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Direct GC–(EI)MS determination of fatty acid alkyl esters in olive oils

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ABSTRACT

A new analytical method for fatty acid alkyl esters (FAAEs) determination by GC–MS in virgin olive oils is proposed. No sample preparation is required and FAAEs are directly thermo-desorbed and cryo-focalised in the cooled injector of a GC–MS (EI) instrument. The analytical conditions were optimized by Design of Experiment (DoE) techniques (an exploratory Plackett–Burman design followed by a factorial design on three selected variables). After the improvement of method performances, several samples of extra virgin and low quality virgin olive oils were analyzed both by the new method and by the Official EU Method of analysis. The application of Principal Component Analysis to the obtained results confirmed that the ability of the proposed method to discriminate between extra virgin and lower quality olive oils is at least equal to that of the Official Method, but the new method is faster, simpler, requires a much lower amount of organic solvents and significantly enhances method repeatability.

1. Introduction

The esterification of free fatty acids (FFA) with low molecular weight alcohols, such as methanol and ethanol, can easily take place in an acid medium. The development of the first order reaction strongly depends on the reagent contents and on temperature [1]. The content of fatty acid alkyl esters (FAAEs) in olive fruits is highly related to their health conditions and is obviously higher if olives undergo hydrolytic and fermentative processes, thus increasing the amounts of both free fatty acids and alcohols. Oils obtained from fermented fruits are low quality virgin olive oils; their unpleasant sensorial features prevent them from being classified as extra virgin olive oils, thus decreasing their commercial value, and can also prevent them from being considered edible without a refining process. Thus, a so-called "soft deodorization", employing temperatures below 100 °C, is often used to correct the sensorial defects of these oils so that they can be used for an illegal non-reported blending with extra virgin olive oils. The soft deodorization does not induce those chemical modifications, such as the formation of *trans* fatty acids, that are commonly used to single out "refined" olive oils [1]; therefore, the availability of reliable markers of this adulteration can be a relevant tool against the increasing presence on the market of soft deodorized olive oils, pure or blended. Thus, several methods were then proposed to single out these oils, including the detection of chlorophyll derivatives [2] and the quantification of CLAs (conjugated linoleic acids) [3] and diglycerides isomers [4], which are generated during soft deodorization. However, these compounds can also be generated during olive oils ageing [1] and the previously mentioned analytical methods failed to discriminate soft deodorized from aged olive oils. In order to detect the presence of low quality and deodorized oils in extra virgin olive oils, the European Union (EU) has recently adopted the Official Method for FAAEs determination by GC–FID [5] previously proposed by the International Olive Council (COI) [6]. The basis of the method is that the soft deodorization process does not lower the content of FAAEs of low quality oils. Unfortunately, this method requires a preliminary separation of FAAEs from the oil by means of a classical glass column chromatography, using silica gel as adsorbent, with hexane and ethyl ether used as eluents. Solvents are finally evaporated by a Rotavapor. This Official procedure is time consuming, needs high quantities of solvents (approximately up to 300 mL total) and also shows low repeatability, as reported in the COI method [6]. Recently, an alternative purification of FAAEs by SPE on silica cartridges was proposed [1,7] and validated [7]: though SPE reduces the solvent amounts to approximately 30 mL, the use of toluene instead of diethyl ether enhances the health concern, without improving repeatability [7]. Furthermore in 2012 the COI proposed a modification to its method that involves a reduction of both the amounts of analyzed samples and the volumes of the employed solvents (approximately 45 mL) [8]. Though COI Decision 21/100-V/2013 [9] recommends to the members the "provisional application" of this method, at present EU has not modified its Official Method.

In this paper a new, fast and simple analytical method for FAAE determination in virgin olive oil by thermo-desorption coupled

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with GC and (EI)MS detector is proposed. Thermo-desorption is a useful technique that allows evaporating and directly transferring several solutes from fats or oils to a chromatographic system, and in the past years it was proposed for the analysis of different compounds in edible oils [10–12]. No sample preparation is required for FAAE determination and the oil sample is directly introduced in the thermo-desorption device coupled with the injector of the GC–MS apparatus. The analytical parameters were optimized by Design of Experiment (DoE) techniques.

The ability of the proposed method to discriminate oil quality is then tested by analyzing two sets of extra virgin and lower quality oils, categorized according to their FAAEs content determined by the EU Official Method. The correlation between the two methods is studied and Principal Component Analysis (PCA) is applied to the results of FAAEs determination obtained by the two methods.

2. Materials and methods

2.1. Instrument

GC–MS analyses were performed by an Agilent 6890 GC equipped with an Agilent 5973 (EI)MS detector (Agilent Technologies, Palo Alto, CA, USA), using the MSD Chemstation Software E.02.01 for data acquisition and processing. The mass-spectrometer interface temperature was set at 250 °C. The temperature of the ion source was 230 °C, electron energy 70 eV and quadrupole temperature 150 °C.

A 30 m × 0.25 mm × 0.25 μm film thickness DB-5MS fused-silica capillary column (J&W, Folsom, CA, USA) was used for the gas chromatographic analysis, at a constant Helium flow rate. A 10 min post-run at 300 °C was always performed.

The injector was a Gerstel CIS4 programmed temperature injector (Gerstel, GmbH, Mülheim an der Ruhr, Germany), which can be cooled down to −150 °C (with liquid nitrogen) and heated up to 350 °C. The injector was coupled by a capillary transfer line with the external thermal extraction unit (TDS2, Gerstel), containing a removable glass tube for the sample desorption.

The oil sample was directly introduced in the TDS2 glass tube and heated. A high Helium flow rate (100 mL/min) allowed transferring the desorbed compounds to CIS4, where they were cryo-trapped and focused on the cooled surface of the empty glass liner.

2.2. Instrument performance improvement

The experiments leading to the improvement of the GC method performances were performed in Total Ion Current (TIC) mode and the optimal setting of the extraction and chromatographic variables was defined by DoE. Previous experiences and preliminary experiments had allowed selecting among the many variables those supposed having a stronger influence on the chromatographic separation: three variables referred to the desorption device, three variables to the cooling injector and four variables to the conditions of GC separation (Table 1). A Plackett–Burman design [13] was used to estimate the actual effect of the 10 selected variables on the chromatographic separation. Since the design for 10 variables requires 12 experiments, thus allowing the estimation of 12 coefficients (one constant + 11 linear terms), a column for an hypothetical (dummy) variable X_{11} was added. In order to have an estimation of the experimental variability, one of the 12 experiments of the experimental matrix has been replicated. The 13 chromatograms have been ranked according to a visual analysis, taking into account peak resolutions, peak areas and analysis time. The ranking has been performed by three operators, independently from each other, and the global response associated to each experiment was the sum of the scores (best = score 13, worst = score 1). The coefficients of the obtained model showed that variables 2, 7 and 10 were the most relevant variables (Fig. 1), and they were further studied by a factorial design (8 experiments + 1 replicate) (Table 2).

2.3. Final analytical conditions

The quantification of FAAEs was obtained by methyl heptadecanoate as internal standard (Fluka, purity > 99%). 1 mL of the standard

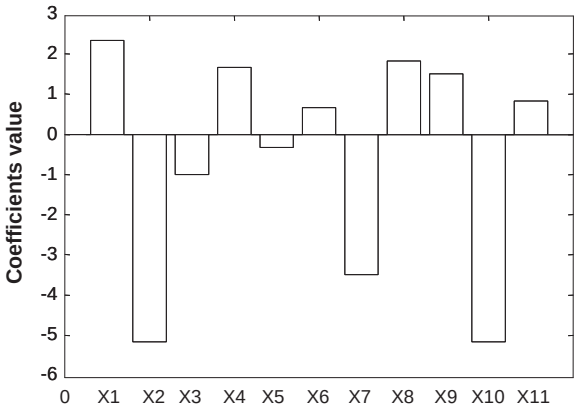

Fig. 1. The coefficients of the model obtained by the Plackett–Burman design.

Table 1
The Plackett–Burman experimental plan. Experiment 10 was performed twice.

Exp. N.	TDS2			CIS4				GC			
	Ramp rate °C/min	Final T °C	Final t min	Cooling T °C	Final T °C	Split ratio	Column flow mL/min	Init. T °C	Ramp rate °C/min	Final T °C	Total score
1	60	250	1	−20	300	100	0.8	40	3	230	**16**
2	20	250	2	−60	300	100	1.2	40	3	200	**11**
3	60	200	2	−20	250	100	1.2	70	3	200	**32**
4	20	250	1	−20	300	50	1.2	70	6	200	**20**
5	20	200	2	−60	300	100	0.8	70	6	230	**23**
6	20	200	1	−20	250	100	1.2	40	6	230	**20**
7	60	200	1	−60	300	50	1.2	70	3	230	**20**
8	60	250	1	−60	250	100	0.8	70	6	200	**32**
9	60	250	2	−60	250	50	1.2	40	6	230	**6**
10a	20	250	2	−20	250	50	0.8	70	3	230	**14**
10b	20	250	2	−20	250	50	0.8	70	3	230	**13**
11	60	200	2	−20	300	50	0.8	40	6	200	**38**
12	20	200	1	−60	250	50	0.8	40	3	200	**28**

Table 2
The values of the variables in the 2^3 factorial design (experiment number 5 was performed twice). Bold characters are used for the 3 variables studied at 2 different levels. Italic singles out the best conditions.

Exp. N.	TDS2			CIS4				GC		
	Ramp rate °C/min	Final *T* °C	Final *t* min	Cooling *T* °C	Final *T* °C	Split ratio	Column flow mL/min	Init. *T* °C	Ramp rate °C/min	Final *T* °C
1	60	**240**	1	−20	300	1:50	**0.8**	70	6	**220**
2	60	**210**	1	−20	300	1:50	**0.8**	70	6	**210**
3	*60*	***240***	*1*	*−20*	*300*	*1:50*	***1.0***	*70*	*6*	***220***
4	60	**240**	1	−20	300	1:50	**0.8**	70	6	**210**
5	60	**240**	1	−20	300	1:50	**1.0**	70	6	**210**
6	60	**210**	1	−20	300	1:50	**0.8**	70	6	**220**
7	60	**210**	1	−20	300	1:50	**1.0**	70	6	**210**
8	60	**210**	1	−20	300	1:50	**1.0**	70	6	**220**

Table 3
The FAAEs retention times and the ions selected for SIM mode.

	t_R		*m/z*	
Methyl palmitate	22.63	270.2 $[M]^+$	74.0	87.0
Ethyl palmitate	23.75	284.3 $[M]^+$	88.0	101.0
Methyl heptadecanoate	24.26	284.2 $[M]^+$	74.0	253.0
Methyl linoleate	25.31	294.2 $[M]^+$	81.0	95.0
Methyl oleate	25.42	296.2 $[M]^+$	74.0	87.0
Methyl stearate	25.88	298.0 $[M]^+$	74.0	87.0
Ethyl linoleate	26.49	308.2 $[M]^+$	263.2	
Ethyl oleate	26.63	310.2 $[M]^+$	88.0	
Ethyl stearate	27.16	312.2 $[M]^+$	88.0	101.0

solution (4.0 mg/100 mL in *n*-heptane) was added to 2 g of oil (exactly weighed at the third decimal digit). After *n*-heptane evaporation in the Rotavapor at 20 °C, 10 μL of oil were introduced in the glass tube of TDS2, which was subsequently inserted in the desorption unit and heated. The starting desorption temperature (20 °C) was raised to 240 °C at 60 °C/min, keeping TDS2 at this temperature for 1 min. In this desorption step, a 100 mL/min Helium flow transferred in the vapor phase in Solvent Vent Mode to the CIS4 injector, cooled at −20 °C, allowing the cryo-focalization of the desorbed analytes. At the end of the desorption step, the glass tube containing the sample was replaced by an empty one (Sample Remove Mode). Then, at the injection time the temperature of the injector was raised at 12 °C/s up to 300 °C, held for 1 min, with a 50:1 split ratio. At the same time, the column temperature, which was previously held constant at 70 °C, was raised to 220 °C with a 6 °C/min rate, holding for 10 min. The analysis was carried out in constant flow mode (Helium flow: 1.0 mL/min) and a 10 min post-run at 300 °C was then performed.

In order to improve method selectivity, SIM mode was then used for the quantification of FAAEs. Table 3 reports the retention times and the selected ions of the detected FAAEs.

The limit of detection for methyl heptadecanoate in oil (i.e. the injected amount of standard that results in a peak being three times higher than the baseline noise measured in SIM in the final instrumental conditions) was 0.01 mg/kg. Recovery tests were performed by thermo-desorbing similar amounts of pure methyl heptadecanoate (100, 50 and 25 μL of the standard solution) and methyl heptadecanoate in virgin olive oils (2, 1 and 0.5 mL of the standard solution added to 2 g of oil followed by solvent evaporation): the recovery of methyl heptadecanoate in oil was >98%. The RSD of the overall method in SIM mode was lower than 2.5% for each FAAE and lower than 2% for the sum of FAAEs. The repeatability ($2.8 \times s$) of the total amount of FAAEs was always lower than 2 mg/kg even considering samples with different FAAEs amounts, and the repeatability of the ratio ethyl esters/methyl esters (FAEEs/FAMEs) was lower than 0.01.

2.4. Method performance evaluation

Forty virgin olive oils of different geographical origin (Italy, Spain, Tunisia, Greece, pure or mixed together) from the 2010 to 2011 and the 2011 to 2012 olive crops were analyzed by the proposed method. Preliminarily, in order to assess their compliance with EU legislation, the oil samples had been analyzed at the Customs Agency laboratory, following EU Regulation 2568/91 and its further amendments [14]. Acidity, peroxide index, UV absorbance, fatty acid and sterol composition, ΔECN42, erythrodiol and uvaol, stigmastadiene, waxes, FAAEs and organoleptic scores (fruity and defect medians) were determined, so that the 40 oil samples were classified as extra virgin olive oils (Table 4) and lower quality virgin olive oils (Table 5).

2.5. Statistical analysis

Statistical analysis was performed in Matlab 6.1 (The MathWorks Inc., Natick, MA, USA), by using routines written by one of the authors; the Passing-Bablok regression [15] was performed by using the free routine Passing Bablok [16] for Matlab.

3. Results and discussion

Starting from the conditions previously reported for the determination of volatile esters in whiskey [17], the preliminary experiments on olive oils showed that the separation of each FAAE from the predominating free fatty acid was difficult in the reported analytical conditions. In fact, FFAs were desorbed in the TDS2 unit and cryofocalised in the CIS4 injector together with FAAEs and the differences among their boiling points did not allow a significant enrichment of FAAE fraction by thermodesorption. Then, a Plackett–Burman design was applied in order to explore the effect of several variables on the resolution between critical pairs and on the whole chromatographic profile. The multivariate approach was preferred to the One Variable at a Time (OVAT) approach since it allows to obtain information of higher quality by performing a lower number of experiments. Previous experiences [18] and preliminary investigations in TIC allowed to single out the 10 variables that were expected to affect at the highest degree the resolution: six were instrumental variables of the TDS2-CIS4 system (three related to the extraction step by TDS2 and three to the injection step by CIS4), and four were GC instrumental parameters. The order of the 13 experiments (12 experiments + 1 replicate, reported in Table 1) was randomized. As described in the Instrument Performance Improvement sub-section, the ranking of the chromatograms was performed by each of the three operators, taking into account both peak resolutions and peak intensities; if

Table 4
The analytical parameters detected for the 20 extra virgin olive oil samples following EU Official Methods of Analysis [5].

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Acidity (%)	0.2	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.4	0.4	0.2	0.5	0.2	0.3	0.2	0.4	0.1	0.3	0.5
Peroxide index (m_{eq} active O_2/kg)	9	11	12	9	12	9	14	13	12	10	13	8	14	9	12	8	10	11	12	9
K_{232}	1.43	2.33	1.95	1.65	2.34	2.09	2.34	2.36	2.33	2.06	2.11	1.35	2.33	1.93	2.45	1.65	2.20	1.50	2.01	2.08
K_{270}	0.10	0.13	0.13	0.13	0.12	0.11	0.11	0.13	0.18	0.11	0.13	0.08	0.12	0.17	0.15	0.10	0.13	0.09	0.13	0.123
Delta $-K$	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	−0.002	−0.004	0.000	0.000	0.000	0.000	0.000	−0.004	0.000	−0.002
Fatty acids (%)[a]																				
myristic	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
linolenic	0.58	0.68	0.65	0.61	0.64	0.70	0.65	0.68	0.66	0.70	0.75	0.60	0.70	0.63	0.69	0.64	0.66	0.62	0.65	0.65
arachidic	0.39	0.42	0.14	0.38	0.39	0.42	0.43	0.43	0.42	0.39	0.45	0.39	0.41	0.36	0.42	0.41	0.41	0.43	0.41	0.41
eicosenoic	0.29	0.22	0.25	0.25	0.23	0.22	0.23	0.21	0.26	0.29	0.28	0.29	0.19	0.23	0.21	0.31	0.20	0.30	0.26	0.23
behenic	0.14	0.12	0.12	0.11	0.11	0.12	0.12	0.12	0.12	0.12	0.15	0.12	0.11	0.10	0.13	0.16	0.12	0.15	0.12	0.13
lignoceric	0.06	0.07	0.06	0.05	0.06	0.07	0.06	0.07	0.06	0.06	0.01	0.05	0.07	0.04	0.08	0.06	0.07	0.06	0.06	0.06
ΔECN42	0.0	−0.1	0.1	0.1	0.2	0.0	−0.1	−0.2	0.1	0.1	0.1	0.0	−0.1	−0.1	−0.3	0.0	−0.1	−0.1	0.1	0.0
FAAEs (mg/kg)	25.2	48.7	70.0	62.2	47.0	29.0	19.1	17.8	41.3	18.5	41.0	10.3	21.0	51.2	26.0	8.4	19.1	10.9	41.8	18.5
FAEEs/FAMEs	0.41	0.69	1.04	1.76	0.77	0.37	0.93	0.75	1.47	0.93	1.04	0.73	0.50	2.54	0.81	0.66	0.56	1.11	0.95	0.73
Sterols (%)[a]																				
cholesterol	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
brassicasterol	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
campesterol	3.4	3.3	3.3	3.4	3.3	3.4	3.5	3.4	3.3	3.6	3.5	3.4	3.2	3.3	3.4	3.6	3.3	3.7	4.0	3.2
stigmasterol	0.6	0.6	0.7	0.9	0.6	0.4	0.4	0.4	0.6	0.7	0.6	0.4	0.4	0.8	0.4	0.8	0.4	0.5	0.5	0.5
beta-sitosterol	95.0	94.9	94.9	94.8	95.1	94.9	94.8	94.9	95.1	94.9	95.0	95.4	95.1	95.0	94.6	94.8	95.1	94.7	94.6	94.9
delta-7-stigmastenol	0.1	0.3	0.2	0.2	0.2	0.3	0.3	0.2	0.3	0.1	0.2	0.1	0.3	0.2	0.3	0.1	0.2	0.2	0.2	0.4
Total sterols (mg/kg)	1465	2124	1678	1232	1666	2114	2009	2194	1650	1570	1733	1425	2349	1253	2108	1469	2166	1299	1618	1868
Erythrodiol and uvaol (%)	1.4	1.6	1.9	1.3	1.3	1.4	1.7	1.5	1.5	0.9	2.1	1.1	1.5	1.3	1.4	1.5	1.7	1.8	1.2	1.4
Stigmastadiene (mg/kg)	0.00	0.01	0.02	0.03	0.01	0.01	0.05	0.00	0.00	0.00	0.01	0.01	0.00	0.02	0.00	0.01	0.01	0.00	0.01	0.00
Waxes (mg/kg)	19	68	72	49	43	44	45	43	51	48	50	55	62	47	47	60	65	27	50	71
Organoleptic evaluation																				
defect median	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
fruit median	3.4	2.9	3.7	3.1	2.8	4.8	3.3	3.4	3.5	3.7	4.6	4.9	4.5	2.8	3.4	5.0	4.3	4.8	3.3	3.9

[a] For fatty acids and sterols only the items subjected to a legal limit are reported.

Table 5
The analytical parameters determined for the 20 low quality virgin olive oil samples following EU Official Methods of Analysis [5].

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Acidity (%)	0.5	0.5	0.6	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.3	0.4
Peroxide index (m_{eq} active O_2/kg)	11	18	11	13	12	14	13	12	13	14	15	12	11	11	14	11	11	10	13	13
K_{232}	2.16	1.90	1.92	2.13	2.00	1.88	1.82	1.77	1.86	1.81	1.81	1.75	1.75	1.78	1.77	1.75	1.89	1.92	1.86	1.96
K_{270}	0.14	0.16	0.13	0.14	0.12	0.16	0.16	0.13	0.12	0.12	0.11	0.13	0.12	0.12	0.13	0.13	0.12	0.16	0.12	0.13
Delta $-K$	0.001	0.000	0.002	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.003	0.000	0.000	0.000	−0.001	0.001	0.000	0.000	0.00	0.00
Fatty acids (%)[a]																				
myristic	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01
linolenic	0.67	0.62	0.69	0.65	0.65	0.62	0.62	0.62	0.62	0.63	0.63	0.64	0.63	0.63	0.63	0.63	0.65	0.65	0.61	0.64
arachidic	0.41	0.39	0.41	0.40	0.40	0.38	0.38	0.38	0.38	0.39	0.39	0.39	0.39	0.38	0.39	0.38	0.40	0.40	0.36	0.39
eicosenoic	0.25	0.24	0.26	0.23	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.25	0.24	0.25	0.24	0.25	0.25	0.24	0.24
behenic	0.12	0.11	0.11	0.11	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.11	0.11	0.10	0.10	0.12	0.12	0.10	0.10
lignoceric	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.05
ΔECN42	0.1	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.2	0.2	0.0	0.0
FAAEs (mg/kg)	130	207	236	107	78	106	99	93	93	92	77	110	113	105	123	105	70	72	105	75
FAEEs/FAMEs	3.4	2.9	3.1	1.8	2.2	2.5	2.2	2.3	2.4	2.3	2.2	1.6	1.5	1.6	1.1	1.8	1.7	1.7	2.0	1.5
Sterols (%)[a]																				
cholesterol	0.1	0.3	0.1	0.1	0.1	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.3	0.1	0.2	0.2	0.2	0.2	0.3	0.2
brassicasterol	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
campesterol	3.3	3.5	3.2	3.3	3.4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.4	3.2	3.4	3.4
stigmasterol	1.0	1.0	1.1	0.9	0.9	1.0	1.0	1.1	1.1	1.1	1.1	1.0	1.1	1.1	1.0	1.0	0.9	0.9	0.8	1.0
beta-sitosterolo	94.8	94.5	94.6	94.8	94.9	94.6	94.8	94.8	94.9	94.8	94.7	94.9	94.7	94.9	94.7	94.8	94.7	94.6	94.0	94.7
delta-7-stigmastenol	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Total sterols (mg/kg)	1615	1318	1639	1636	1763	1343	1404	1346	1362	1382	1337	1386	1409	1388	1348	1336	1483	1580	1321	1354
Erythrodiol and uvaol (%)	1.7	1.6	1.5	1.4	1.6	1.3	1.2	1.3	1.3	1.3	1.2	1.4	1.3	1.3	1.5	1.4	1.6	1.5	1.2	1.3
Stigmastadiene (mg/kg)	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.03	0.02	0.01	0.02	0.01	0.02
Waxes (mg/kg)	64	66	88	64	58	56	53	47	47	47	46	55	74	50	42	43	49	49	49	43
Organoleptic evaluation																				
defect median	–	3.4	2.3	1.1	1.1	1.4	1.1	1.4	2.1	0.9	2.1	2.1	2.0	2.1	2.1	2.1	1.2	1.1	2.6	1.3
fruit median	2.3.	1.5	2.2	1.7	2.7	2.2	2.1	2.2	2.2	2.4	2.5	2.4	2.6	2.4	2.6	2.4	2.2	2.5	2.1	2.3

[a] For fatty acids and sterols only the item subjected to a legal limit are reported.

they were the same for two experiments, a higher score was given to the faster one.

The coefficients of the model obtained by the experiments of the Plackett–Burman design (Fig. 1) showed that the TDS2 final temperature (variable 2), the Helium flow in the chromatographic column (variable 7) and the final temperature of the GC oven (variable 10) had a significant effect and deserved a further investigation by a factorial design. The other experimental parameters did not significantly influence the instrumental performance and thus they were set at the most convenient values on the basis of analysis time and cost.

The coefficient of the three selected variables had a minus sign in the Plackett–Burman model, meaning that a better performance was obtained when they were at the lower level. This would suggest to move the experimental domain even further in that direction, but the two temperatures (variables 2 and 10) could not be lowered below 210 °C, since this would lead to a difficult quantification of the lower peaks. In the eight experiments of the following factorial design (Table 2) the overall peak resolution was often more than satisfactory. The conditions of the third experiment, that allowed obtaining also the best sensitivity, were chosen as the final analytical conditions and are reported with italic characters in Table 2.

As far as the MS spectrometer is concerned, for FAAEs quantification the SIM acquisition parameters were carefully investigated in order to obtain a correct profiling of each peak and to enhance method repeatability: to this purpose, both peak width and cycles/s were considered, so that more than 20 points/peak were obtained. Moreover, to enhance the method sensitivity, each FAAE was detected as the sum of its molecular peak and one or two characteristic ions with high abundance (Table 3).

The ability of the proposed method to discriminate between high- and low-quality virgin olive oils was then tested by the analysis of 40 samples of virgin olive oils. Samples were preliminarily characterized for their chemical–physical and organoleptic parameters following the EU Official Methods reported in regulation 2568/91 [14] and its further amendments. All the analyzed samples conformed with the analytical parameters provided by EU regulation for virgin olive oils [5], but their content of FAAEs, determined by the Official Method and their organoleptic scores [5], allowed to single out only 20 extra virgin oils. The remaining samples had FAAEs amounts (total FAAEs or FAEEs/FAMEs ratio) and/or organoleptic scores that stood for lower quality virgin olive oil (Tables 4 and 5).

Preliminarily the amounts of FAAEs obtained by the proposed method and by the Official method were compared by Passing-Bablok regression, since classical univariate regression, being based on the assumption that the errors of x-values are negligible, cannot be correctly applied to method comparison. Fig. 2a shows the model obtained for the total FAAEs ($y=-2.65+2.71x$). The two methods were not identical (slope significantly $\neq 1$, intercept $\neq 0$), but the correlation between the detected amounts was very high ($r=0.97$), as clearly shown in Fig. 2a. The differences are not surprising if considering the lack of the FAAEs standards and the consequent application, in both methods, of the same response

Table 6
The amounts of FAAEs determined by the proposed TDS-GC–MS method. *MeP*, Methyl palmitate; *EtP*, Ethyl palmitate; *MeL*, Methyl linoleate; *MeO*, Methyl oleate; *MeS*, Methyl stearate; *EtL*, Ethyl linoleate; *EtO*, Ethyl oleate; *EtS*, Ethyl stearate.

	MeP	*EtP*	*MeL*	*MeO*	*MeS*	*EtL*	*EtO*	*EtS*	*ΣFAAEs*	*FAEEs/FAMEs*
1	0.58	0.60	0.12	0.05	0.13	0.03	0.41	0.13	2.05	1.34
2	4.22	5.78	1.18	2.55	0.67	0.28	1.99	0.61	17.3	1.00
3	3.14	5.89	0.71	2.80	0.79	0.19	2.96	1.45	17.93	1.41
4	1.63	3.88	0.43	2.60	0.73	0.21	4.27	2.59	16.32	2.04
5	4.21	5.23	1.16	3.04	1.13	0.23	2.44	1.20	18.64	0.95
6	2.36	2.41	0.82	1.14	0.36	0.18	0.93	0.17	8.37	0.79
7	2.46	2.08	0.56	1.48	1.34	n.d.	0.96	n.d.	8.88	0.52
8	2.51	2.18	0.60	0.76	0.39	0.12	0.78	0.24	7.58	0.78
9	3.20	4.31	0.72	2.94	0.79	0.25	2.52	1.22	15.95	1.09
10	2.18	2.29	0.72	2.49	0.74	0.10	1.58	0.42	10.52	0.72
11	1.65	3.93	0.42	2.64	0.74	0.21	4.32	2.62	16.53	2.04
12	0.93	0.62	0.25	1.11	0.31	0.03	0.62	0.10	3.97	0.53
13	3.52	1.94	0.94	1.67	1.64	0.01	0.68	0.01	10.39	0.34
14	3.23	6.68	0.62	4.03	1.09	0.22	4.86	2.49	23.21	1.59
15	2.25	2.18	0.78	1.23	0.56	0.12	0.60	0.37	8.13	0.68
16	0.65	0.57	0.22	0.71	0.19	0.05	0.37	0.10	2.86	0.61
17	3.21	2.11	1.29	1.75	0.76	0.15	0.72	0.28	10.27	0.47
18	0.61	0.44	0.21	0.69	0.19	0.04	0.29	0.12	2.58	0.53
19	2.78	2.60	0.87	2.48	0.83	0.14	1.54	0.46	11.69	0.62
20	3.25	2.00	1.13	2.16	0.76	0.18	1.08	0.21	10.77	0.47
21	5.95	19.2	1.37	6.36	1.7	0.88	11.00	4.88	51.36	2.34
22	7.67	30.31	1.70	8.14	2.19	1.27	17.10	7.08	75.46	2.83
23	7.95	28.97	1.71	8.54	2.51	1.64	17.91	8.42	77.65	2.75
24	5.54	13.73	1.48	5.15	1.55	0.55	7.69	3.50	39.19	1.86
25	5.13	11.89	1.34	4.74	1.58	0.73	6.12	2.76	34.28	1.68
26	6.02	12.54	0.80	6.4	1.84	0.01	7.76	3.99	39.36	1.61
27	5.75	12.12	1.10	6.49	1.82	0.62	7.55	3.58	39.03	1.57
28	6.37	13.63	1.07	6.68	1.88	0.14	7.66	3.62	41.05	1.56
29	6.08	12.81	0.76	6.20	1.89	0.64	8.01	4.10	40.49	1.71
30	5.21	12.83	0.88	6.12	1.66	0.36	6.67	3.08	36.81	1.65
31	5.16	12.98	0.88	6.09	1.52	0.79	8.16	3.77	39.35	1.88
32	5.45	12.54	1.07	6.23	1.77	0.75	8.07	3.69	39.57	1.73
33	6.18	13.51	1.01	6.17	1.71	0.33	7.74	3.37	40.03	1.65
34	6.28	13.09	0.82	6.65	1.59	0.69	8.20	4.19	41.51	1.71
35	6.51	13.81	1.20	7.31	2.22	0.70	8.80	4.16	44.71	1.60
36	5.82	12.59	1.02	6.40	1.80	0.60	7.76	3.74	39.73	1.64
37	7.48	14.15	1.82	6.86	2.07	0.60	7.51	3.52	44.01	1.41
38	4.77	8.80	1.45	4.85	1.62	0.75	5.75	2.35	30.34	1.39
39	5.13	12.29	0.71	6.94	1.73	0.26	8.19	3.67	38.92	1.71
40	4.46	9.29	0.61	4.70	1.51	0.01	5.85	3.15	29.58	1.63

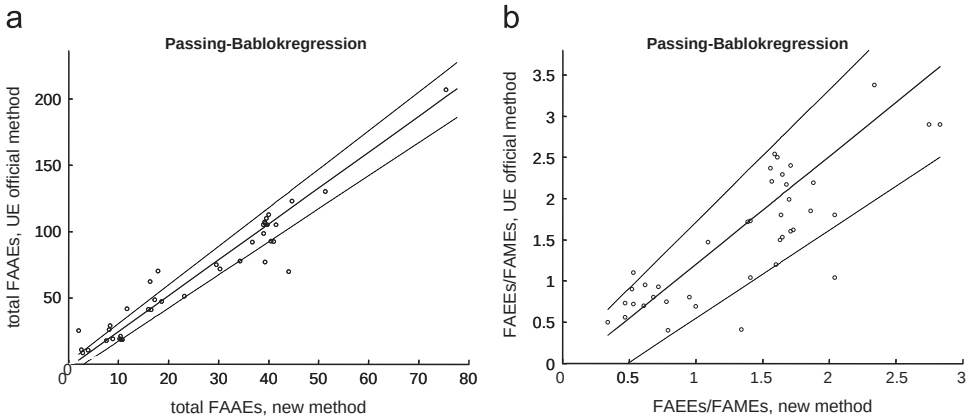

Fig. 2. Plots of the Passing-Bablok regressions. The dashed lines represent the 95% confidence interval. Total FAAEs (a); FAEEs/FAMEs (b).

factor to each FAAE. Moreover, Dodds et al. [19] have shown that assuming that the FID response of all FAMEs is equivalent is not completely acceptable, and this finding is certainly extensible to all FAAEs. As a consequence, the results obtained by FID cannot be considered as an absolute reference, in spite of the fact that they are conventionally considered true. On the other hand, since MS-SIM detector is more selective than FID, possible additive interferences were by far less likely with the new method [19] and SIM detection is more and more fit for the analysis of complex biological samples. On the basis of the regression line, the upper limit of 75 mg/kg, which is set by EU Official Method for reliable extra virgin olive oils, would correspond to about 25–30 mg/kg with the proposed method, and the limit of 75 mg/kg is obviously no more suitable. The new limit is to be precisely defined by a larger experimentation, but it should be emphasized that this does not involve a lower sensitivity of the new method, since SIM detection leads to a very low noise and the limit of quantification was really very low.

As far as samples 33, 35 and 40 are concerned, it is interesting to note that the combination of FAAEs contents and of FAEEs/FAMEs ratios, as determined by the Official Method, would allow to classify them as extra-virgin, though their sensorial defects stood for lower quality oils. On the contrary the FAAEs amounts by the new method (Table 6) are in accordance with the organoleptic determination, with samples 33, 38 and 40 close to the upper value of the proposed limit.

As far as the ratio between FAEEs and FAMEs is concerned, the results obtained with the two methods were not significantly different ($y=-0.11+1.31x$), with slope not significantly $\neq 1$ and intercept not significantly $\neq 0$ (Fig. 2b); nevertheless the correlation was lower than that for total FAAEs ($r=0.82$).

In order to confirm that the proposed method had at least the same ability of the EU Official Method to discriminate extra virgin olive oils from lower quality oils, the 40 oil samples were divided into two categories on the basis of EU Regulation [5] (category 1: extra virgin olive oils; category 2: lower quality virgin olive oils). Two data sets were obtained: set A had 40 rows (the 40 samples) and 12 columns (the amounts of each alkyl ester, their total sum, the sum of FAEEs, the sum of FAMEs and the FAAEs/FAMEs ratio, determined by the proposed method); set B had 40 rows (the 40 samples) and 6 columns (the sum of methyl and ethyl C16, the sum of methyl and ethyl C18, their total sum and the FAAEs/FAMEs ratio, determined by EU Official Method). Principal Component Analysis (PCA) was then performed on the two data sets, after autoscaling. Figs. 3 and 4 report the scores (a) and the loadings (b) on the first two PCs obtained for set A and B, respectively. As far as set A is concerned (Fig. 3), the full separation of categories 1 and 2 on PC1–PC2 plane (93% of the explained variance) (Fig. 3a) confirmed that the proposed method is suitable for singling out low quality virgin olive oils from extra virgin olive oils. PC1 explained 87% of variance and the variable loadings on it (Fig. 3b) were very similar, meaning that the variables were highly correlated. It is interesting to note that the sum of alkyl esters (variable 9), which provided almost the same information of the other variables on PC1, would be enough to separate the 2 categories and that FAAEs/FAMEs (variable 12) did not appear relevant to the separation. Nevertheless it must be remembered that this ratio is proposed by

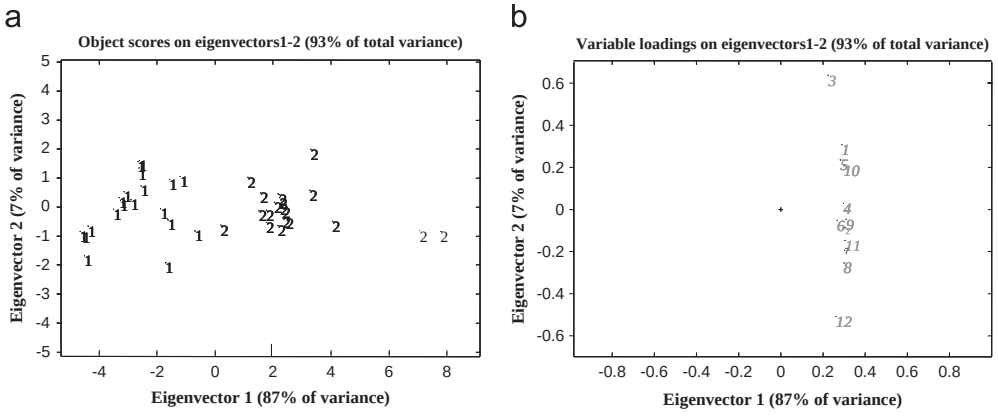

Fig. 3. Score (a) and loading (b) plots on the first two PCs obtained for set A (TDS/CIS4 method). Scores (a): 1, extra virgin olive oils; 2, low quality virgin olive oils. Loadings (b): 1, methyl palmitate; 2, ethyl palmitate; 3, methyl linoleate; 4, methyl oleate; 5, methyl stearate; 6, ethyl linoleate; 7, ethyl oleate; 8, ethyl stearate; 9, sum of alkyl esters (FAAEs); 10, sum of methyl esters (FAMEs); 11, ethyl esters (FAEEs); 12, FAAEs/FAMEs.

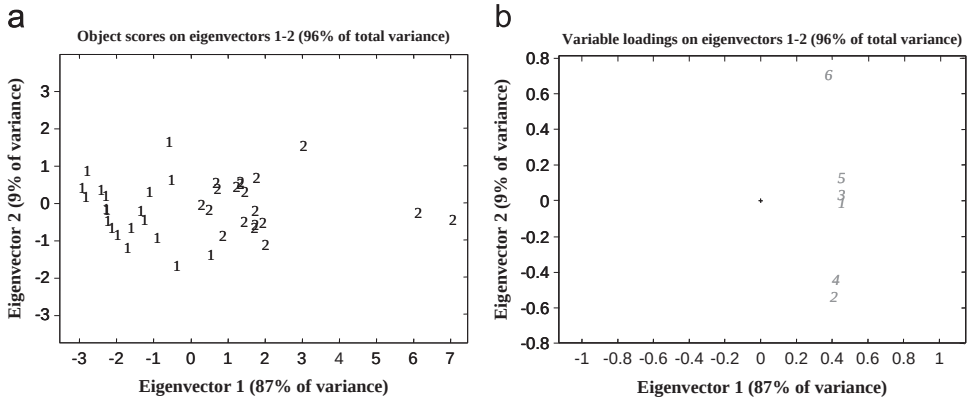

Fig. 4. Score (a) and loading (b) on the first two PCs obtained for set B (EU Official Method [5]). Scores (a): 1, extra virgin olive oils; 2, low quality virgin olive oils. Loadings (b): 1, sum of alkyl esters; 2, C16 methyl esters; 3, C16 ethyl esters; 4, C18 methyl esters; 5, C18 ethyl esters; 6, FAAEs/FAMEs.

EU regulation only for samples exceeding the limit value of total FAAEs for extra virgin olive oils and that this ratio is considered quite variable, and thus not significant, for high quality extra virgin olive oils. Very similar results were obtained for set B (Fig. 4), though it is possible to note (Fig. 4a) that the separation between categories 1 and 2 is slightly less sharp, with the plane PC1–PC2 explaining 96% of the total variance. Also in this case variables were highly correlated (Fig. 4b) and the FAEEs/FAMEs ratio (variable 6) is not more informative than the total FAAEs amount (variable 1).

4. Conclusions

The application of PCA to the data obtained by the proposed method showed that the samples of the two types (1, extra virgin olive oils; 2, low quality virgin olive oils) lie in two well-defined regions of the space, and that the distance among the two groups is not smaller than what is obtained on the data of the Official Method. On the basis of these results, it is possible to confirm that the proposed method for the detection of FAAEs shows a discriminating ability that is at least equal to that of the Official EU Method. Further research efforts are advisable in order to confirm the proposed limit to the total FAAEs content for extra virgin olive oils according to the new method. Anyway, simulations performed with a bootstrapping technique showed a very high consistency of the statistical parameters. Moreover, though the method repeatability was excellent, a collaborative study on the reproducibility of the proposed method seems to be advisable.

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