HUMAN HEALTH

ENVIRONMENTAL HEALTH

OLIVE OIL REFERENCE BOOK

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OLIVE OIL

Olive oil: an introduction

Olive oil is the product of transformation obtained from the olive fruit (Olea europaea). Its origins date back over 6000 years ago, when the cultivation of this plant began to spread throughout the Mediterranean: numerous and beneficial therapeutic, nutritional and cosmetic properties were attributed to the olive oil. Still today, the creation and consumption of olive oil are concentrated in the Mediterranean countries, but the export volumes to other countries are continuously growing in the last years (especially for extra virgin olive oil).

The extra virgin olive oil is a very important element of the Mediterranean diet, as it makes a large uptake of substances such as polyunsaturated fatty acids, vitamins and antioxidants, which play an important nutraceutical role (nutritional + pharmaceutical) in the organism.

Depending on olive processing and qualitative characteristics of the finished product, olive oil is classified into different product classes established by EC Regulation 61/2011.

Mechanical extraction - (virgin oils)	Chemical and physical extraction - (refined oils)
Extra virgin olive oil	Refined olive oil
Virgin olive oil	Olive oil (mix of virgin + refined oils)
Lampante olive oil	Crude olive-resiude oil
	Refined olive-residue oil
	Olive residue oil

Table 1: Product classes of olive oils

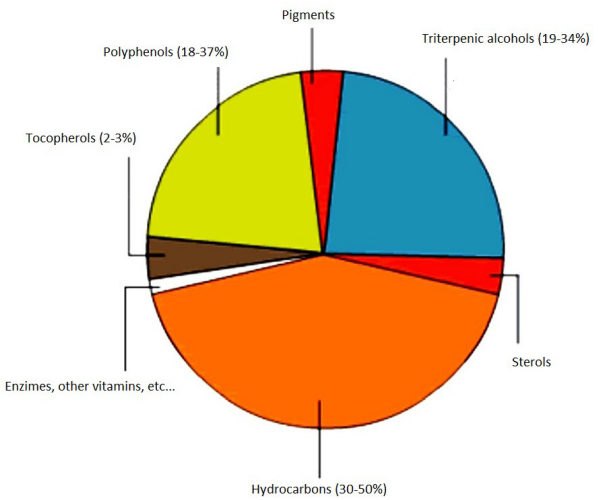
For the classification of the product within the different classes, the Regulation 61/2011 sets out a number of chemical parameters, in conjunction with organoleptic analysis (panel test), in order to determine which class an olive oil belongs to. It should be noted that because of its high market value, the extra virgin olive oil is a product that is most subject to adulteration and fraudulent sophistications, which undermine the quality attributes and sometimes even the safety of use.

Therefore, the quality parameters covered by the law shall guarantee the authenticity of the oil first, and its quality in the broadest sense, which depends on the quality of olives, milling technologies and mode of preservation.

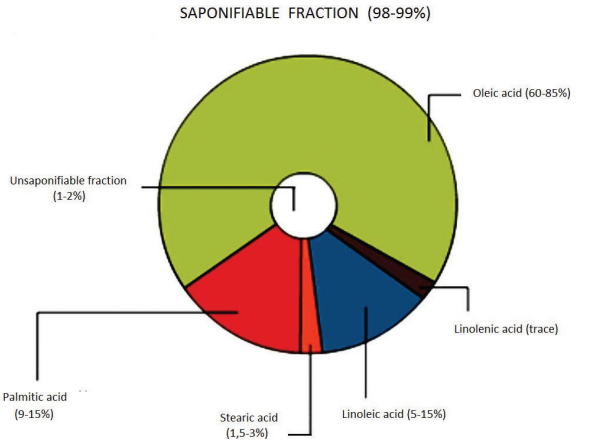
From a chemical point of view, olive oil can be divided into two fractions, depending on their behavior in presence of a strong alkaline solution (NaOH or KOH) and heating, i.e. saponifiable and unsaponifiable fractions:

- **Saponifiable** (98% to 99% of total weight), composed of substances able to form soaps in the conditions mentioned; this fraction include free fatty acids or esterified fatty acids with glycerol to form triglycerides, diglycerides and monoglycerides, containing for 75% to 85% unsaturated fatty acids (mainly oleic and linoleic acids) and for 15% to 25% saturated fatty acids (palmitic and stearic acids).

- **Unsaponifiable** (1-2%), formed by micro-components that do not form soaps in the conditions mentioned; even where present in small quantities, this fraction is very important from a nutritional and analytical point of view to check the authenticity of the oil and its stability; it contains mostly sterols, fat-soluble vitamins, waxes, aliphatic alcohols, aromatic compounds and antioxidants.



Picture 1: Chemical composition of Unsaponifiable fractions of olive oil



Picture 2: Chemical composition of Unsaponifiable and Saponifiable fractions of olive oil

In the EU Regulation 61/2011 the analytical parameters and threshold values for individual product classes are listed (Table 1), together with the scores to be obtained in the organoleptic evaluation carried out by means of panel test (for virgin oils). The physiochemical parameters established by the Regulation are as follows:

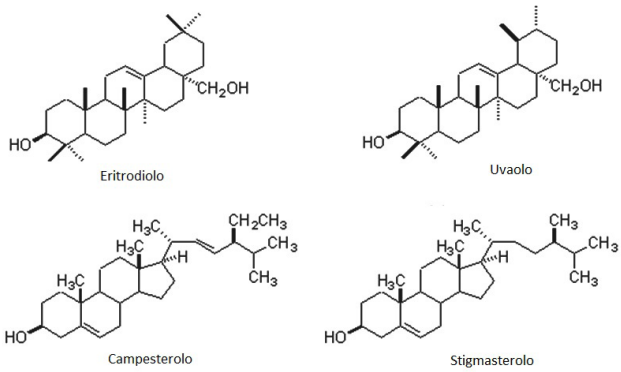
1. **Free acidity**: It is an indirect measure, carried out by a simple acid-base titration of free fatty acid content in oil. It is expressed as a percentage of oleic acid and since these molecules are produced by triglycerides hydrolysis, their content increases if oil and/or olives preservation was not appropriately managed.

2. **Peroxide number**: It is a measure of the oxidative state of the oil, performed by titration with iodine and thiosulphate and expressed as mg of O2 absorbed by oil. The oxidation involves the formation of peroxides, which affect the stability of the product promoting hydrolytic rancidity. High values of this parameter are indicative of an improper olive manipulation or preservation management.

3. **K (UV-Vis)**: It measures the oil absorption in the UV to highlight the addition of refined oils, since during the refining process double bonds present in the polyunsaturated fatty acids change their position and form triens and diens conjugates (resulting in an increasing in the A270 and the A232 nm respectively); the K value is rather a relative measure of the absorbance peak at 270 against the entire UV spectrum. High values of these three parameters are indicative of an addition of refined oil to virgin or extra virgin oils.

4. **Acid composition** (Fatty Acid Methyl Esters, FAME): Through GC-FID technique is possible to determine the relative abundance of fatty acids in oil after triglyceride hydrolysis and subsequent saponification in basic medium (to obtain the corresponding methyl esters). The presence of fatty acids normally absent in the olive (e.g. behenic acid, arachidic acid, etc…) is indicative of adulteration of the product with oils obtained from different seeds.

5. **Total Sterols, Erythrodiol and Uvaol**: It recovers the unsaponifiable fraction and separates the sterol fraction by preparative chromatography. It should then be performed a silanization reaction to analyze the sterolic composition by means of GC-FID. The sterol profile is species-specific, so adulterations of the product with other oils are easily detectable. The erythrodiol and uvaol content is higher in olive-residue oils due to the chemical extraction by solvent and a high content of these two compounds is indicative of adulteration caused by pomace oils or oils from different species.



Picture 3: Structure of sterol characteristics in olive oil

6. **Trans isomers of fatty acids**: The normal configuration of double bonds in unsaturated fatty acids is the cis. The processes of bleaching and deodorization modify the configuration forming trans isomers that can be quantified by GC-FID on a suitable capillary column.

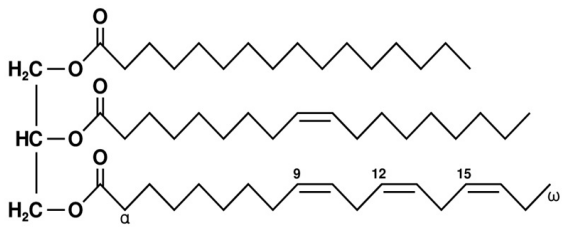
7. **ΔECN42**: This method is based on the separation and quantification of the various triglycerides present in oil The triglycerides composition is a function of primary fatty acid composition of oil; thus the expected composition of triglycerides can be calculated starting from the fatty acid composition (GC-FID of FAMEs).

The expected value is compared with the value measured by HPLC-RI for some of the triglyceride classes (in particular those with equivalent number of carbons equal to 42, such as the tri-linolein). Differences between expected and measured concentration values allow to identify adulterations with other oils having a different triglycerides composition.

8. **3.5-Stigmastadiene**: This compound is formed during the refining processes from sterols. Its determination in virgin olive oil by means of preparative chromatography and subsequent analysis by GC-FID allows detecting the presence of refined oils, even in very low concentrations.

9. **2-glyceryl monopalmitate**: The biosynthesis of triglycerides occurs through site-specific enzymes, which starting from free fatty acids and glycerol form the triglyceride. In virgin olive oils only about 2% of palmitic acid present is bonded on position 2, while in oil artificially esterified the bonding with glycerol occurs in a random manner and significantly increase this percentage. The concentration of 2-glyceryl monopalmitate is determined after site-specific enzymatic digestion of triglycerides (which eliminates fatty acids in position 1 and 3) and subsequent GC-FID analysis of monoglycerides.

10. **FAMEs/FAEEs** (methyl and ethyl esters of fatty acids): The presence of FAMEs and FAEEs in oil is indicative of an occurred reaction of free fatty acids with methanol (formed by degradation of cell walls) or ethanol (formed during fermentation processes). Their presence indicates an incorrect manipulation of olives or a slight oil deodorization (to eliminate off-odors produced by microorganisms). Their content can be measured without carrying out any oil saponification and using appropriate preparative chromatography, performed prior to the analysis by GC.



Picture 4: Triglyceride consisting of Palmitic acid, Oleic acid and Linolenic acid

11. **Waxes**: The wax content is higher in olive-residue oil, since these compounds are found mainly in olive stone and their extraction is increased using organic solvents. Waxes are simultaneously determined by GC-FID analysis of FAMEs and FAEEs and an excessive concentration of these compounds indicates an adulteration with pomace oils. Waxes are also an important parameter in discriminating crude olive-residue oil from lampante olive oil, combining their concentration with aliphatic alcohols content.

12. **Aliphatic alcohols**: The measure of total aliphatic alcohols, in combination with those of waxes, uvaol and erythrodiol allow distinguishing crude olive-residue oil from lampante olive oil. These molecules are mainly extracted in oils obtained by the use of solvents (such as the case of pomace oil).

Abbreviation	Double Bonds	Common Name	Chemical Formula
C 14:0	0	Myristic acid	$CH_3 - (CH_2)_{12} - COOH$
C 16:0	**0**	**Palmitic acid**	**$CH_3 - (CH_2)_{14} - COOH$**
C 18:0	**0**	**Stearic acid**	**$CH_3 - (CH_2)_{16} - COOH$**
C 20:0	0	Arachid acid	$CH_3 - (CH_2)_{18} - COOH$
C 22:0	0	Behenic acid	$CH_3 - (CH_2)_{20} - COOH$
C 16:1	1	Palmitoleic acid	$CH_3 - (CH_2)_7 - CH = CH - (CH_2)_5 - COOH$
C 18:1	**1**	**Oleic acid**	**$CH_3 - (CH_2)_7 - CH = CH - (CH_2)_7 - COOH$**
C 18:2	**2**	**Linoleic acid**	**$CH_3 - (CH_2)_4 - CH = CH - CH_2 - CH= CH - (CH_2)_7 - COOH$**
C 18:3	3	Linolenic acid	$CH_3 - CH_2 - CH = CH - CH_2 - CH= CH - CH_2 - CH = CH - (CH_2)_7 - COOH$

Table 2: Examples of some fatty acids (in bold are those most common in olive oil)

Parameter	Limit	Analytical Method	Technique	Column	App #
FAMEs/FAEEs	∑ FAMEs + FAEEs ≤ 75 mg/Kg Or ∑ FAMEs + FAEEs between 75 and 150 mg/Kg if FAEEs/FAMEs ≤ 1,5	Annex II EU Regulation 61/2011	GC (FID)	N932-6274: Elite 5 HT, 15 m, 1 µm, 0.32 mm	010211_01
WAXES	≤250 mg/Kg	COI/T.20/Doc. 11/Rev2/2001 OR Annex XVII EU Regulation 2568/1991	GC (FID)	N932-6274: Elite 5 HT, 15 m, 1 µm, 0.32 mm	010211_01
3.5-Stigmastadien	≤0.10 mg/Kg	Annexes V and VI EU Regulation 2568/1991	GC (FID)	N931-6076: Elite 5; 30m, 0.25µm, 0.25mm	010207_01
Total Sterols (Also referred to the % composition of major sterol characteristics)	≥1000 mg/Kg	Annexes V and VI EU Regulation 2568/1991	GC (FID)	N931-6076: Elite 5; 30m, 0.25µm, 0.25mm	010212_01
Erythrodiol + Uvaol	≤4.5% of total sterols	Annexes V and VI EU Regulation 2568/1991	GC (FID)	N931-6076: Elite 5; 30m, 0.25µm, 0.25mm	010212_01
Acidic composition (FAME)	It is expected a maximum % composition for 6 fatty acids	Annex X EU Regulation 2568/1991 OR COI/T.20/Doc.24/2001	GC (FID)	N931-6413: Elite Wax, 30 m; 0.50 µm, 0.32 mm	010206_01
TRANS Isomers	Isomers ∑ 18:1 ≤ 0.05% (tot FAME) and isomers ∑ 18:2 + isomers ∑ 18:3 ≤ 0.05%	Annex X EU Regulation 2568/1991 OR COI/T.20/Doc.17/2001	GC (FID)	N931-6508: Elite PE-23 (cis/trans); 60 m; 0.25 µm, 0.25 mm (allows FAME analysis)	010206_01
ΔECN42	Difference between HPLC ECN42 and theoretical ECN42 ≤ 0.2%	Annex XVIII EU Regulation 2568/1991 OR COI/T.20/Doc.20/2001	HPLC (RI GC (FID)	For GC: N931-6413: Elite Wax, 30 m; 0.50 µm, 0.32 mm For LC: N9303514 - Analytical C18 5 µm; length 250 mm x 4.6 mm	010202_01
2-glyceryl monopalmitate	≤0.9% if C16:0 ≤14% or ≤1% if C16:0 ≥ 4%	Annex VII EU Regulation 2568/1991 OR COI/T.20/Doc.23/2006	GC (FID)	N932-6274: Elite 5 HT, 15 m, 1 µm, 0.32 mm	010203_01
K (UV-VIS)	K_{232} ≤ 2.5, K_{270} ≤0,22 , ΔK≤0.01	Annex IX EU Regulation 2568/1991 OR COI/T.20/Doc.19/2001	UV-VIS	//	008298A_01
Aliphatic alcohols (*)	Yes	COI/T.20/Doc.26 OR Annex XIX EU Regulation 2568/1991	GC (FID)	N931-6076: Elite 5; 30m, 0.25 µm, 0.25 mm	010204_01
Group separation of Total Sterols and Aliphatic Alcohols			HPLC	Analytical Silica 5um; 250mm x 4.6mm Part No. N9303526	010205_01

Table 3: Quality parameters set out by EU Regulation 2568/1991 and 61/2011
(*) Analysis is needed to distinguish crude olive-residue oil from lampante olive oil

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APPLICATION BRIEF

Gas Chromatography

Analysis of FAMEs – FAEEs and quantification of waxes

Introduction

The chemical and physical properties of these classes of compounds allow obtaining an effective separation in a single chromatographic run. The Fatty Acid Methyl Esters (FAMEs) and Fatty Acid Ethyl Esters (FAEEs) may be indicative of an incorrect handling/ processing of the raw material (olives) or deodorization processes in which the oil may be subjected.

The content in Waxes is normally higher in olive pomace as mainly contained in the stone and principally extracted with the use of solvents in the extraction process.

The extent of these two parameters allows differentiating the olive oil from the olive pomace oil or, in case of extra virgin olive oils, identifying oils obtained by blending with lampante or deodorized virgin oils.

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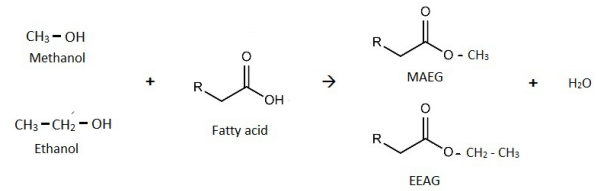
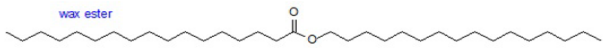
Principle of the Method

The FAMEs and FAEEs are naturally formed by reaction of free fatty acids with methanol or ethanol within badly maintained olives or low quality oils obtained therefrom.



Picture 1: Esterification reaction

The waxes contained in the olive oil are synthesized from the plant by esterification of fatty acids with long chain aliphatic alcohols. The aliphatic chains present all an even number of carbon atoms.



Picture 2: Wax

Instruments

- Gas Chromatograph with Clarus 580 GC with PPC
- On-column or PSS Injector
- FID Flame Ionization Detector
- TotalChrom Computing and Instrumentation Management Software
- N932-6274 Column: Elite 5 HT, 15 m, 1 µm, 0.32 mm

Picture 3: GC Clarus 580

Sample Preparation

It should be used two internal standards: Methyl heptadecanoate (for FAMEs and FAEEs) and Lauryl arachidate (for Waxes).

The fraction containing FAMEs, FAEEs and Waxes is obtained from the oil by passing it through a preconditioned packed silica gel column with n-hexane and then eluted with n-hexane/ethyl ether 99/1. The solvent in the collected fraction is eliminated with a Rotavapor and the residue re-suspended with 2-4 ml of heptane (A Solution).

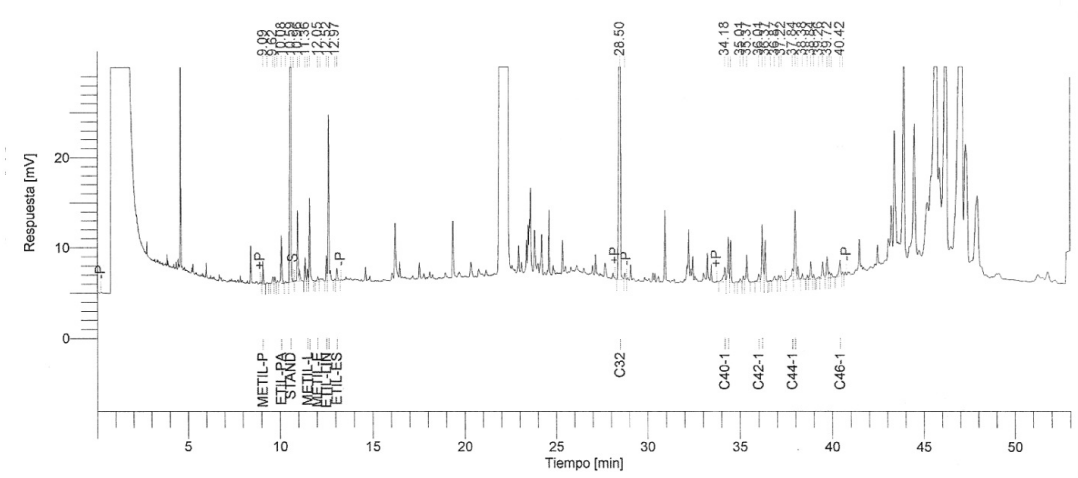
Analytical Method

- Injection volume: 1.5 µl of A Solution
- Injector temperature: 5°C above the oven temperature
- Heating Ramp: 80°C (held 1 min)

  80°C – 120°C (30°C/min)

  120°C – 340°C (5°C/min)

  340°C (held 10')



Informe Ceras - Esteres

Tiempo [min]	Area [uV*sec]	Area [%]	Mg Ceras	Mg Esteres	Nombre Componente
9.087	7079.48	0.73	0.0000	4.3978	Metil-palmitato
10.081	19021.01	1.96	0.0000	11.8159	Etil-Palmitato
10.588	321954.85	33.18	----------	0.0000	Standar
11.500	4045.93	0.42	0.0000	1.2567e-08	Metil-Linoleato
11.613	30948.55	3.19	0.0000	19.2254	Metil-Oleato
12.052	2242.47	0.23	0.0000	6.9652e-09	Metil-Estearato
12.519	9195.41	0.95	0.0000	2.8561e-08	Etil-Linoleato
12.631	66769.61	6.88	0.0000	41.4776	Etil-Oleato
13.082	5382.20	0.55	0.0000	1.6717e-08	Etil-Estearato
28.496	374952.08	38.64	----------	0.0000	C32
34.381	33430.76	3.45	17.8320	0.0000	C40
36.201	39511.24	4.07	21.0754	0.0000	C44
37.993	45696.08	4.71	24.3744	0.0000	C42
40.419	10142.63	1.05	5.4101	0.0000	C46
	970372.30	100.00	68.6918	76.9168	

Picture 4: Analysis of FAMEs, FAEEs (first portion of the chromatogram) and Waxes (final part).

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010211_01

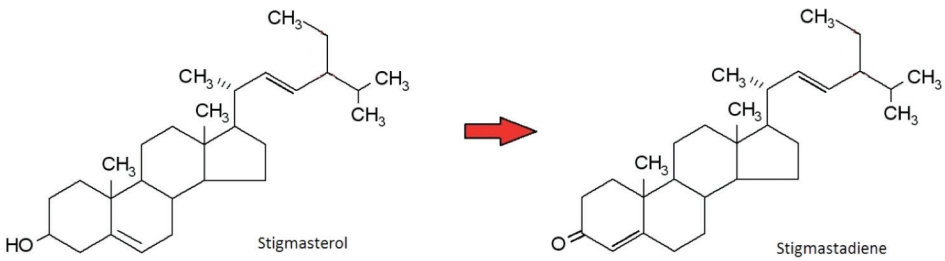
APPLICATION BRIEF

Gas Chromatography

Determination of 3,5-Stigmastadiene Content

Introduction

The stigmastadiene is a compound that is formed in small quantities starting from stigmasterol during the refining processes of oils carried out thermally or with the use of active carbons. In virgin olive oils, this compound is practically absent and therefore its determination allows to detect the presence of refined oils (olive, olive-residue, sunflower, palm, etc.) in virgin olive oils, even if they're added in small quantities.



Principle of the Method

After isolation of the unsaponifiable fraction, the latter is fractioned by chromatography on preparative silica gel column to recover the fraction containing the steroids. The recovered fraction is subjected to derivatization and subsequently analyzed by GC-FID to quantify the 3,5-stigmastadiene. The method can be reliably applied to all vegetable oils, but its accuracy is good only if the stigmastadiene content lies between 0.01 and 4.0 mg/kg. The procedures for sample preparation and analysis are described in detail in Annex XVII of EU Regulation 2568/91.

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Sample Preparation

Start with 20 grams of oil, then add an appropriate amount of 3,5-cholestadiene (internal standard) and 75 mL of 10% KOH in ethanol. After the saponification is completed, the unsaponifiable fraction is recovered in hexane and passed on a silica gel packed column, eluting with 1 mL/min of hexane. The first fraction (about 25-30 mL) containing the saturated hydrocarbons is eliminated, while the next 40 mL fraction containing the steroidic-hydrocarbons is recovered and concentrated by rotavapor until a volume of 200 µL.

Table 1. Instrumentation.

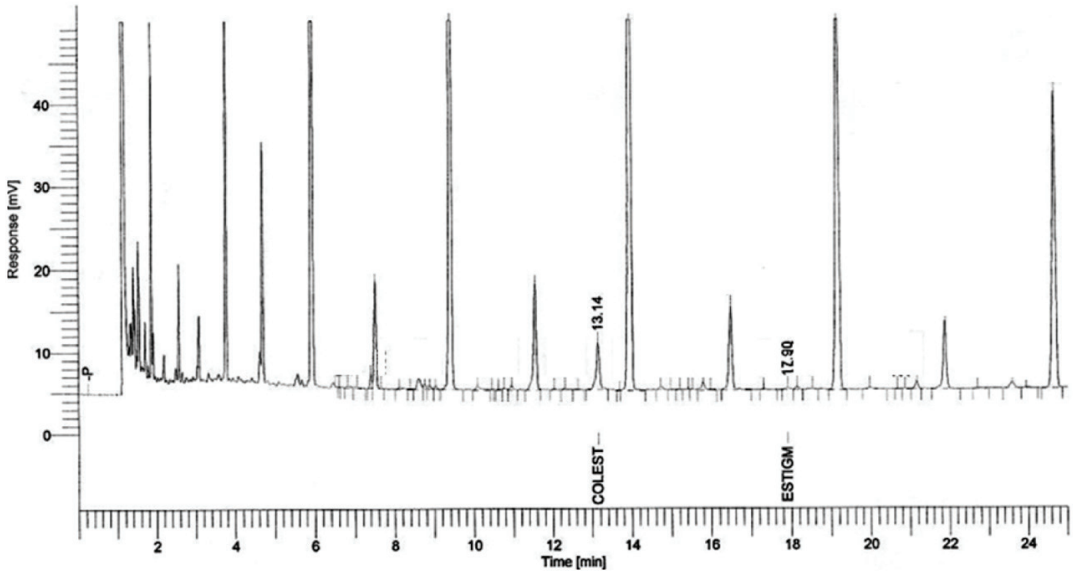
Gas Chromatograph:	Clarus 580 GC with PPC
Injector:	Split/Splitless Injector
Detector:	FID Flame Ionization Detector
Software:	TotalChrom® Computing and Instrumentation Management Software
Columns:	Elite 5, 30 m; 0.25 µm, 0.25 mm Part No. N9316076

Table 2. Analytical Method.

Injection Volume:	1.0 µL
Injector Temperature:	300 ˚C
Column Temperature:	320 ˚C
Heating Ramp:	235 ˚C (held 6 min) 2 ˚C/min up to 285 ˚C 285 ˚C (held 10 min)
Carrier Flow:	40 psi (Split = 20 mL/min)

Chromatogram

The internal standard peak appears after about 13 min, whereas the experimental conditions employed present a retention time of 17.9 minutes for the 3,5-stigmastadiene. This compoound is often associated with small quantities of its isomer, but usually during the analysis, these two compounds originate a single chromatographic peak. If two peaks are obtained, the result should be given as the sum of the two areas and expressed, referring to the internal standard, as mg/kg.



Time [min]	Area [µV·s]	Area [%]	Norm Area [%]	Component Name
13.145	34916.53	94.73	94.73	COLESTADIENO
17.901	1941.64	5.27	5.27	ESTIGMASTADIENO
	36858.17	100.00	100.00	

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010207_01

APPLICATION BRIEF

Gas Chromatography

Total Sterols and Uvaol + Erythrodiol

Introduction

The sterol composition is a sort of fingerprint of olive oils. Sterols to be determined are those designed by EU Regulation 2568/1991- All. V (see Table n. 1).

Furthermore, the same Regulation establishes the method and the limit for the determination of two specific sterols (App. VI): Uvaol and Erythrodiol.

Erythrodiol (commonly understood as the set of erythrodiol and uvaol diols) is a constituent of the unsaponifiable fraction, characteristic of some types of fats. Its concentration is significantly higher in the olive oil resulting from chemical extraction, if compared to other oils that contain it (pressed olive oil, grapeseed oil). Therefore its determination can be used to detect the presence of refined oil in virgin olive oil.

Although the regulation provides a specific analysis for each of these two parameters (Sterols and Uvaol/Erythrodiol), it is possible to quantify the content in a single chromatographic run.

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Table 1: Sterolic compounds detectable in olive oil.

Peak	Identification	
1	cholesterol	Δ-5-cholesten-3β-ol
2	cholesterol	5α-cholestan-3β-ol
3	brassicasterol	[24S]-24-methyl-Δ-5,22-cholestadien-3β-ol
4	24-methylene-cholesterol	24-methylene-Δ-5,24-cholesten-3β-ol
5	campesterol	[24R]-24-methyl-Δ-5-cholesten-3β-ol
6	campesterol	[24R]-24-methyl-cholestan-3β-ol
7	stigmasterol	[24R]-24-ethyl-Δ-5,22-cholastadien-3β-ol
8	Δ-7-campesterol	[24R]-24-methyl-Δ-7-cholesten-3β-ol
9	Δ-5,23-stigmastadienol	[24R,S]-24-ethyl-Δ-5,23-cholestadien-3β-ol
10	chlerosterol	[24S]-24-ethyl-Δ-5,25-cholastadien-3β-ol
11	β-sotosterol	[24R]-24-ethyl-Δ-5-cholestan-3β-ol
12	sitostanol	24-ethyl-cholestan-3β-ol
13	Δ-5-avenasterol	[24Z]-24-ethylidene-5-cholesten-3β-ol
14	Δ-5,24-stigmastedienol	[24R,S]-ethyl-Δ-5,24-cholestadien-3β-ol
15	Δ-7-stigmastenol	[24R,S]-24-Ethyl-Δ-7,24-cholestadien-3β-ol
16	Δ-7-avenasterol	[24Z]-24-ethyliden-Δ-7-cholesten-3β-ol

Principle of the Method

The sterol determination is carried out by gas chromatography using FID as detector.

The analysis is not possible on untreated sample, but should be carried out on a purified extract of the unsaponifiable fraction obtained by two steps briefly summarized below.

Instrumentation

- Gas Chromatograph with Clarus 580 GC with PPC
- Split/Splitless Injector
- FID Flame Ionization Detector
- TotalChrom Computing and Instrumentation Management Software
- N931-6076: Elite 5; 30m, 0.25µm, 0.25mm

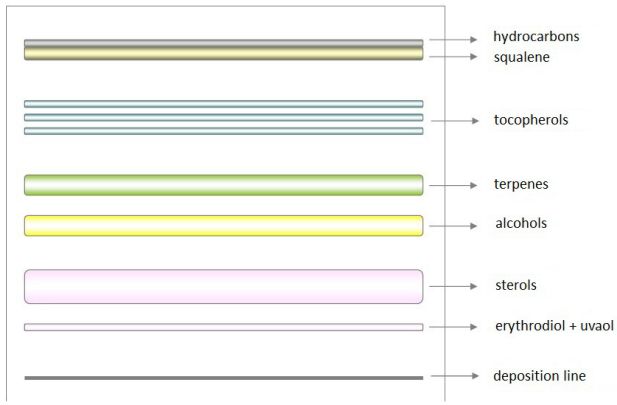
Picture 1: Clarus 580 GC

Sample Preparation

For the analysis of the oil sample must be suitably prepared as summarized below:

1. The oil (with α-cholestanol added as internal standard) is subjected to saponification with 2 N KOH in ethanolic solution; the unsaponifiable matter is extracted with ethyl ether and recovered after phase separation.

2. The sterol fraction is separated from the extract by thin-layer chromatography (TLC), using basic silica gel plates and an eluent phase of hexane/ethyl ether 65/35 or benzene/acetone 95/5. The bands obtained are highlighted by spraying the plate with 2',7'-dichlorofluorescein; sterols are then recovered from the silica gel by scraping its corresponding and from the plate.



Since sterols are not enough volatile to be directly analyzed in gas chromatography, after recovery from TLC plate a to trimethylsilyl derivatization is required prior to the GC-FID analysis; the reaction occurs with (CH3)3Si-Cl trimethylsilyl chloride in pyridine:

R-OH + (CH3)3Si-Cl ⟹ RO-Si(CH3)3

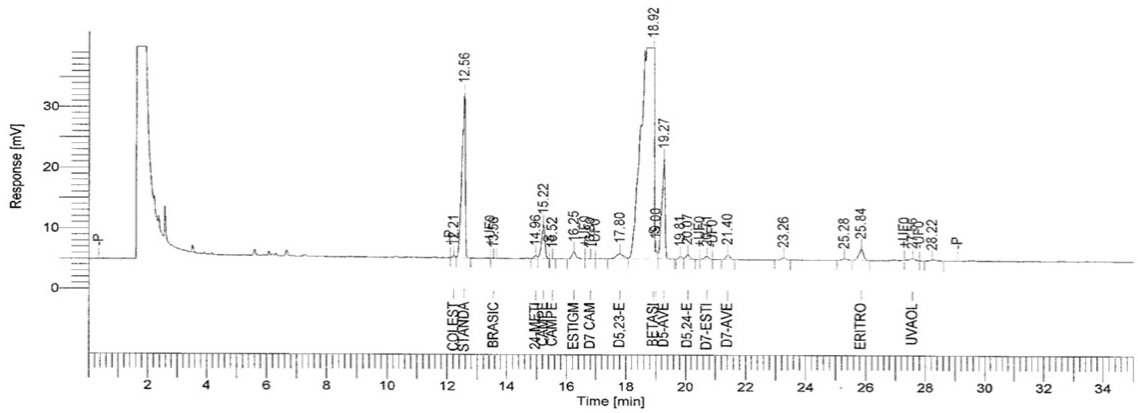
Analytical Method

Injection volume: 1,0 µl

Injector temperature: 290°

Column temperature: 300°C

Heating Ramp: 270°C (held 30 min)



Picture 2: The chromatogram obtained, with the peaks of sterols indicated in Table 1 as well as those of Uvaol and Erythrodiol (in the last part of the chromatogram). The 12.56' peak is referred to the α-cholestanol internal standard.

Group Report For : ERI+UVA

Pico #	Tiempo [min]	Area	Area [%]	BL	ISTD Amt. Ratio	Nombre Componentes
1	12.214	2007.02	0.13	BV	1.8683	Colesterol
3	13.557	257.41	0.02	MM	0.2396	Brasicasterol
4	14.963	3455.54	0.23	BV	3.2167	24-metilencolesterol
5	15.224	44296.74	2.96	VB	41.2350	Campesterol
6	15.520	234.40	0.02	*BB	0.2182	Campestanol
7	16.250	11737.39	0.78	BB	10.9261	Estigmasterol
8	16.800	489.80	0.03	MM	0.4559	D7 campesterol
	19.000	1396687.88	93.27		1300.1508	B-SITOSTEROL
15	20.715	5067.99	0.34	MM	4.7177	D7-estigmastenol
16	21.399	8247.20	0.55	BB	7.6772	D7-avenasterol
19	25.843	20704.83	1.38	VB	19.2737	ERITRODIOL
20	27.563	4219.17	0.28	MM	3.9276	UVAOL
		1497405.36	100.00		1393.9069	

$$\% \text{ of sterol } x = \frac{A_x}{\Sigma A} \cdot 100$$

A_x = peak area for x;

ΣA = total peak area for sterols.

Picture 3: Percentage of individual sterols in the total sample (excluding erythrodiol and uvaol).

Group Report For : ESTEROLES

Pico #	Tiempo [min]	Area	Area [%]	BL	ISTD Amt. Ratio	Nombre Componentes
1	12.214	2007.02	0.14	BV	1.8683	Colesterol
3	13.557	257.41	0.02	MM	0.2396	Brasicasterol
4	14.963	3455.54	0.23	BV	3.2167	24-metilencolesterol
5	15.224	44296.74	3.01	VB	41.2350	Campesterol
6	15.520	234.40	0.02	*BB	0.2182	Campestanol
7	16.250	11737.39	0.80	BB	10.9261	Estigmasterol
8	16.800	489.80	0.03	MM	0.4559	D7 campesterol
	19.000	1396687.88	94.85		1300.1508	B-SITOSTEROL
15	20.715	5067.99	0.34	MM	4.7177	D7-estigmastenol
16	21.399	8247.20	0.56	BB	7.6772	D7-avenasterol
		1472481.37	100.00		1370.7056	

$$\text{Erythrodiol } \% = \frac{A_1 + A_2}{A_1 + A_2 + \Sigma A_{sterols}} \times 100$$

A_1 = peak area for erythrodiol

A_2 = peak area for uvaol

$\Sigma A_{sterols}$ = total peak area for sterols

Picture 4: Percentage of Uvaol and Erythrodiol of the total sterols.

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010212_01

APPLICATION BRIEF

Gas Chromatography

Determination of the Acidic Composition of Triglycerides and Trans Fatty Acids

Introduction

The saponifiable fraction of olive oil is composed primarily of triglycerides, consisting of one molecule of glycerol on which are esterified up to three fatty acid chains (all with an even number of carbon atoms) saturated and unsaturated. Since the biosynthesis of fatty acids within the plant is variable depending on the species considered, the presence of fatty acids normally absent in the olive oil is indicative of adulteration of the product with oils obtained from different seeds. In particular, the relative percentages of myristic, linolenic, arachidic, eicosenoic, behenic and lignoceric acids are measured, as specified in EC Regulation 2568/91. The percentage composition of the other fatty acids commonly present in olive oil (oleic, linoleic, palmitic, etc.) is also used for determining the ECN42, in the calculation of theoretical composition of triglycerides. The chromatographic separation on an appropriate capillary column allow to separate and quantify not only the normal (cis) fatty acid, but also their *trans*-isomers. These compounds are important markers of heat treatments (deodorization) or refining (e.g. activated carbons). The official methods of this analysis are described in Annex X of EU Regulation 2568/91.

Principle of the Method

Before proceeding to the determination of fatty acids by GC-FID, a glycerides hydrolysis should be performed to cleave the free fatty acids linked to glycerol. In this way, the fatty acids can be transformed (by a process of trans-esterification with methanol in basic conditions) in their respective methyl esters, which have a higher volatility and a lower polarity.

Methylated fatty acids can be injected into the column, separated and detected with FID. In this way it is possible to obtain a profile of the acidic composition of the oil in terms of fatty acid-derived methyl esters (FAMEs). The identification and quantification of individual fatty acids are carried out by comparing areas and retention times with those of the internal standard.

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Sample Preparation

A possible sample preparation is described in the A Method (cold trans esterification) reported in the Annex X B of EC Regulation 2568/91 and can be summarized in the following steps:

1. Weigh 0.1 g of sample
2. Add 2 mL of heptane
3. Add 0.2 mL of 2 N methanolic KOH
4. Shake and recover the supernatant after stratification of the phases.

Table 1. Instrumentation.

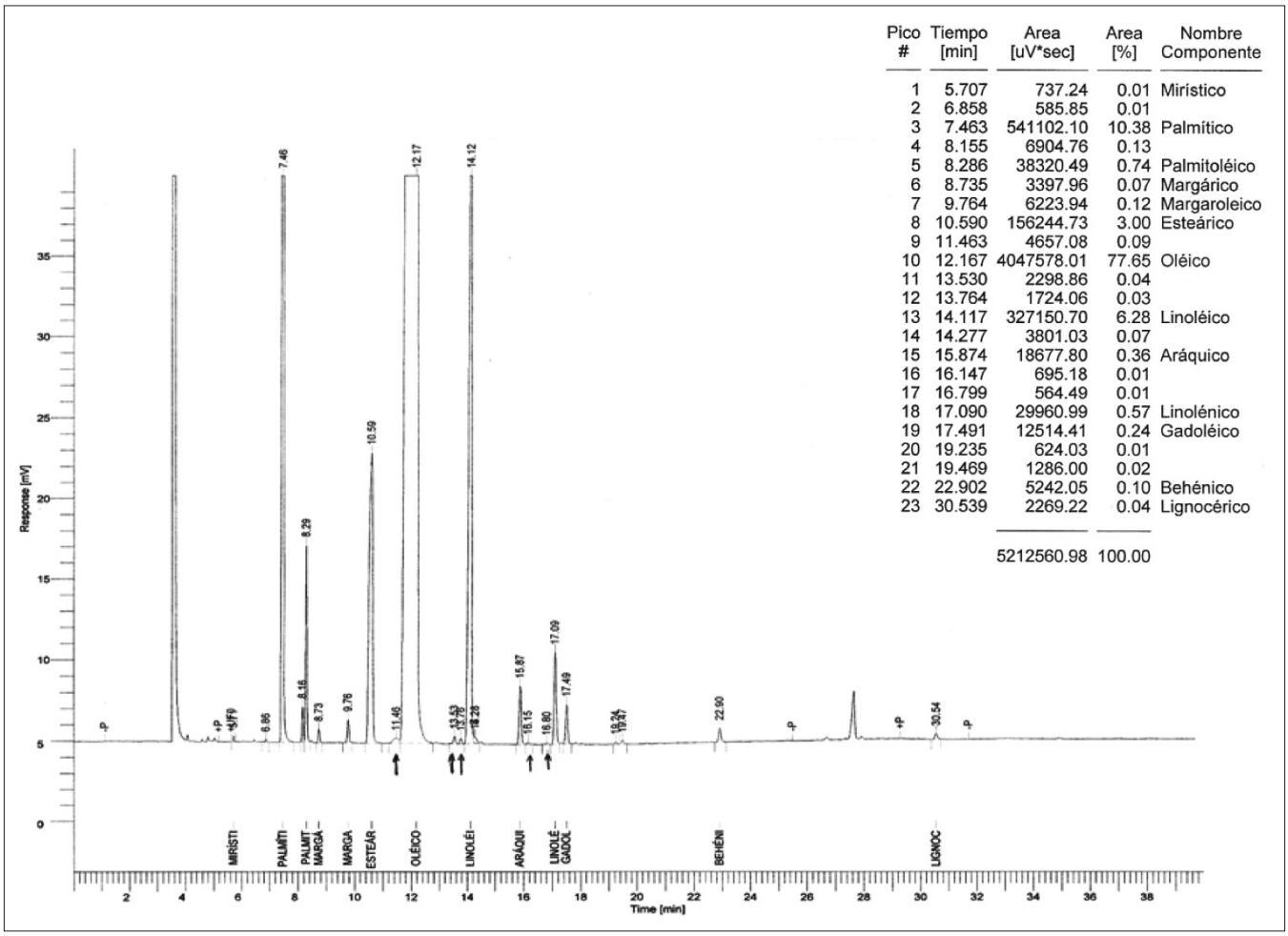
Gas Chromatograph:	Clarus 580 GC with PPC
Injector:	Split/Splitless Injector
Detector:	FID Flame Ionization Detector
Software:	TotalChrom® Computing and Instrumentation Management Software
Columns:	Elite Column, 60 m; 0.25 µm, 0.25 mm Part No. N9316508

Table 2. Analytical Method.

Injection Volume:	1.0 µL
Injector Temperature:	200 °C
Column Temperature:	300 °C
Heating Ramp:	170 °C (held 13 min) 1.5 °C/min up to 190 °C 190 °C (held 10 min)

From the chromatogram analysis, it is possible to calculate the percentage composition of some Fatty Acids as required by the Regulation (Myristic, Linolenic, Arachidic, Eicosenoic, Behenic, and Lignoceric Acids). It is also possible to determine and express their concentration as a percentage of total fatty acids to calculate:

- The sum of TRANS isomers of Oleic Acid (C18:1);
- The sum of TRANS isomers of Linoleic and Linolenic Acids (C18:2 and C18:3).



Pico #	Tiempo [min]	Area [uV*sec]	Area [%]	Nombre Componente
1	5.707	737.24	0.01	Mirístico
2	6.858	585.85	0.01	
3	7.463	541102.10	10.38	Palmítico
4	8.155	6904.76	0.13	
5	8.286	38320.49	0.74	Palmitoléico
6	8.735	3397.96	0.07	Margárico
7	9.764	6223.94	0.12	Margaroleico
8	10.590	156244.73	3.00	Esteárico
9	11.463	4657.08	0.09	
10	12.167	4047578.01	77.65	Oléico
11	13.530	2298.86	0.04	
12	13.764	1724.06	0.03	
13	14.117	327150.70	6.28	Linoléico
14	14.277	3801.03	0.07	
15	15.874	18677.80	0.36	Aráquico
16	16.147	695.18	0.01	
17	16.799	564.49	0.01	
18	17.090	29960.99	0.57	Linolénico
19	17.491	12514.41	0.24	Gadoléico
20	19.235	624.03	0.01	
21	19.469	1286.00	0.02	
22	22.902	5242.05	0.10	Behénico
23	30.539	2269.22	0.04	Lignocérico
		5212560.98	100.00	

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010206_01

APPLICATION BRIEF

Liquid Chromatography

Determination of ΔECN42 and Trilinolein Content

Introduction

The composition of triglyceride is a function of elementary acidic composition of the oil. By means of suitable mathematical calculations, it is possible to obtain the expected (or theoretical) composition of triglycerides, starting from the acid composition (GC-FID of methyl esters). The trilinolein content, calculated by theoretical method and measured by HPLC-RI, allows calculating the ΔECN42, i.e. the percentage difference between these two values. According to Reg. 61/2011 about the extra virgin olive oil, this difference should not exceed 0.2%.

Principle of the Method

Comparing the expected value with the measured one obtained by HPLC-RI for some of the classes of triglycerides, it is possible to identify adulteration of the oil with other oils having a higher content of linolenic acid (seed oils). For olive oil, this type of analysis is focused only on certain characteristic categories of triglycerides, i.e. those having an equivalent number of carbon atoms (ECN)
equal to 42. The ECN is calculated by counting the carbon atoms which constitute fatty acids and subtracting 2 for each double bond contained in the chain. The possible triplets of fatty acids forming a molecule with ECN equal to 42 are listed in the Fig 1. Usually they originate only 3 peaks in HPLC.

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Triacilgliceroli con ECN42
LLL
PoLL e isomero di posizione LPoL
OLLn e isomeri di posizione OLnL e LnOL
PoPoL e isomero di posizione PoLPo
PoOLn e isomeri di posizione OPoLn e OLnPo
PLLn e isomeri di posizione LLnP e LnPL
PoPoPo
SLnLn e isomero di posizione LnSLn
PPoLn e isomeri di posizione PLnPo e PoPLn

Fig. 1 Po = palmitoleic; P = palmitic; S = stearic; O = oleic; L = linoleic; Ln = linolenic.*

The percentage difference between the theoretical content (calculated from the acidic composition assayed as described previously) and the actual content of triglycerides with ECN42 (measured by HPLC-RI) provides the ΔECN42 parameter, which falls between the quality parameters listed in EC Regulation 61/2011. In case of adulteration of olive oils (naturally lacking in linolenic acid) with other oils rich in linolenic acid (seed oils), the ΔECN42 value increases because the determined trilinolein content is higher than that calculated by theoretical method from the acid composition.

Sample Preparation

For the calculation of the acidic composition it should proceed exactly as described for the method of the methyl esters of fatty acids, as reported in Annex X of the Reg. 2568/91 and previously described for the determination of fatty acids (FAMEs).

For the quantitative determination of triglycerides, the sample must be suitably prepared for HPLC-RI analysis. 2.5 grams of oil are weighed and diluted in 20 mL of elution mixture 87:13 (v/v) of petroleum ether and ethyl ether. This compound is then purified on silica gel eluting 150 mL of eluent mixture, evaporated to dryness with a rotavac, and then weighed and re-suspended in acetone (up to a 5% concentration). The extract thus obtained can be injected into the HPLC-RI system for the separation and quantification of triglycerides. A faster preparation protocol that can be used for routine analysis of edible oil (when official method is not required) is based simply on the dilution of 0.5 g of filtered oil sample into 10 mL of proprionitrile and direct injected in HPLC system.

Instrumentation

- Flexar HPLC
- Flexar RI Detector
- TotalChrom® Computing and Instrumentation Management Software
- N9303514 – Analytical C18 5 µm; length 250 mm x 4.6 mm; 110Å Carbon load 13%; end capped

Analytical method

Injection volume:	20 µL
Column temperature:	room temperature
Solvents:	acetone (A):acetonitrile (B)
Gradient:	isocratic 50:50
Flow:	1.5 mL/min

For the determination of triacylglycerols with ECN42, the first group of three peaks (See Fig.2 and Fig.3) representing them should be well separated. The content of triglycerides ECN42 is expressed as percentage of total triglycerides present (up to ECN 54).

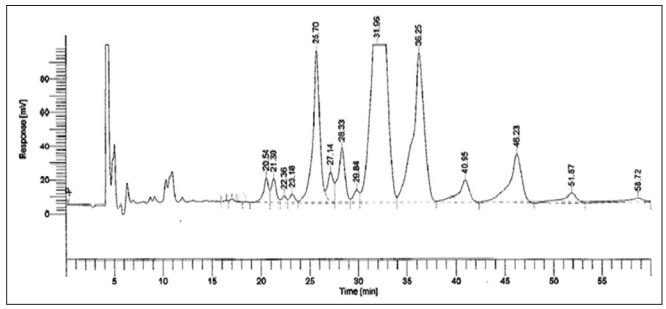

Fig. 2 Chromatogram

Pico #	Tiempo [min]	Area [uV*sec]	Area [%]
5	20.537	545274.77	1.38
6	21.298	503325.06	1.27
7	22.359	127740.75	0.32
8	23.184	187789.49	0.47
9	25.697	4523907.92	11.43
10	27.143	689213.50	1.74
11	28.331	1543488.30	3.90
12	29.838	312383.93	0.79
13	31.965	19457911.25	49.14
14	36.249	7235378.36	18.27
15	40.946	1075540.16	2.72
16	46.228	2521006.47	6.37
17	51.867	514188.93	1.30
18	58.722	265018.64	0.67
		39596276.23	100.00

Fig. 3 Peack table

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010202_01

APPLICATION BRIEF

Gas Chromatography

Determination of 2-Glyceryl monopalmitate

Introduction

The biosynthesis of triglycerides is a process mediated by the action of site-specific enzymes operating the esterification of glycerol with the fatty acid chains. The specificity in the action of these enzymes implies that, in the triglycerides of olive oils, only about 2% of glycerides linked in position 2 of glycerol are represented by palmitic acid. In the case of esterified oils artificially processed, the attack occurs in a nonspecific random manner resulting in a substantial increase of this percentage.

Principle of the Method

The concentration of 2-glyceryl monopalmitate is determined after enzymatic digestion of triglycerides with pancreatic lipase (Fig. 1), which hydrolyzes only the ester bonds in positions 1 and 3, leaving intact the bond in position 2 of glycerol. The GC-FID analysis allows quantifying the percentage of 2-glyceryl-monopalmitate among all the 2-monoglycerides obtained from the digestion. The identification of the compounds takes place by comparison of retention times with those of the reference standard.

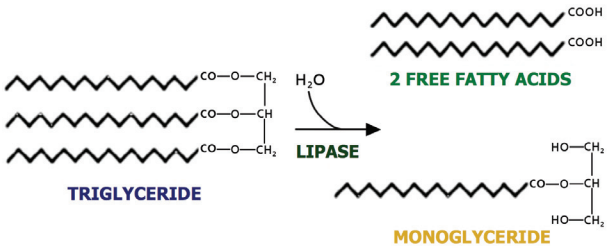

Fig. 1 Triglycerides Enzymatic Digetion

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Sample Preparation

The preparation of the sample consists of several steps described in detail in Annex VII of EC Regulation 2568/91. The basic steps are summarized below:

- If the oil to be analyzed does not have a free acidity of more than 3%, it is possible to proceed with the sample preparation without performing any neutralization;
- 1 gram of oil is diluted in 10 mL of n-exane/diethyl ether 87/13, to be subsequently loaded (1 mL) on a 500 mg SPE cartridge. After this, an elution with 4 mL of n-hexane/ethyl ether 9/1 is performed and the eluate is dried in a nitrogen stream;
- The enzymatic digestion occurs by adding a suitable buffer and the pancreatic lipase, working at 40 °C under shaking;
- The glyceride fraction is recovered in 1 mL of ethyl ether by extraction and centrifugation of the lysate;
- 100 µL of supernatant are subjected to silanization to make the 2-monoglycerides volatile; then, after recovering the analytes in 5 mL of hexane, it is possible to perform the GC-FID analysis.

Table 1. Instrumentation.

Gas Chromatograph:	Clarus® 580 GC with PPC
Injector:	On-Column or PPS Injector
Detector:	Flame Ionization Detector (FID)
Software:	TotalChrom® Computing and Instrumentation Management Software
Columns:	Elite 5-HT (15 m x 0.32 mm I.D. x 1 µm film) Part No. N9326274

Table 2. Analytical method.

Injection volume:	0.5 - 1.0 µL (on column)
Injector temperature:	< 68 °C (temperature of solvent evaporation)
Column temperature:	350 °C
Heating Ramp:	60 °C (1 min) Up to 180 °C (15 °C/min) Up to 340 °C (5 °C/min) 340 °C for 13 min
Flow:	20 psi

Chromatogram

From the chromatogram (Fig. 2) it is possible to obtain the peak area relative to the 2-glyceryl monopalmitate and the result is expressed as a percentage area of the total of areas represented by monoglycerides in the sample. The EU Regulation 61/2011 indicates for the quality of extra-virgin olive oil a maximum permissible concentration limit equal to 0.9% (where palmitic acid represents a percentage less than 14% of all fatty acids present), or a percentage of 1% where the palmitic acid is present in a content exceeding 14% of total fatty acids.

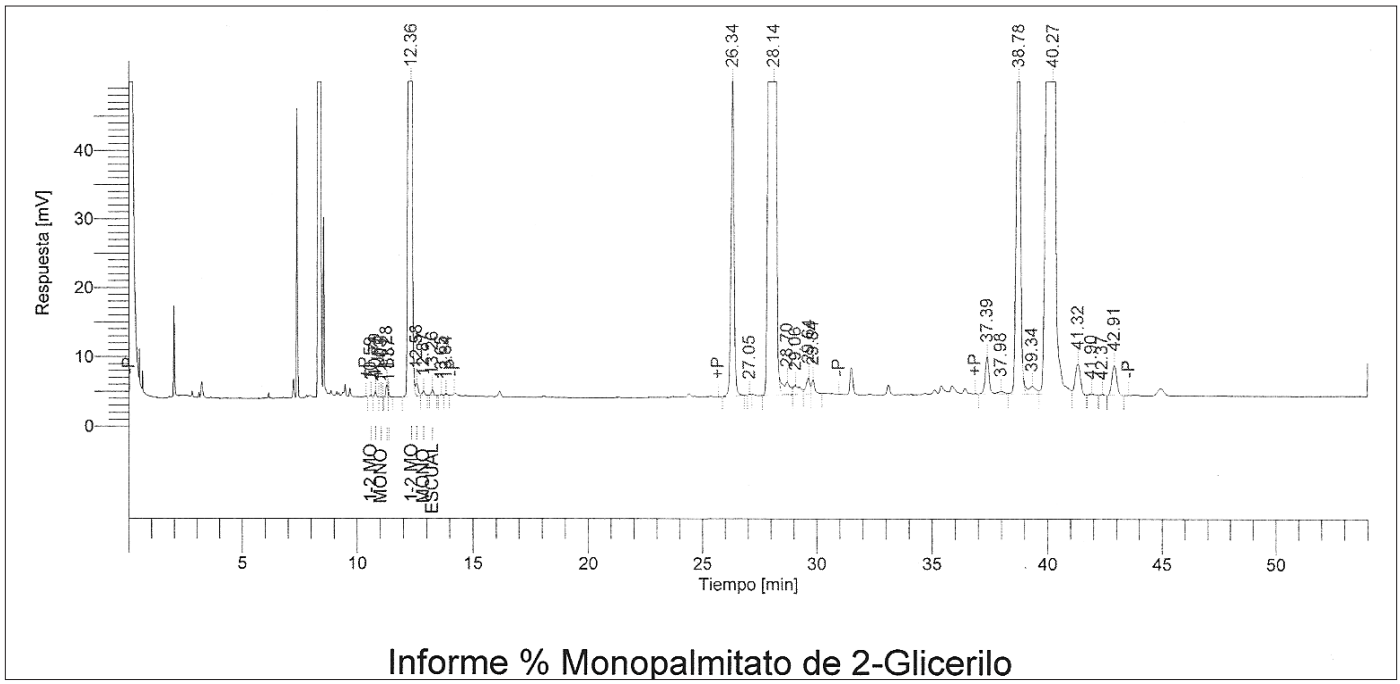

Fig. 2 Chromatogram

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010203_01

APPLICATION NOTE

UV/Vis Spectroscopy

Author

Jorge Puente

PerkinElmer
Spain

Determination of Olive Oil Purity and Degree of Oxidation using the LAMBDA XLS

Introduction

Olive oil is well established in the food industry. Demand continues to grow not only because of its distinct flavor, but also because of an increased awareness of its health benefits. In fact, the FDA allows producers of olive oil to place a health claim on their products because there is some scientific evidence to support a risk reduction of coronary heart disease by consuming a higher proportion of monounsaturated fat in one's diet. This is significant because olive oil is considerably rich in monounsaturated fats, most notably oleic acid. It is therefore of interest to producers to know the quality of the oil, its state of preservation and changes brought about in it by technological processes. EEC Regulation 2568/91 (1991) outlines the method for measuring olive oil purity, using a UV spectrophotometric technique.

The quality of the olive oil is studied by measuring the characteristics of the absorption bands between 200 and 300 nm. These are frequencies related to conjugated diene and triene systems. A low absorption in this region is indicative of a high quality extra virgin olive oil, whereas adulterated/refined oils show a greater level of absorptions in this region.

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Instrument

The PerkinElmer® LAMBDA™ XLS UV/Vis spectrophotometer, shown in Figure 1, is a stand-alone, robust scanning spectrophotometer with no moving parts and a unique Xenon® Lamp Source (XLS) with a typical lifetime of 5 years. The system produces reliable and accurate oil purity results in relation to EEC Regulation 2568/91 (1991) in seconds.

The LAMBDA XLS is equipped with a large LCD screen making running methods and viewing data easier. Results can be printed, stored, or exported into Microsoft® Excel® for use on your personal computer.

Method

Olive oil samples were diluted in iso-octane (2,2,4-trimethylpentane). All samples were measured in matched, synthetic fused silica cuvettes (10 mm is the recommended pathlength) running a solvent blank as a reference.

Absorption measurements for purity determination were made at 232, 266, 270 and 274 nm. K values were calculated according to the equation shown in Figure 2.

The purity of olive oil can be determined from three parameters:

- K232 Absorbance at 232 nm
- K270 Absorbance at 270 nm
- Delta K (Figure 3)

The LAMBDA XLS was used to collect UV data from 4 x different label graded commercial olive oil samples.

Results

The results collected using the LAMBDA XLS for the 4 x different graded olive oils showed correct alignment with the EEC Regulation limits (Table 1).

Conclusion

The LAMBDA XLS is a reliable and cost effective system appropriate for compliance with EEC Regulation 2568/91 (1991); the standard method for measuring olive oil purity, using a UV spectrophotometric technique.

Table 1. Measured and Permitted K Values of Commercial Olive Oils.

	Measured K values				
Olive oil sample type	**K232**	**K266**	**K270**	**K274**	**Delta K**
'Extra virgin' sample 1	1,897	0,151	0,148	0,135	0,005
'Extra virgin' sample 2	1,717	0,201	0,189	0,173	0,002
'Virgin' sample 3	1,436	0,240	0,248	0,223	0,016
'Olive oil' sample 4	3,000	0,640	0,832	0,458	0,283
Maximum Permitted Values (EEC Commission number 2568/91)					
Extra virgin olive oil	≤ 2,4		≤ 0,20		≤ 0,01
Virgin olive oil	≤ 2,5		≤ 0,25		≤ 0,01
Olive oil	≤3.3		≤ 1,0		≤ 0,13

Figure 1. LAMBDA XLS UV/Vis Spectrophotometer.

$$K\lambda = \frac{Abs\lambda}{D \times L}$$

D = Dilution gr/L

L = cuvette pathlength

Figure 2. K equation for λ nm.

$$\text{Delta K} = K_{270} - \frac{K_{266} + K_{274}}{2}$$

Figure 3. Delta K equation.

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008298A_01 Mar 2012 Printed in USA

APPLICATION BRIEF

Gas Chromatography

Determination of Aliphatic Alcohols

Introduction

The aliphatic alcohols are contained in the unsaponifiable fraction of olive oil. These molecules have an even number of carbon atoms (from 20 to 30) mostly located inside the stone and are partially extracted by mechanical processes. Their content in the oil may be increased where extraction is performed with solvents (as in the case of refined oil) and therefore the concentration of these compounds, in combination with other parameters (erythrodiol, uvaol and wax content) allows to distinguish crude olive-residue oil from lampante oil. The methods of preparation and analysis are described in detail in Annex XIX of EC Regulation 2568/91.

Principle of the Method

Oil is added with 1-eicosanol as internal standard prior to proceed to cold saponification with potassium hydroxide. The unsaponifiable fraction is then recovered with ethyl ether, and the fraction of the alcohol is subsequently separated by thin-layer chromatography. After recovery from TLC plate the alcohols are derivatized to trimethylsilyl ethers and analyzed by capillary gas chromatography.

Sample Preparation

It starts from 5 grams of oil, to which is added an appropriate amount of 1-eicosanol (internal standard); the sample is then added with 50 mL of 2N KOH in ethanol and the saponification reaction is completed through stirring and water additions. Finally, the unsaponifiable fraction is extracted in ethyl ether. The fraction obtained is dried and resuspended in chloroform in order to reach a concentration equal to about 5% (m/v). 100 µL of the extract thus obtained are subjected to thin-layer chromatography using as mobile phase a 65:35

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(v/v) hexane/ethyl ether solution to separate the fraction of the aliphatic alcohols. After plate development with 2'7'-dichlorofluorescein, the alcohol band can be recovered from the plate to carry out the derivatization process with pyridine-hexamethyldisilazane-trimethylchlorosilane 9:3:1 (v/v/v) in order to obtain trimethylsilyl ethers, and then proceed to perform the gas chromatographic analysis on the same column used for the determination of sterols and erythrodiol.

Table 1. Instrumentation.

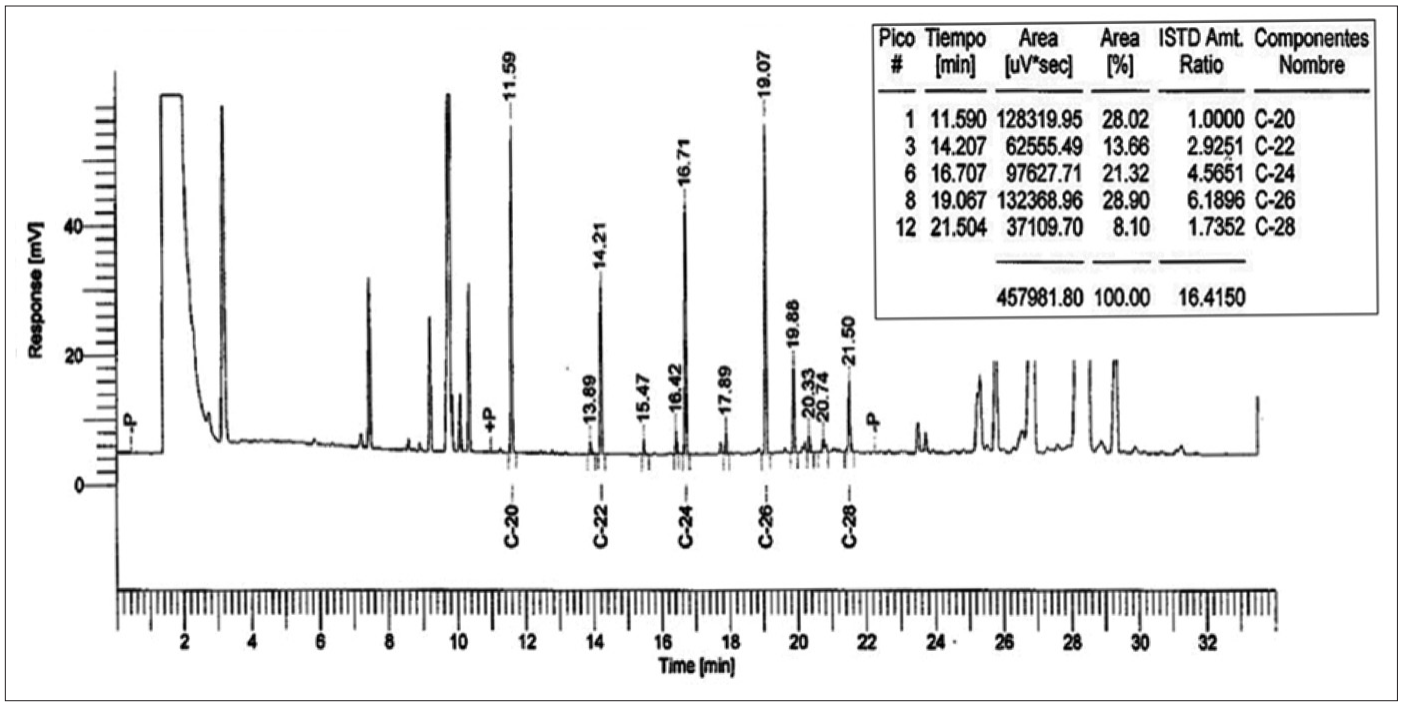
Gas Chromatograph:	Clarus 580 GC with PPC
Injector:	Split/Splitless Injector
Detector:	FID Flame Ionization Detector
Software:	TotalChrom Computing and Instrumentation Management Software
Columns:	Elite 5; 30 m, 0.25 µm, 0.25 mm Part No. N9316076

Table 2. Analytical Method.

Injection Volume:	1.0 µL
Column Temperature:	290 °C
Column Temperature:	300 °C
Heating Ramp:	190 °C (held 2 min) Up to 295 °C (6 °C/min) 295 °C (held 14 min)

Chromatogram

The chromatogram obtained by GC-FID is similar to that reported in the following figure. The internal standard peak (C 20) has a retention time of 11.59 minutes in the operating conditions applied. The content of total aliphatic alcohols is quantified as the sum of the C-22, C-24, C-26 and C-28 peaks, and expressed as equivalent mg of standard per kg of sample.



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010204_01

APPLICATION BRIEF

Liquid Chromatography

Group Separation of Total Sterols and Aliphatic Alcohols in Olive Oil by HPLC

Introduction

Some official analytical methods (e.g. determination of total Sterols and Aliphatic Alcohols) within the sample preparation provide a separation phase performed through thin-layer chromatography with the purpose of separating, recovering and submitting to analysis the fractions of interest.

The thin-layer chromatography (TLC), while being recognized as a valid separation method, requires some skill by the operator (deposition of the sample, selection and recovery of the fractions) and can not be easily automated.

The separation of some fractions can be easily obtained using an HPLC system (e.g. the same used for the ΔECN42 parameter) equipped with a suitable column and an appropriate fraction collector.

Principle of the Method

The fractions of aliphatic alcohols and that of Total Sterols contained in the unsaponifiable fraction are separated and collected automatically and then analyzed according to specific procedures.

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Sample Preparation

The sample undergoes saponification process as previously described for aliphatic alcohols. The unsaponifiable fraction is recovered and diluted to 5% in n-hexane/ethyl ether 50/50, filtered and then injected in the HPLC system to separate and collect the different classes of compounds that compose the unsaponifiable fraction.

Table 1. Instrumentation.

Liquid Chromatograph:	HPLC Flexar®
Injector:	Manual or Automatic Sample Injection System
Detector:	Refractive Index (RI) Detector
Sampler:	Fraction Collector Part No. N0911044
Software:	TotalChrom Computing and Instrumentation Management Software
Columns:	Brownlee™ Analytical Silica Column Part No. N9303526

Table 2. Analytical Method.

Injection Volume:	200 µL
Column Temperature:	Room Temperature
Solvents:	n-hexane and Ethyl Ether
Gradient:	Isocratic 50:50
Flow:	1.2 mL/min
Detector:	Refraction Index
Column:	Analytical Silica 5 um; 250 mm x 4.6 mm Part No. N9303526

Chromatogram

Applying the experimental conditions previously described (Tables 1 and 2) is possible to collect two fractions (See Fig.1) that contain two of the major components of the unsaponifiable fraction:

- from 5 to 9.5 minutes the aliphatic alcohols can be recovered;
- from 9.5 to 25 minutes the sterols fraction, comprehensive of erytrodiol and uvaol can be recovered.

The other components of the unsaponifiable fraction are eluted in the first part of the chromatograms and are not separated with this protocol.

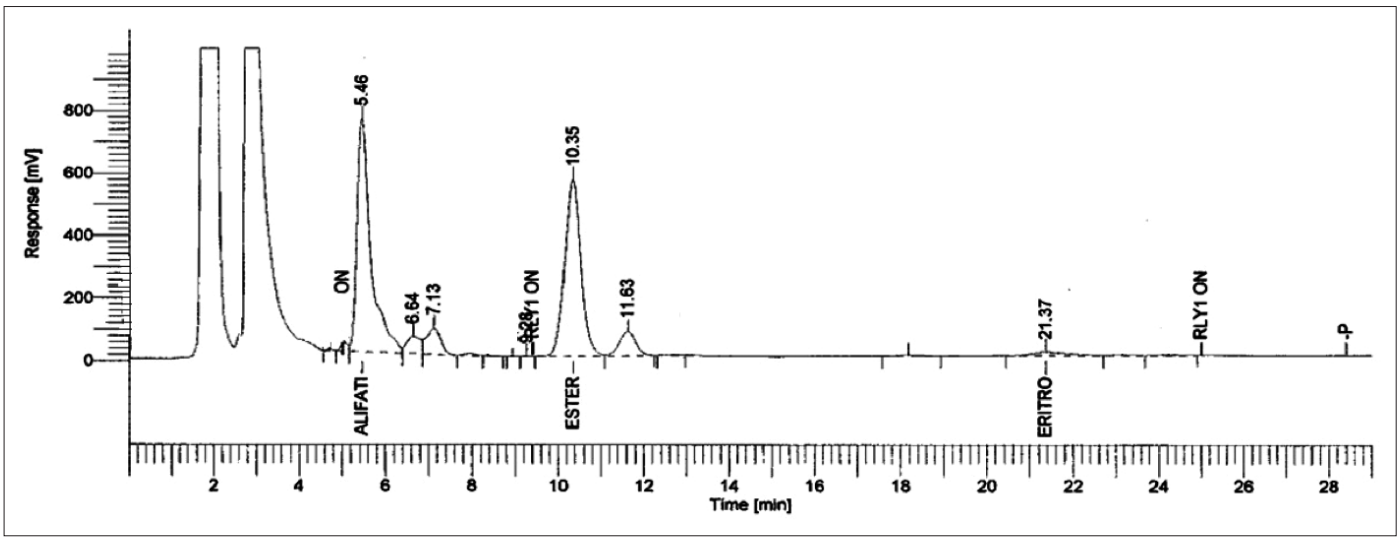

Fig. 1 Chromatogram

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