Sylvaner wines were extracted and the extracts were hydrolyzed using an appropriate glycosidase enzymatic preparation, as reported previously (*29*). The aglycones released were semiquantified as 4-nonanol equivalents, used as an internal standard. That showed that the Sylvaner wine contained a low level of isobutanoic acid aglycone (4.5 μg/L of 4-nonanol equivalent), whereas it was not even detected in the Muscadet wine extract, which was consistent with the low levels of acid aglycons reported previously in Melon B. grapes (*29*). Thus, this route could not explain the much higher amounts of ethyl esters of branched fatty acids produced during wine aging.

CONCLUSION

The results of the model experiments performed with addition of labeled compounds to wine have shown that the acid−ester equilibrium was the most effective in generating the branched fatty acid ethyl esters from their corresponding acids during wine aging. Their esterification molar ratios, very low in young wine, increased significantly with aging, but this upward trend did not reach the equilibrium at the end of the model aging experiment. On the other hand, the formation of branched short-chain fatty acids by the Strecker degradation of their corresponding amino acids or from grape glycoconjugates could not explain the much higher amounts of their corresponding ethyl esters produced during wine aging.

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