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Determination of free fatty acids and their ethyl esters in musts and wines

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Abstract

White must and wine fatty acids are present mainly in the free form, or esterified as ethyl esters, and both contribute to flavor and foam properties of wine. A reliable and sensitive analytical procedure for analyzing the free fatty acids (C6–C18) and their ethyl esters has been developed. Sulfuric acid (3%) in methanol was selected as a derivative reagent, and optimal derivatization conditions were established (3 h at room temperature). This reagent gives total methylation of fatty acids and partial transesterification of ethyl esters to methyl esters and, through the study of this transesterification, free and bound fractions were determined satisfactory. © 1997 Elsevier Science B.V.

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1. Introduction

Fatty acids in wine come from the firm tissues of the grapes [1]. However, the greatest amount of fatty acid is formed during alcoholic fermentation, as fatty acids can also be released by yeast [2–5]. Fatty acids of wine occur in two forms: free (C*n*: fatty acid with *n* carbon number) or bound, mainly as ethyl esters (C*n*E: ethyl ester of fatty acid C*n*) since ethanol is the main alcohol. Both forms contribute to flavor: the volatile fatty acids [1,6] and the fruity ethyl esters [1], directly, and the unsaturated fatty acids as precursors of aldehydes and alcohols of six carbons with herbaceous flavor [1,7] indirectly. Moreover, in sparkling wines, the fatty acids influence foam formation and stability [4,8,9]. Finally, medium-chain fatty acids are toxic to both yeast and malolactic bacteria [10,11]. They could be used to reduce the doses of sulfur dioxide required in accordance with toxicological and safety recommendations. On the other hand, their presence inhibits fermentation and can cause its premature termination [10].

Several authors have indicated difficulties in determining, with the same analytical method, fatty acids, free and bound as ethyl esters, volatiles and non-volatiles [7,12], at the different concentration levels at which they occur in musts and wines. Furthermore, several juice and wine compounds (phenols, flavor compounds, etc.) interfere with the analysis of the fatty acids. The usual analytical methods for fatty acids [1,2,4,5,11] include extraction, derivatization to methyl esters, (C*n*M: methyl ester of fatty acid C*n*), and gas chromatography (GC). Derivatization is suitable for the less volatile free fatty acids ($n>10$). However, the conditions (reagent, time and temperature) could lead to the

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transesterification of ethyl esters to methyl esters [12]. In the determination of total fatty acids, the ethyl esters should be totally transesterified [1,2,11]. For determination of free fatty acids, the methods described use thin-layer chromatography (TLC) [5], or saponification [4] to separate the free and bound fatty acids of the sample. Later, the two fractions are studied, separately, by GC.

These methods are long and laborious, and it is not always clear whether the fatty acid concentrations belong only to the free fraction of fatty acids. Some methods [1,6,7] avoid the derivatization reaction, but there is interference from other compounds of the sample (phenols, aroma compounds). Moreover, the reliability of the method was not evaluated in any of these studies.

The aim of the current study was to find a single analytical method for determination, in musts and wines, of free fatty acids, ranging from caproic acid (C6) to stearic acid (C18). For the determination of volatile fatty acids (C2–C10), other more simple methods are described [6,13]. Factors that influence the formation of methyl esters were studied: the derivatization, acid or base catalyzed, the time of contact between fatty acids and derivatization reagent and the temperature of reaction. The selected derivatization procedure produces partial transesterification of ethyl esters to methyl esters. Therefore, in samples containing ethyl esters, the methyl ester chromatographic peaks include the free fatty acids from the sample and the fatty acids released from the ethyl esters. For free-fatty-acid determination, it is essential to determine the amount of fatty acid released from transesterification and apply a suitable correction. The study of this partial transesterification allows the determination, in the same chromatogram, of both free and bound fatty acids, such as ethyl esters. Moreover, method validation (detection and quantification limits, reproducibility and recovery) was performed for two winemaking products representing different matrices: musts and wines.

2. Experimental

2.1. Chemicals

Standards of fatty acids, methyl esters and ethyl esters (Sigma) of purities higher than 98% were used. The solvents, methanol and hexane, were Pestipur (SDS, Peypin, France). Internal standard (I.S.) solutions: solution A for wine: fatty acids (C*n*) and ethyl esters (C*n*E) in methanol: C7, C13, C17, C9E and C15E at 3.5, 1.5, 1.0, 0.9 and 0.15 g/l, respectively. Solution B for must: fatty acids and ethyl esters in methanol: C7, C13, C17, C9E and C15E at 3.5, 1.5, 1.0, 0.04 and 0.01 g/l, respectively.

2.2. Equipment

A Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detection (FID) system was used. The capillary column was a Supelcowax 10 with PEG 20M stationary phase (30 m×0.25 mm, 0.25 μm). An HP 3396 A integrator was used.

2.3. Conditions

2.3.1. Sample preparation

Must and wine samples were defrosted overnight at 4°C; 50 μl of internal standard (I.S.) solutions (A for wines and B for musts) was added to 50 ml of sample; 2 g of NaCl was added to the sample to avoid formation of emulsion; each sample was divided into three aliquots for triplicate analysis.

2.3.2. Extraction

Each aliquot was extracted batch-wise with 3×5 ml of hexane, by shaking for 1 min in a closed mixer tube; the organic and aqueous phases were separated by centrifugation at 700 *g* for 1 min; the hexane layers were combined in a vial, dried with anhydrous sodium sulfate, and transferred to a concentration tube; the volume was reduced to approximately 1 ml under a gentle stream of nitrogen.

2.3.3. Derivatization

A 1-ml volume of the derivatization reagent (3% H_2SO_4 in methanol) was added to 1 ml of concentrated extract; the mixture was shaken for 30 s in a mixer, and left for 3 h at ambient temperature. Then the hexane layer, which contained methyl and ethyl esters, was removed from the reagent layer, transferred twice to a cap Pyrex tube with anhydrous

sodium sulfate, and stored in vial at −20°C until GC analysis.

2.3.4. Chromatographic conditions

The final extract was concentrated, under a stream of nitrogen, to 0.1 ml; 1 μl of extract was injected (split 60:1); temperature was held at 50°C for 10 min, raised to 220°C at 2°C/min and then held for 15 min; injector and detector were both at 250°C. The carrier gas was helium, at 1 ml/min. A chromatogram of a wine sample is shown in Fig. 1, together with the identification of the peaks.

2.4. Relative response factors of fatty acids as methyl esters

Three internal standards were used (C7, C13 and C17); the relative response factors were calculated for each methyl ester, in relation to all three internal standards (Table 1). The relative response factors were established from a standard solution of methyl esters in hexane; this solution was prepared three times, and three dilutions were prepared from each solution. The concentration range obtained is shown in Table 1. Two aliquots of 1 ml of each dilution were concentrated to 0.1 ml and 1 μl was injected; for every methyl ester, $n=18$ (3 solutions×3 dilutions×2 aliquots).

2.5. Relative response factors of ethyl esters

The C9E for C6E, C8E and C10E, and the C15E for the remaining ethyl esters were used as ethyl ester I.S.; the relative response factors were calculated with a standard solution of ethyl esters in hexane prepared in triplicate; from every solution, four dilutions were prepared. The concentration range studied is shown in Table 1. Internal standards were added: 0.896 mg/l of C9E and 0.145 mg/l of C15E for the two most concentrated dilutions, and 0.045 and 0.015 mg/l of C9E and C15E, respectively, for the two less concentrated dilutions; two aliquots of 1 ml of every dilution were concentrated to 0.1 ml and 1 μl was injected; for every ethyl ester $n=24$ (3 solutions×4 dilutions×2 aliquots).

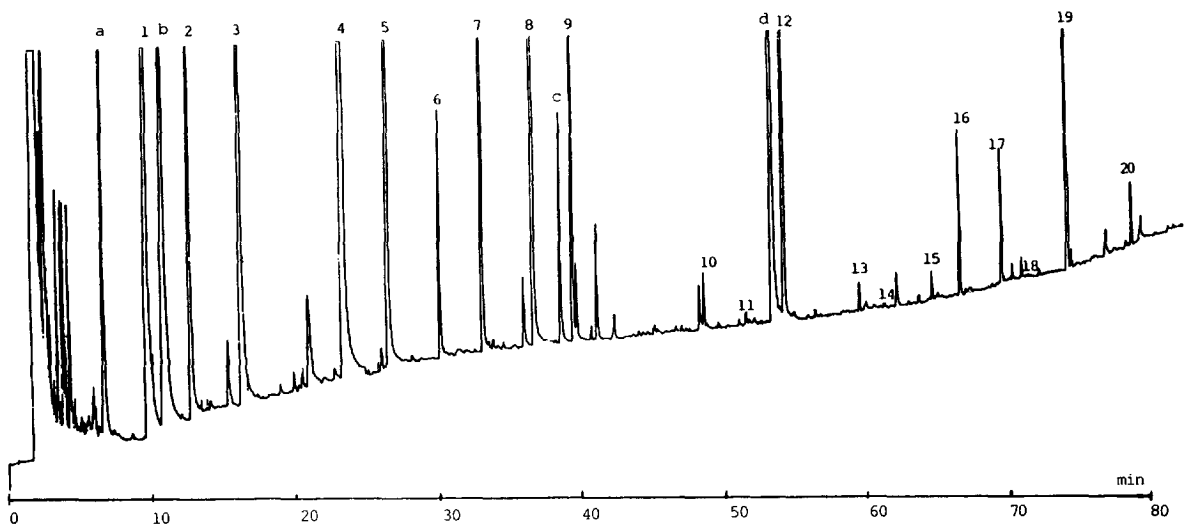

Fig. 1. Chromatogram of a wine sample obtained with the proposed method: reagent H_2SO_4 in methanol at room temperature for 3 h; 1 μl of extract was injected (split 60:1), temperature was held at 50°C for 10 min, raised to 220°C at 2°C/min and then held for 15 min, injector and detector were both at 250°C, and the carrier gas was helium, at 1 ml/min. Peaks: 1 C6M; 2 C6E; 3 C7M (I.S.); 4 C8M; 5 C8E; 6 C9M; 7 C9E (I.S.); 8 C10M; 9 C10E; 10 C12M; 11 C12E; 12 C13M (I.S.); 13 C14M; 14 C14E; 15 C15M; 16 C15E (I.S.); 17 C16M; 18 C16E; 19 C17M (I.S.); 20 C18M. Tentatively, by GC–MS: c=decenoic acid ethyl ester; and by retention time: a=isoamyl acetate; b=isoamyl alcohols; d=2-phenylethanol. C*n*M: Fatty acid methyl ester; C*n*E: fatty acid ethyl ester.

Table 1
Factors of response of methyl and ethyl esters, relative to internal standards

	I.S.[a]	Concentration interval (mg/l)	Relative response factor[b]
Methyl ester			
	C7M		0.962
C6M	C13M	1.570–8.960	1.159
	C17M		1.002
	C7M		0.764
C8M	C13M	1.580–15.820	1.119
	C17M		0.971
	C7M		0.813
C10M	C13M	1.990–11.380	1.043
	C17M		0.892
	C7M		0.745
C12M	C13M	0.400–2.900	1.000
	C17M		0.93
	C7M		0.685
C14M	C13M	0.220–2.420	1.023
	C17M		0.953
	C7M		0.686
C16M	C13M	0.180–0.980	1.043
	C17M		0.944
	C7M		0.846
C18M	C13M	0.180–1.000	1.283
	C17M		0.934
Ethyl ester			
C6E	C9E	0.361–5.412	1.391
C8E	C9E	0.025–8.028	1.074
C10E	C9E	0.010–3.130	1.048
C12E	C15E	0.004–0.098	0.787
C14E	C15E	0.004–0.091	0.975
C16E	C15E	0.016–1.456	1.083
C18E	C15E	0.047–0.442	1.022

[a] I.S. concentration: C7M=2.38, C13M=1.30 and C17M=0.90 mg/l; C9E=0.896 and 0.045 mg/l, C15E=0.145 and 0.015 mg/l.
[b] For methyl esters, slope of equation: C*n*MC/ISC=(C*n*MA/ISA)*b*+*a*.
C*n*MC: methyl ester concentration (mg/l); ISC: internal standard concentration (mg/l); C*n*MA: methyl ester area; ISA: internal standard area.
For ethyl esters, slope of equation: C*n*EC/ISC=(C*n*EA/ISA)*b*+*a*.
C*n*EC: ethyl ester concentration (mg/l); C*n*EA: ethyl ester area.

2.6. Study of derivatization

The derivatization reagents, 3% H_2SO_4 in methanol and 0.5 *M* sodium methoxide in methanol were studied, under the same conditions of derivatization, with six aliquots (three aliquots for each reagent) of 10 ml of model solution (Table 2). Time and temperature conditions of the selected reagent (3% H_2SO_4 in methanol) were studied: a wine sample was extracted six times: two extracts were derivatized for 3 h, another two for 2 h, always at room temperature, and the last two were derivatized for 20 min at 100°C (Table 2).

2.7. Transesterification study

To correct for the error due to transesterification, a possible relation between ethyl esters I.S. transesterification and ethyl ester transesterification was studied: an ethyl ester solution in hexane was prepared three times. From each solution, eight different dilutions were prepared obtaining a wide concentration range (Table 3). For the four most diluted ethyl esters solutions, I.S. were added to the following concentrations: C9E=0.045 mg/l and C15E=0.0145 mg/l; and for the four most concentrated solutions: C9E=0.896 mg/l and C15E=0.145 mg/l. Two aliquots of 1 ml of every dilution were derivatized with H_2SO_4 in methanol as described in Section 2.3. For every ethyl ester, a linear equation was found that relates ethyl ester transesterification with the initial concentration (Table 3). For every equation n=48 (3 solutions×8 dilutions×2 derivatized aliquots).

2.8. Accuracy in the transesterification of ethyl esters

Fatty acids, ethyl esters and internal standard solution in hexane were prepared twice, with similar concentrations as samples. Three aliquots of 1 ml of every standard solution were derivatized according to the method described in Section 2.3. The free fatty acid and ethyl ester concentrations were calculated using transesterification equations.

2.9. Validation of proposed method

2.9.1. Detection and quantification limits

Three aliquots of 50 ml of Milli-Q water were analyzed in triplicate, with increasing detector sen-

Table 2
Study of derivatization

Compound	Comparison between acid and base catalyzed derivatization in a model solution[a]			Influence of derivatization conditions with H_2SO_4 methanol in wine	
	Concentration (mg/l)	Acid-catalyzed 3% H_2SO_4 in methanol Recovery (%) ($n=3$)	Base-catalyzed 0.5 *M* sodium methoxide Recovery (%) ($n=3$)	Room temperature[b], 3 h Mean (mg/l)±S.D.[b] ($n=2$)	Temperature[b] 100°C, 20 min Mean (mg/l)±S.D.[b] ($n=2$)
C6M	8.74	95	106	4.025±0.280	4.803±0. 375
C8M	13.67	103	109	6.446±0.199	11.949±0.430
C10M	2.11	139	173	1.273±0.063	3.095±0.160
C12M	0.15	181	335	0.061±0.006	0.117±0.010
C14M	0.03	177	303	0.005 (<DL)	–
C16M	0.23	131	157	0.226±0.015	0.410±0.035
C18M	0.12	134	149	0.191±0.024	0.302±0.028
C6E	1.04	73	0	0.950±0.076	–
C8E	1.33	76	0	4.385±0.331	–
C10E	1.77	65	0	1.346±0.083	–
C12E	0.53	70	0	0.072±0.004	–
C14E	0.08	70	0	–	–
C16E	0.06	68	0	0.276±0.028	–
C18E	0.07	79	0	0.349±0.040	–

[a] Hydroalcoholic solution 10% (v/v) with glucose: 2 g/l, tartaric acid: 10 g/l, fatty acids, ethyl esters and I.S. (C17: 0.96 mg/l; C9E: 0.80 mg/l).
[b] C13 and C9E as I.S..

sitivity; nine blanks were obtained and, in each one, ten peaks around the integration zone corresponding to every fatty acid and ethyl ester were quantified. With the average value and the standard deviation (S.D.) of every compound concentration ($n=9$ chromatograms×10 peaks=90) the detection (DL) and quantification (QL) limits were calculated (Table 4) for each fatty acid and ethyl ester, using the following equations [14]: DL=$x\pm3$ S.D. and QL=$x\pm10$ S.D..

Table 3
Linear equations for ethyl ester transesterification

Ethyl ester	Concentration interval (mg/l)	Equation[a]	
		a	*b*
C6E	0.146–16.236	0.033	0.458
C8E	0.025–24.084	0.034	0.801
C10E	0.010–8.622	0.050	1.141
C12E	0.001–0.119	0.022	0.854
C14E	0.002–0.091	0.009	0.970
C16E	0.016–1.638	0.072	0.945
C18E	0.005–0.442	0.112	1.108

[a] Equation: $CnE_{nt}A/IS_{nt}A=a+(CnE/ISC)b$
$CnE_{nt}A$: concentration of ethyl ester (mg/l); $IS_{nt}A$: area of internal standard non transesterified; CnE: concentration of ethyl ester (mg/l); ISC: I.S. concentration C9E=0.896 and 0.045 mg/l; C15E=0.145 and 0.015 mg/l; *a*: intercept; *b*: slope.

2.9.2. Precision

Chromatographic injection repeatability and method reproducibility were studied and expressed as relative standard deviations (R.S.D.s).

2.9.3. Chromatographic repeatability

One of the extracts of wine was injected six times ($n=6$).

2.9.4. Method reproducibility

Three aliquots of 50 ml of the same sample (must and wine) were treated as described in Section 2.3. See Table 5 for results.

2.9.5. Accuracy

This was expressed as the percentage recovery. For free fatty acids recovery in must, three aliquots of 50 ml of must were treated as described in Section 2.3; another three aliquots of 50 ml of the same must were spiked with 50 μl of fatty acid standard

Table 4
Detection (DL) and quantification (QL) limits of fatty acids and ethyl esters

Fatty acid	DL (mg/l)	QL (mg/l)	Ethyl ester	DL (mg/l)	QL (mg/l)
C6	0.010	0.028	C6E	0.013	0.039
C8	0.005	0.015	C8E	0.003	0.008
C10	0.006	0.017	C10E	0.001	0.003
C12	0.016	0.046	C12E	0.004	0.011
C14	0.006	0.016	C14E	0.006	0.018
C16	0.005	0.015	C16E	0.008	0.022
C18	0.067	0.196	C18E	0.066	0.197

solution in methanol. The concentrations added are shown in Table 6. For free fatty acid and ethyl ester recovery in wine, three aliquots of 50 ml of wine were treated as described in Section 2.3; another three aliquots of 50 ml of the same wine were spiked with 50 μl of ethyl ester standard solution in methanol, and with 50 μl of fatty acid standard solution in methanol. The concentrations added are shown in Table 6. For the more volatile fatty acids (C6, C8 and C10) the recovery was studied at two concentration levels.

3. Results and discussion

It was decided to use three internal standards: the C7, C13 and C17. Relative response factors of each methyl ester in relation to three internal standards (C7, C13 and C17) were calculated as slope of equations shown in Table 1. The correlation coefficients were higher than 0.999 for all the methyl esters. The fatty acid recovery values and standard deviations, in must and wine, were used to select the most appropriate internal standard for each fatty acid (Table 6). Therefore, for every fatty acid, three recovery values were obtained, depending on the internal standard used in quantification; for each fatty acid, the best recovery values and their standard deviations established the C7 I.S. to quantify C6 and C8, the C13 I.S. for C10, C12 and C14, and the C17 I.S. for C16 and C18 as the most appropriate internal standards.

Table 5
Fatty acid and ethyl ester reproducibility

	Must			Wine		
	Mean (mg/l) ($n=9$)	S.D.	R.S.D. (%)	Mean (mg/l) ($n=9$)	S.D.	R.S.D. (%)
Fatty acid						
C6	0.063	0.007	10.4	4.434	0.231	5.2
C8	0.027	0.004	13.3	12.086	0.469	3.9
C10	0.013	0.001	10.1	2.967	0.149	5.0
C12	0.166	0.016	9.4	0.473	0.042	8.9
C14	0.057	0.006	11.1	0.064	0.007	10.2
C16	0.875	0.059	6.8	0.422	0.028	6.7
C18	0.143 (<QL)	0.012	8.4	0.110 (<QL)	0.010	9.4
Ethyl ester						
C6E	0.013 (<DL)	–	–	1.441	0.107	7.4
C8E	0.003 (<DL)	–	–	2.073	0.121	5.8
C10E	0.001 (<DL)	–	–	0.614	0.019	3.1
C12E	0.004 (<DL)	–	–	0.085	0.005	6.4
C14E	0.006 (<DL)	–	–	0.011	0.001	10.4
C16E	0.008 (<DL)	–	–	0.046	0.004	9.1
C18E	0.066 (<DL)	–	–	0.029 (<DL)	–	–

Table 6
Recovery of fatty acids and ethyl esters in must and wine

	Wine			Must		
	Amount in wine (mg/l) mean±S.D. ($n=9$)	Amount added (mg/l)[a]	Recovery[b] (%), mean±S.D. ($n=9$)	Amount in must (mg/l) mean±S.D. ($n=9$)	Amount added (mg/l)	Recovery[b] (%), mean±S.D. ($n=9$)
Fatty acid						
C6	4.434±0.231	4.970	102±5[c]	0.063±0.007	0.046	87±9
		2.512	92±12[d]			64±13
			79±9[e]			49±10
C8	12.086±0.469	9.830	99±2	0.027±0.004	0.017	100±6
		5.150	95±6			85±9
			83±7			70±8
C10	2.967±0.149	3.042	106±7	0.013±0.001	0.012	110±2
		1.502	99±3			97±6
			90±8			68±8
C12	0.473±0.042	0.192	126±15	0.166±0.016	0.178	115±7
		0.195	109±10			93±6
			104±12			79±10
C14	0.064±0.007	0.019	122±15	0.057±0.006	0.033	108±10
		0.020	107±8			99±5
			111±8			83±8
C16	0.422±0.028	0.240	67±4	0.875±0.059	0.475	104±13
		0.242	114±8			102±10
			102±3			86±5
C18	0.109±0.010	0.096	83±10	0.143 (<QL)	0.097	134±22
		0.094	132±19			113±17
			107±8			93±4
Ethyl ester						
C6E	1.441±0.107	0.697	114±6	–	–	–
C8E	2.073±0.121	1.204	93±6	–	–	–
C10E	0.614±0.019	0.390	83±5	–	–	–
C12E	0.085±0.005	0.083	88±5	–	–	–
C14E	0.011±0.001	0.019	100±11	–	–	–
C16E	0.046±0.004	0.030	129±12	–	–	–
C18E	0.029 (<DL)	0.254	126±22	–	–	–

[a] Higher value $n=3$, lower value $n=6$.
[b] Recovery (%)=(amount founded·100)/(amount in sample+amount added).
For every fatty acid, the I.S. used was [c] C7, [d] C13 and [e] C17.

The C9E I.S. (for C6E, C8E and C10E) and the C15E I.S. (for C12E, C14E, C16E and C18E), were used as internal standards at two concentrations, to improve quantification of ethyl esters that have a wide concentration range, depending on the sample (Table 1). The linearity of the relative response factors of ethyl esters was satisfactory, with correlation coefficients always higher than 0.999.

The results of the use of the derivatization reagents, H_2SO_4 and sodium methoxide in methanol, are shown on Table 2: when sodium methoxide in methanol was used, the concentrations were higher for all the fatty acids, with recovery values greater than 100%, and ethyl esters disappeared. Thus, sodium methoxide in methanol causes total transesterification of ethyl esters to methyl esters. On the other hand, the use of H_2SO_4 in methanol causes partial transesterification. To determinate free fatty

acids using this base-catalyzed reagent, two determinations would be necessary in the same sample. One determination, with derivatization, to quantify total fatty acids; and another determination, without derivatization, to quantify ethyl esters. The total fatty acids minus the fatty acids bound as ethyl esters, would be the free fatty acids. This option was ruled out, because it is longer and more laborious than the method proposed above. Moreover, the higher ethyl ester peaks (beyond C12E) were usually masked by the peaks of non-methylated lower free fatty acids, that appear as wide bands, at the end of the chromatogram.

The proposed derivatization of free fatty acids with (3%) H_2SO_4 in methanol, for 3 h at room temperature produced total methylation of free fatty acids and partial ethyl ester transesterification, releasing the fatty acids and converting them to methyl esters (Table 2). To determine free fatty acids it was necessary to study transesterification. Thus, the free fatty acids and their ethyl esters could be determined with a single chromatogram.

The minimal reaction time with (3%) H_2SO_4 in methanol was 3 h, so if the reaction lasted 2 h free fatty acids appeared non-derivatized. Moreover, if the temperature was raised to 100°C and the time was reduced to 20 min, transesterification was total (Table 2).

In the partial transesterification study, using standard solutions of ethyl esters, for every ethyl ester, a linear equation (Table 3) was found, with satisfactory linearity ($r>0.99$). These equations were obtained over a wide concentration range; therefore, the different levels of ethyl esters in samples were taken into account:

$$\frac{C\mathit{n}E_{nt}A}{IS_{nt}A} = \frac{C\mathit{n}E}{ISC} \cdot b + a \quad (1)$$

where $C\mathit{n}E_{nt}A$ is the area of non-transesterified ethyl ester in the chromatogram, $IS_{nt}A$ is the area of non-transesterified internal standard, $C\mathit{n}E$ is the concentration of ethyl ester, ISC is the concentration of ethyl ester internal standard, a is the intercept and b is the slope.

In samples, the concentration of ethyl ester ($C\mathit{n}E$) was obtained from Eq. (1):

$$C\mathit{n}E = \frac{C\mathit{n}E_{nt}A}{IS_{nt}A} - a \cdot \frac{ISC}{b} \quad (2)$$

The concentration of free fatty acids was obtained as follows: from $C\mathit{n}E$, calculated according to Eq. (2), the corresponding area ($C\mathit{n}EA$) was calculated using the expression of relative response factor (Table 1). This area was used to determine the transesterified ethyl ester area ($C\mathit{n}E_tA$), using the expression:

$$C\mathit{n}E_tA = C\mathit{n}EA - C\mathit{n}E_{nt}A \quad (3)$$

Using fatty acid relative response factors (Table 1), the transesterified ethyl ester area ($C\mathit{n}E_tA$) was converted to transesterified fatty acid concentration ($C\mathit{n}_tC$). In addition, the methyl ester area obtained on the chromatogram was converted to fatty acid concentration ($C\mathit{n}C$). $C\mathit{n}C$ is the sum of the free fatty acid concentration ($C\mathit{n}FC$) and the concentration of the transesterified fatty acid from the corresponding ethyl ester ($C\mathit{n}_tC$). Therefore, free fatty acid concentration was obtained from the expression:

$$C\mathit{n}FC = C\mathit{n}C - C\mathit{n}_tC \quad (4)$$

The accuracy of transesterification Eq. (1) and Eqs. (2)–(4), in ethyl esters and free fatty acid quantification, expressed as percentage of recovery, shows the difference between the concentrations of the solution standard prepared and the concentrations calculated from Eqs. (1)–(4). Every ethyl ester was quantificated with an accuracy near to 100% (91–107%). For fatty acids, the accuracy was satisfactory (86–107%), except for the most volatile C6 (80%).

For every free fatty acid and ethyl ester, the detection limits were lower than 0.02 mg/l, except for C18 and C18E, (about 0.07 mg/l) (Table 4). The quantification limits were less than 0.05 mg/l except for C18 and C18E with values of 0.20 mg/l. The detection and quantification problems of C18 and C18E could be due to their retention time: they appear at the end of the chromatogram, where the artifacts and impurities increased and the resolution was not optimal.

The repeatability of chromatographic injection was very high for all the fatty acids and ethyl esters (R.S.D.<3%), with the exception of C18 (15.5%), C16E (6.8%) and C18E (83.3%). The wine used to determine repeatability had a lower concentration of

C18 (0.135 mg/l) than its quantification limit (0.196 mg/l). The C18E concentration was below the detection limit, and therefore its R.S.D. was higher that of the other ethyl esters. The reproducibility values of the method, expressed as R.S.D. (Table 5), were compared with the interval of intra-laboratory precision obtained by applying the equation of Horwitz [15]. This author defines an equation, which depends on the concentration of the substance determined, to calculate the precision interval that would be acceptable. All the R.S.D. values (Table 5) were inside the Horwitz interval, and, therefore, satisfactory. In wine, C18E repeatability was not determined because the concentration in the sample was below its detection limit.

The recovery percentages were satisfactory for all the fatty acids, in both must and wine, with values between 86 and 109% (Table 6). Moreover, they were precise. In wine, the recovery values of the ethyl esters ranged between 83 and 129%, and were also precise (Table 6). The recovery value of C16E was the worst, but its concentration in wine was low. The C18E recovery was calculated from the amount added to wine, since the initial concentration in the sample was below the detection limit.

4. Conclusions

A single analytical procedure was developed for the determination of free fatty acids (C6–C18), and the fatty acids bound as ethyl esters (C6E–C18E). The derivatization reagent selected, (3%) H_2SO_4 in methanol at room temperature for 3 h, produced total methylation of free fatty acids, although it also causes partial transesterification. A corrective equation was obtained that allows calculation of the initial concentration of every ethyl ester, the concentration of fatty acid attributable to transesterification, and the concentration of free fatty acids. The C13 I.S. was the most suitable for quantification of medium-chain fatty acids (C10, C12 and C14), the C7 I.S. for the more volatile fatty acids (C8 and C10) and the C17 I.S. for the longer chain fatty acids (C16 and C18). Validation of the method (detection and quantification limits, reproducibility and accuracy) shows that the procedure was satisfactory for all the fatty acids and their ethyl esters in must and wine. In this method, the study of transesterification was laborious, but, once developed, its application in samples was easier and faster than the other methods reported in the literature, and more information was furnished. The method may also be used to evaluate other flavor compounds that appear on the chromatogram (Fig. 1). These compounds are mainly masked in the procedures that do not include derivatization. The identification and possible quantification of such compounds is in progress.

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