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Review

Chromatographic analysis of unsaponifiable compounds of olive oils and fat-containing foods

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Abstract

The analysis of the ''minor components'' present in food lipids is usually hampered by the large diversity of compounds found in this fraction. High-purity degree reagents and solvents, good collection techniques and highly sensitive analysis are required in order to accurately identify and quantify these components. Chromatographic techniques have proven to be particularly suitable for these determinations, especially capillary gas chromatography. This study reports several analytical cases of the main classes of components of the unsaponifiable matter obtained from olive oils or food matrices. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Olive oil; Food analysis; Fats; Lipids

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1. Introduction

The study of a fraction of any real system can be performed by analyzing the matrix either in its original form or after being subjected to prefractionation and/or purification methods, depending on its chemical and physical properties. Over the last few years, the direct analytical determinations have become more popular, since they do not require a large number of sample manipulations, thus reducing the

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artifact formation; spectroscopy analytical techniques, such as IR and NMR, are used for this scope. On the other hand, sample fractionation selectively modifies the original composition of the matrix and can generate compounds that can interfere with the analysis, providing a wrong picture of the composition; furthermore, serial fractionation often does not grant a complete description of the real composition. Nevertheless, artifact formation can be diminished by taking into account several analytical precautions, as well as by introducing some corrections to limit the problems.

The analytical control in the lipid field was launched several years ago [1] but, since 1950, the methods of analysis registered a drastic change, due to the introduction of chromatography. The use of chromatographic techniques for lipid studies, especially gas chromatography (GC), has become more and more important because it can be applied to the quality control of many food matrices, providing

Table 1
Legal limits and restrictions for the different categories of pressed olive oils [2]

Parameter	Pressed olive oil			
	Extra virgin olive oil	Virgin olive oil	Current virgin olive oil	Lampant virgin olive oil
Acid value (%)	≤1.0	≤2.0	≤3.3	>3.3
Peroxide value (mequiv. O_2/kg)	≤20	≤20	≤20	>20
Halogenated solvents (ppm)	≤0.20	≤0.20	≤0.20	>0.20
Waxes (ppm)	≤250	≤250	≤250	≤350
Saturated fatty acids of 2-monoglycerides (%)	≤1.3	≤1.3	≤1.3	≤1.3
Erythrodiol+uvaol (% of sterols)	≤4.5	≤4.5	≤4.5	≤4.5
Difference between ECN42 (HPLC) and ECN42 (calculated)[a]	≤0.2	≤0.2	≤0.2	≤0.3
Cholesterol (% of sterols)	≤0.5	≤0.5	≤0.5	≤0.5
Brassicasterol (% of sterols))	≤0.1	≤0.1	≤0.1	≤0.1
Campesterol (% of sterols)	≤4.0	≤ 4.0	≤4.0	≤4.0
Stigmasterol (% of sterols)	<Camp.[b]	<Camp.	<Camp.	–
Sitosterol (% of sterols)[c]	≥93.0	≥93.0	≥93.0	≥93.0
7-Stigmastenol (% of sterols)	≤0.5	≤0.5	≤0.5	≤0.5
Total sterols (ppm)	≥1000	≥1000	≥1000	≥1000
Stigmastadienes (ppm)	≤0.15	≤0.15	≤0.15	≤0.50
Myristic acid (%)	≤0.05	≤0.05	≤0.05	≤0.05
Linolenic acid (%)	≤0.9	≤0.9	≤0.9	≤0.9
Arachidic acid (%)	≤0.6	≤0.6	≤0.6	≤0.6
Eicosenoic acid (%)	≤0.4	≤0.4	≤0.4	≤0.4
Behenic acid (%)	≤0.2	≤0.2	≤0.2	≤0.2
Lignoceric acid (%)	≤0.2	≤0.2	≤0.2	≤0.2
Trans isomers $C_{18:1}$ (%)	≤0.05	≤0.05	≤0.05	≤0.10
Trans isomers ($C_{18:1}$+$C_{18:2}$) (%)	≤0.05	≤0.05	≤0.05	≤0.10
UV absorption (*K* at 232 nm)	≤2.50	≤2.60	≤ 2.60	≤ 3.70
UV absorption (*K* at 270 nm)	≤0.20	≤0.25	≤0.25	>0.25
UV absorption (*K* at 270 nm after alumina treatment)	≤0.10	≤0.10	≤0.10	≤0.11
$\Delta K = [K268 - (K262 + K274)/2]$	≤0.01	≤0.01	≤0.01	–
Panel test	≥6.5	≥5.5	≥3.5	<3.5

[a] ECN 42 = Equivalent chain number at 42 carbon number (amounts of all triglycerides with 42 carbon atoms, considered without glycerine carbon atoms). ECN 42 (HPLC) and the calculated ECN 42 were determined by means of HPLC analysis and the fatty acid composition, respectively.

[b] Camp. = Campesterol.

[c] 5,23-Stigmastadienol + clerosterol + sitosterol + sitostanol + 5-avenasterol + 5,24-stigmastadienol.

reliable qualitative and quantitative results in short analysis times. In particular, GC has been useful for detecting the presence of other fat substances in olive oils, even at very low concentration levels. Tables 1 and 2 summarize the legal limits and restrictions for the different categories of olive oils [2], whereas Table 3 reports the minimum amount of forbidden oils (expressed as percentages) that can be detected in illegal virgin olive oil mixtures with different analytical techniques that have been set up for this scope [3].

2. Food lipids

Triglycerides represent the main lipid fraction of foods, whereas the rest (the so-called ‘‘minor com-

Table 2
Legal limits and restrictions for the different categories of refined olive oils [2]

Parameter	Refined olive oils				
	Refined olive oil	Olive oil	Crude husk olive oil	Refined husk olive oil	Husk olive oil
Acid value (%)	≤0.5	≤1.5	≥2.0	≤0.5	≤1.5
Peroxide value (mequiv. O_2/kg)	≤5	≤15	–	≤5	≤15
Halogenated solvents (ppm)	≤0.20	≤0.20	–	≤0.20	≤0.20
Waxes (ppm)	≤350	≤350	–	–	>350
Saturated fatty acids of 2-monoglycerides (%)	≤1.5	≤1.5	≤1.8	≤2.0	≤2.0
Erythrodiol+uvaol (% of sterols)	≤4.5	≤4.5	≥12	≥12	>4.5
Difference between ECN42 (HPLC) and ECN42 (calculated)[a]	≤0.3	≤0.3	≤0.6	≤0.5	≤0.5
Cholesterol (% of sterols)	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Brassicasterol (% of sterols)	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1
Campesterol (% of sterols)	≤4.0	≤4.0	≤4.0	≤4.0	≤4.0
Stigmasterol (% of sterols)	<Camp.[b]	<Camp.	–	<Camp.	<Camp.
Sitosterol (% of sterols)[c]	≥93.0	≥93.0	≥93.0	≥93.0	≥93.0
7-Stigmastenol (% of sterols)	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Total sterols (ppm)	≥1000	≥1000	≥2500	≥1800	≥1600
Stigmastadienes (ppm)	–	–	–	–	–
Myristic acid (%)	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05
Linolenic acid (%)	≤0.9	≤0.9	≤0.9	≤0.9	≤0.9
Arachidic acid (%)	≤0.6	≤0.6	≤0.6	≤0.6	≤0.6
Eicosenoic acid (%)	≤0.4	≤0.4	≤0.4	≤0.4	≤0.4
Behenic acid (%)	≤0.2	≤0.2	≤0.3	≤0.3	≤0.3
Lignoceric acid (%)	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
Trans isomers $C_{18:1}$ (%)	≤0.20	≤0.20	≤0.20	≤0.40	≤0.40
Trans isomers ($C_{18:1}+C_{18:2}$) (%)	≤0.30	≤0.30	≤0.10	≤0.35	≤0.35
UV absorption (*K* at 232 nm)	≤3.40	≤3.30	–	≤5.50	≤5.50
UV absorption (*K* at 270 nm)	≤1.20	≤1.00	–	≤2.50	≤2.00
UV absorption (*K* at 270 nm after alumina treatment)	–	–	–	–	–
$\Delta K = [K268 - (K262 + K274)/2]$	≤0.16	≤0.13	–	≤0.25	≤0.20
Panel test	–	–	–	–	–

[a] ECN 42 = Equivalent chain number at 42 carbon number (amounts of all triglycerides with 42 carbon atoms, considered without glycerine carbon atoms). ECN 42 (HPLC) and the calculated ECN 42 were determined by means of HPLC analysis and the fatty acid composition, respectively.
[b] Camp = Campesterol.
[c] 5,23-Stigmastadienol + clerosterol + sitosterol + sitostanol + 5-avenasterol + 5,24-stigmastadienol.

ponents'') are constituted by liposoluble compounds and by other components that can derive from triglycerides. The following classes of compounds can be included in the category of minor components: diglycerides (DGs), monoglycerides (MGs), free fatty acids (FFAs), *trans* fatty acids (TFAs), oxygenated fatty acids (OFAs), cyclic fatty acids, non-linear fatty acids (branched fatty acids), dimeric fatty acids, unsaponifiable compounds, polyphenols, cholesterol oxidation products (COPs) and phospholipids (PLs). Some of these components are present in relatively high quantities in some matrices, but they do not exceed 2% of the total lipid composition.

The minor components of the unsaponifiable fraction are useful for recognizing the origin of the lipids from which they were extracted [4–34], since the qualitative and quantitative distribution of many compounds is characteristic from the lipid source. In the case of oleaginous, they display a very distinctive profile of the unsaponifiable fraction, which is thus utilized as a fingerprint for their identification.

The main classes of compounds that constitute the unsaponifiable fraction of food are listed as follows: hydrocarbons, carotenes, tocopherols, tocotrienols, linear fatty alcohols, triterpenic alcohols, methyl sterols and sterols. Carotenoid pigments, triterpenic dialcohols, diterpenic alcohols and phytol are also unsaponifiable components, but they are not always present in all food matrices.

Table 3
Maximum analytical sensitivity of several conventional methods for the detection of forbidden oils (expressed as percentage), that are present in virgin olive oil mixtures [3]

Oil	Analytical determination								
	Sterols	Fatty acids	LLL[a]	Steradienes		*trans* isomers	UV absorption	Waxes	Erythrodiol
				Mean	Max.				
Cottonseed	9	4	3	0.2	0.3	–	–	–	–
Peanut	5	2	–	0.7	0.3	–	–	–	–
Cocoa	5	5	–	nd[b]	nd	–	–	–	–
Safflower	0.3	–	1	nd	nd	–	–	–	–
Coconut	–	0.1	–	nd	nd	–	–	–	–
Rapeseed	0.5	3	–	1.2	2.0	–	–	–	–
Wheat germ	0.4	–	–	nd	nd	–	–	–	–
Sunflower	0.7	–	1.5	1.0	7.0	–	–	–	–
Sunflower (high oleic)	0.7	20	–	1.0	4.0	–	–	–	–
Sunflower (high oleic desterolized)	–	20	–	0.04	0.05	5.0	–	–	–
Jojoba	2	–	–	nd	nd	–	–	–	–
Linseed	3	0.4	–	nd	nd	–	–	–	–
Corn	1	–	3	1.0	2.0	–	–	–	–
Olive (physically refined)	–	–	–	0.7	1.0	–	15	–	–
Olive (chemically refined)	–	–	–	3	15	–	30	–	8
Husk olive	–	–	–	0.5	2.0	–	–	6–15	–
Palm	8	3	–	1.0	1.5	–	–	–	–
Palm kernel	–	0.1	–	nd	nd	–	–	–	–
Grapeseed	7	–	1.5	0.2	0.4	–	–	–	–
Sesame	1.5	–	4	nd	nd	–	–	–	–
Soybean	2	3	3	1.2	2.0	–	–	–	–
Tea	2	–	–	nd	nd	–	–	–	

[a] LLL = Trilinolein.
[b] nd = Not determined.

3. Analysis of the unsaponifiable components

In the refined oils, the amount of unsaponifiable matter is about 1% of the total lipid composition, whereas in other matrices it can be 3–4 times higher. The analysis of these types of components requires a collection via saponification, followed by thin-layer chromatography (TLC) fractionation of the different classes present in the extract (hydrocarbons, carotenes, tocopherols and tocotrienols, linear alcohols, triterpenic alcohols, methyl sterols, sterols and triterpenic dialcohols) (Fig. 1) [35]. It should be pointed out that the same preparative procedure is, in fact, utilized for the analysis of all unsaponifiable components. The purified fractions are analyzed as trimethylsilyl (TMS) derivatives (except for the hydrocarbons) in non-polar capillary gas chromatography (cGC) columns.

A direct analysis of the silylated unsaponifiable components obtained from different food products was performed on a thermostable polar cGC column (65% phenyl–35% dimethylpolysiloxane); this allowed a fast screening of the components of the lipid fraction that are considered to be the fingerprint of a natural matrix (Fig. 2) [36]. Using this methodology, it is possible to detect the presence of small quantities of husk oil in the new or rectified olive oils (Fig. 3) [37], as well as to establish the quality of the oil with respect to oxidation [37]. Another application is the determination of the amount of two different types of coffees (Robusta and Arabica) in a commercial coffee mixture (containing about 5–7% Robusta coffee) [38] by means of the GC trace of the unsaponifiable matter of coffee lipids (Fig. 4) [39].

4. Hydrocarbons

Hydrocarbons in the natural lipid systems are present in quite small amounts (≤0.2% of the total lipids); the only exception is virgin olive oil, which contains about 0.5% and is mainly constituted by squalene. Hydrocarbons are formed by a homologous series of linear compounds that are mainly saturated chains of 15–33 carbon atoms; in food matrices, most of the hydrocarbons have an odd number of

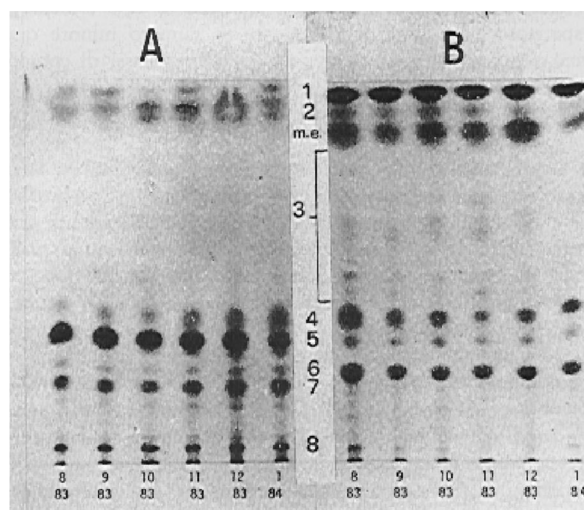

Fig. 1. TLC fractionation of the unsaponifiable matter of the lipids obtained from the exocarp (A) and the endocarp–mesocarp (B) of the oil drupes at different ripening stages (from August to January). Spot identification: (1) hydrocarbons; (2) carotenes; (m.e.) fatty acid methyl esters (unsaponifiable impurity); (3) tocopherols and tocotrienols; (4) linear and triterpenic alcohols (4,4′-dimethylsterols); (5) 4-methylsterols; (6) sterols (desmethylsterols); (7) triterpenic dialcohols; (8) triterpenic dialcohols (methyl esters) (unsaponifiable impurity). The mobile phase was *n*-hexane–diethyl ether (7:3, v/v); the TLC layer was sprayed with a saturated solution of $K_2Cr_2O_7$ in H_2SO_4 (80%) and then carbonized at 130°C/25 min. (From Ref. [35], with permission).

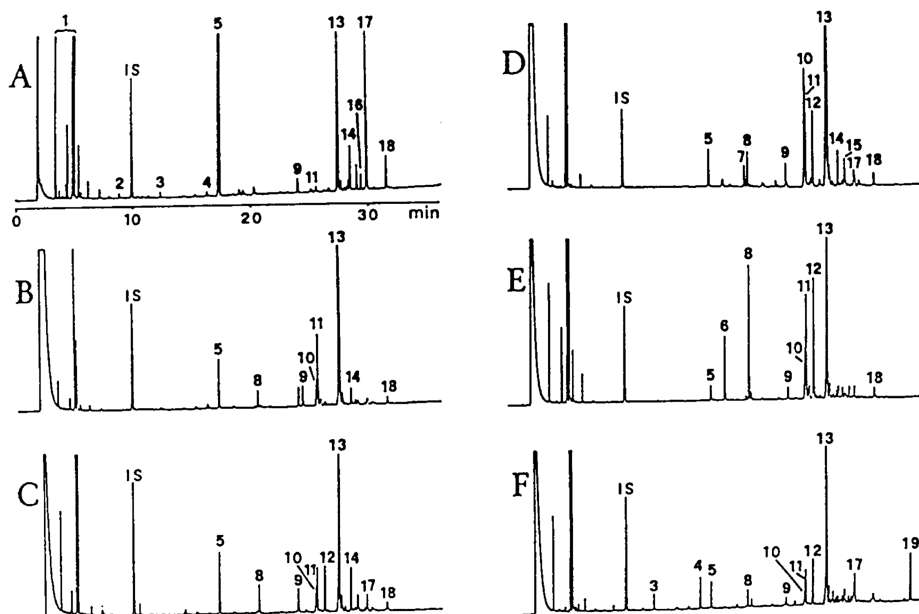

Fig. 2. cGC analysis of the TMS derivatives of the unsaponifiable components of different oils. Trace identification: (A) olive oil; (B) hazelnut oil; (C) peanut oil; (D) corn oil; (E) soybean oil; (F) grapeseed oil. Identification of the main peaks: (1) free fatty acids, IS = internal standard (squalane); (2) docosanol; (3) tetracosanol; (4) hexacosanol; (5) squalene; (6,7) and (8) unknown; (9) α-tocopherol; (10) identified as 22,23-dihydrobrassicasterol (tentative) [36]; (11) campesterol; (12) stigmasterol; (13) sitosterol; (14) 5-avenasterol; (15) cycloartenol; (17) 24-methylenecycloartanol; (18) citrostadienol; (19) oleanolic acid. Oils were injected into a thermostable polar cGC column (65% phenyl–35% dimethylpolysiloxane) (25 m×0.25 mm I.D., 0.1 μm film thickness), with a temperature program from 200 to 300°C at 3°C/min and a helium flow-rate of 0.8 ml/min. (From Ref. [36], with permission).

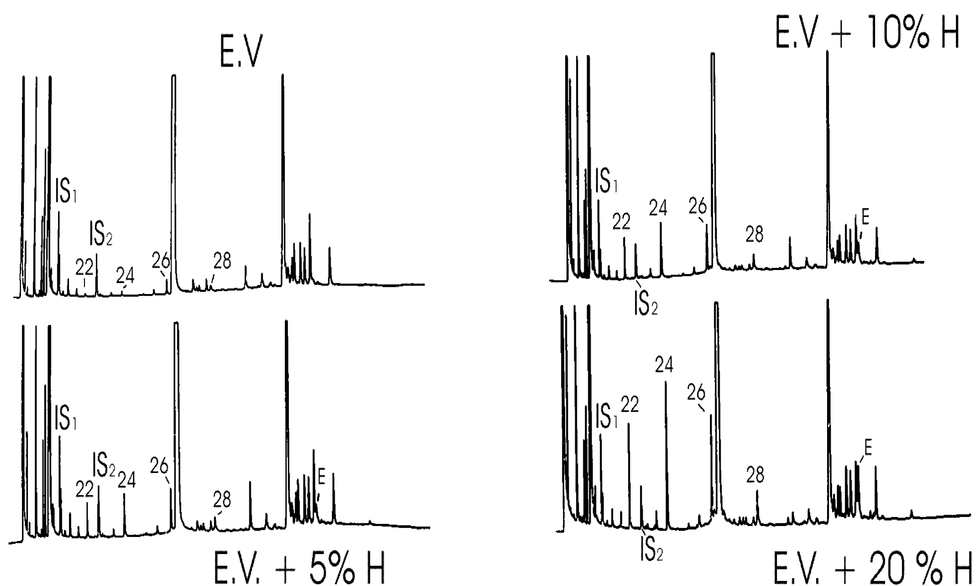

Fig. 3. cGC traces of the TMS derivatives of the unsaponifiable extravirgin olive oil (EV) mixed with different percentages of husk oil (H) (5%, 10% and 20%). Peak identification: IS_1, internal standard (eicosanol); 22, docosanol; IS_2, internal standard (squalane); 24, tetracosanol; 26, hexacosanol; 28, octacosanol; E, erythrodiol. Oils were injected into a thermostable polar cGC column (65% phenyl–35% dimethylpolysiloxane) (25 m×0.25 mm I.D., 0.1 μm film thickness), with a temperature program from 200 to 300°C at 3°C/min and a helium flow-rate of 0.8 ml/min. (From Ref. [37], with permission).

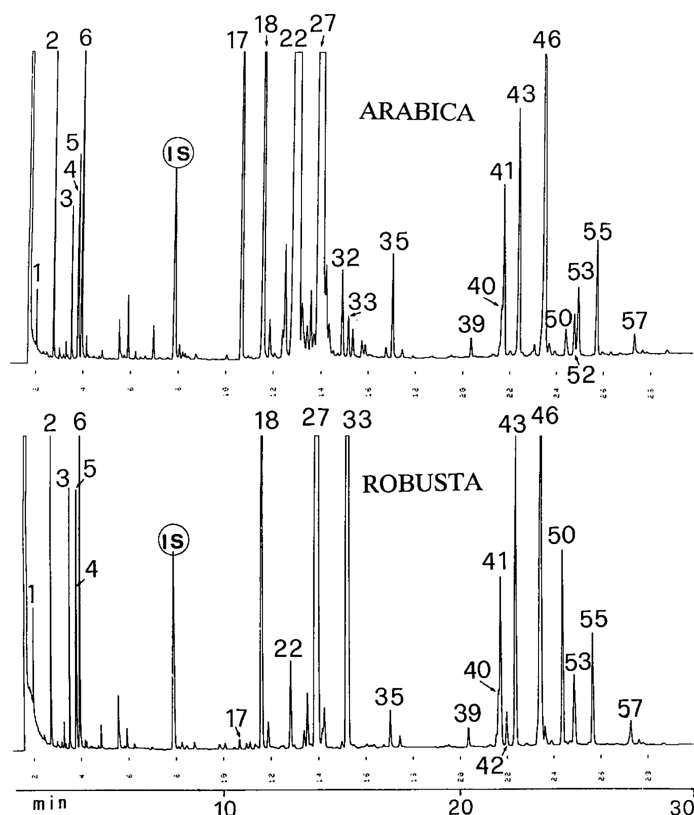

Fig. 4. cGC traces of the unsaponifiable fraction of the Arabica and Robusta coffee varieties. Identification of the main components: (1–4) fatty acids, IS = internal standard (squalane); (17) cafestol dehydration product; (18) kaweol dehydration product; (22) kaweol; (27) cafestol; (33) 16-*O*-methylcafestol; (40) 22, 23-dihydrobrassicasterol (tentative) [38]; (41) campesterol; (43) stigmasterol; (46) sitosterol; (50) 5-avenasterol; (53) cycloartenol; (55) 24-methylenecycloartanol; (57) citrostadienol. The unsaponifiable fractions were injected into a thermostable polar cGC column (65% phenyl–35% dimethylpolysiloxane) (25×0.25 mm I.D., 0.1 μm film thickness), with a temperature program from 200 to 300°C at 5°C/min and a helium flow-rate of 1 ml/min. (From Ref. [39], with permission).

carbon atoms. There are also small amounts of ramified compounds present, which exhibit *iso* and *anteiso*structures [40–42].

Silica TLCs of natural lipids using a mobile phase of *n*-hexane–diethyl ether (7:3, v/v) provide a good separation of hydrocarbons, which elute just after the solvent front. A characteristic GC trace of the TLC hydrocarbon band obtained from natural lipids is shown in Fig. 5 [42]. One interesting case is the beeswax, which displays a notorious quantity of odd hydrocarbons [43]. Some unsaturated cyclic hydrocarbons, known as sterenes, exhibit a steroideal structure and could be useful as tracers of the refining technological step [44,45], since they are not present in the natural lipids and derive from the dehydration of the corresponding sterols.

In some unrefined squeezed oils, small quantities of pollutants can be found (pesticides, volatile organic compounds, chlorinated hydrocarbons, aromatic hydrocarbons, etc.); in fact, small quantities of benzene (about 1 ppm) have been detected in virgin olive oils by using cGC [46]. On the other hand, in the TLC fractionation, the polycyclic aromatic hydrocarbons (PAHs), which originate from environmental pollution, elute together with the other hydrocarbons. However, when these substances are analyzed by cGC, the PAHs peaks overlap with those of the high-molecular weight hydrocarbons, thus rendering it impossible to obtain a reliable quantification of both types of components; high-performance liquid chromatography (HPLC) would be, in this case, a suitable analytical choice, since it has proven

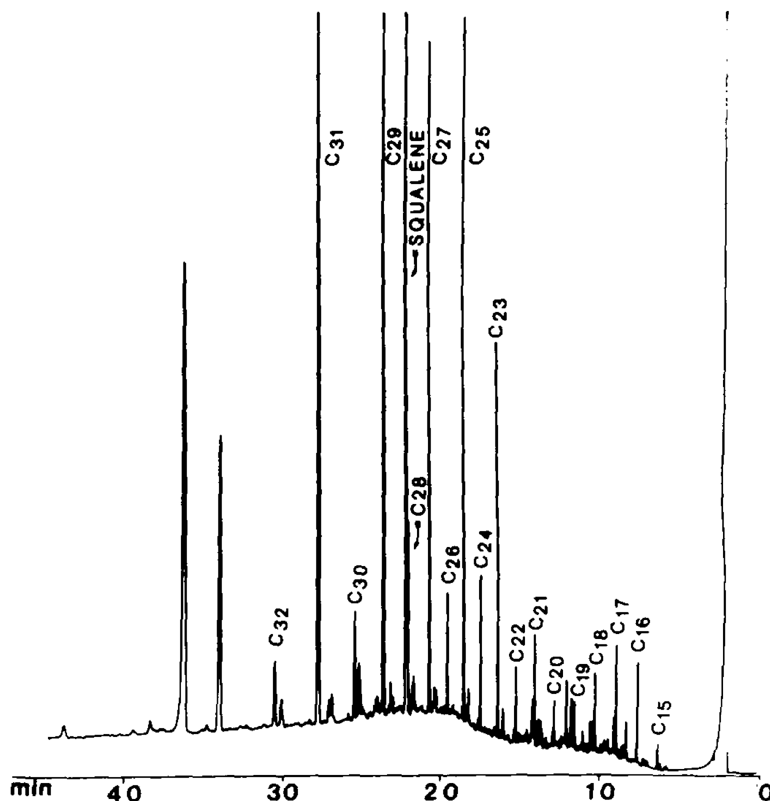

Fig. 5. Analysis of the hydrocarbon fraction of the unsaponifiable matter collected by TLC obtained from the barley kernels. The C_n components correspond to linear hydrocarbons with n number of total carbon atoms. The hydrocarbon fraction was injected into a OV-1 glass capillary column (30 m×0.32 mm I.D., 0.1–0.15 μm film thickness) with a temperature program from 180 to 270°C at 8°C/min and a helium flow-rate of 1.5 ml/min. (From Ref. [30], with permission).

successfully to separate PAHs from the other hydrocarbons [47].

During the refining process, the unsaturated hydrocarbons, such as squalene isomerize, generating a large number of isomers, such as squalene. The presence of these isomers can be detected by cGC [48].

5. Carotenes

These compounds are thermolabile substances regardless of the molecular structure they have (hydrocarbon chain with or without oxygen atoms) [49]. The analysis of carotenes is usually performed by HPLC, since GC degrades the compounds, thus being able to quantify only their pyrolysis products.

6. Tocopherols and tocotrienols

These compounds (see Tables 4 and 5 and Fig. 6) [50–57] display antioxidant properties and they are active as vitamins (vitamin E), which makes them particularly important for human health. Tocotrienols are present in small amounts in food lipids, except for palm oil [54,55], grapeseed oil [53] and the annatto lipid fraction [56], which have a relatively high content of these active components.

The determination of the tocopherols and tocotrienols as TMS derivatives can be performed on a short cGC column of medium polarity (OV-17) in short time of analysis. A very good separation of the two classes of components is actually achieved (Fig. 7) [28].

On the other hand, in the preparative TLC for the

Table 4
Trivial and IUPAC names of tocopherols

Trivial name	IUPAC name	Abbreviation	MS Ref.[a]
5-Methyltocol	2,5-Dimethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	5-T	[50,51]
7-Methyltocol	2,7-Dimethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	7-T	[50,51]
δ-Tocopherol (8-methyltocol)	2,8-Dimethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	δ-T	[52]
5,7-Dimethyltocol	2,5,7-Trimethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	5,7-T	[50,51]
β-Tocopherol (5,8-dimethyltocol)	2,5,8-Trimethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	β-T	[52]
γ-Tocopherol (7,8-dimethyltocol)	2,7,8-Trimethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	γ-T	[52]
α-Tocopherol (5,7,8-trimethyltocol)	2,5,7,8-Tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol	α-T	[52]

[a] Reference that includes the mass spectra identification.

fractionation of the unsaponifiable, the tocopherols and tocotrienols coelute with other compounds of similar polarity. One of these substances are the epoxy-squalenes, which are structurally similar to the squalene but they have an oxyranic ring [31]; the epoxy-squalenes derive from the oxidation of squalene, which can be of enzymatic or chemical origin, as in the olive husk oil [37]. In oil samples extracted from olives harvested at different ripening stages, the level of epoxy-squalenes vary. Since they were practically absent in the oils obtained from mature olives, it can be stated that the epoxy-squalenes are precursors to the steroid biosynthesis, i.e. lanosterol in the animal systems and cycloartenol in the vegetal ones.

7. Linear and triterpenic alcohols

Linear alcohols (see Table 6 and Fig. 8) [58–69] are constituted by a homologue series of primary fatty alcohols that generally contain 20–32 carbon

Table 5
Trivial and IUPAC names of tocotrienols

Trivial name	IUPAC name	Abbreviation	MS Ref.[a]
5-Methyltocotrienol	2,5-Dimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	5-T-3	
7-Methyltocotrienol	2,7-Dimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	7-T-3	
δ-Tocotrienol (8-methyltocotrienol)	2,8-Dimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	δ-T-3	
5,7-Dimethyltocotrienol	2,5,7-Trimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	5,7-T-3	
β-Tocotrienol (5,8-dimethyltocotrienol)	2,5,8-Trimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	β-T-3	
γ-Tocotrienol (7,8-dimethyltocotrienol)	2,7,8-Trimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	γ-T-3	[53]
α-Tocotrienol (5,7,8-trimethyltocotrienol)	2,5,7,8-Tetramethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	α-T-3	[53]

[a] Reference that includes the mass spectra identification.

Fig. 6. Chemical structure of main tocopherols and tocotrienols.

atoms; however, some of them, such as phytol, are generated as artifacts of lipid saponification. The triterpenic alcohols (see Table 6 and Fig. 8), also known as 4,4′-dimethylsterols, have a steroid structure and are present at different levels in all vegetal lipids [12,17,28,70–78].

Both linear and triterpenic alcohols are more polar than tocopherols and are often badly separated in silica TLC; no analytical problems, however, are observed for their determination by cGC. In any case, a better TLC separation could be achieved by using a multiple development technique with a slightly different mobile phase for the second development [79]. Fig. 9 shows the cGC trace of the TMS derivatives of the linear and triterpenic alcohols fraction obtained from a flaxseed oil [80].

The effects of refining and industrial hydrogenation on the structural modifications of linear and triterpenic alcohols have also been studied [76,81–85]. Isomerization due to the presence of a double bond in position 24 or to a 3-carbon atom ring, is the main consequence of these technological steps (Figs. 10 and 11). Differences between the triterpenic alcohol fractions obtained from corn oil before and after hydrogenation are evinced in Fig. 12. The analysis of these fractions were performed on two types of cGC columns of different polarity [85].

8. Methylsterols (4-methylsterols)

The amount of methylsterols (see Table 7 and Fig.

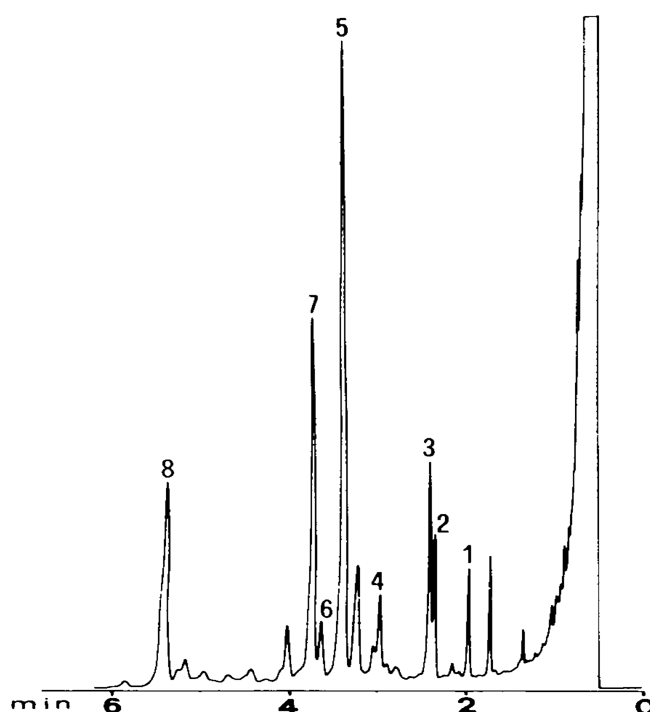

Fig. 7. cGC trace of a tocopherol and tocotrienol mixture obtained from palm and grapeseed oils. Peak identification: (1) δ-tocopherol; (2) β-tocopherol; (3) γ-tocopherol; (4) δ-tocotrienol, (5) α-tocopherol; (6) β-tocotrienol; (7) γ-tocotrienol; (8) α-tocotrienol. This fraction was injected into a OV-17 open tubular supported glass column (15 m×0.25 mm I.D., 0.1–0.15 μm film thickness supported on $BaCO_3$) at 240°C with a helium flow-rate of 2.5 ml/min. (From Ref. [28], with permission).

Table 6
Trivial and IUPAC names of some 4,4′-dimethylsterols.

Trivial name	IUPAC name	Conf.[a]	MS Ref.[b]
Cycloartenol	9β,19-Cyclo-5α-lanost-24-ene 3β-ol		[17,61,62]
α-Amyrin	5α-Urs-12-ene 3β-ol		[17]
β-Amyrin	5α-Olean-12-ene 3β-ol		[17]
Butyrospermol	5α-Eupha-7,24(25)-diene 3β-ol		[17,67–70]
24-Methylenecycloartanol	24-Methylen-9β,19-cyclo-5α-lanost-24-ene 3β-ol		[64]
24-Methylenelanost-9(11)-enol	24-Methylen-lanost-9(11)-ene 3β-ol		[72]
Parkeol	5α-Lanosta-9(11),24-diene 3β-ol		[60,17]
Cyclolaudenol	24-Methyl-9β,19-cyclo-5α-lanost-24-ene 3β-ol	24S	[65,66,73]
Cycloartanol	9β,19-Cyclo-5α-lanostane 3β-ol		[59,63]
Lupeol	5α-Lup-20(29)-ene 3β-ol		[17]
Cyclobranol[c]	24-Methyl-9β,19-cyclo-5α-lanost-24-ene 3β-ol		[79]
Lanostenol	5α-Lanost-8-ene		[73]

[a] Conf = Configuration.
[b] Reference that includes the mass spectra identification.
[c] Compound originated by 24-methylenecycloartanol isomerization.

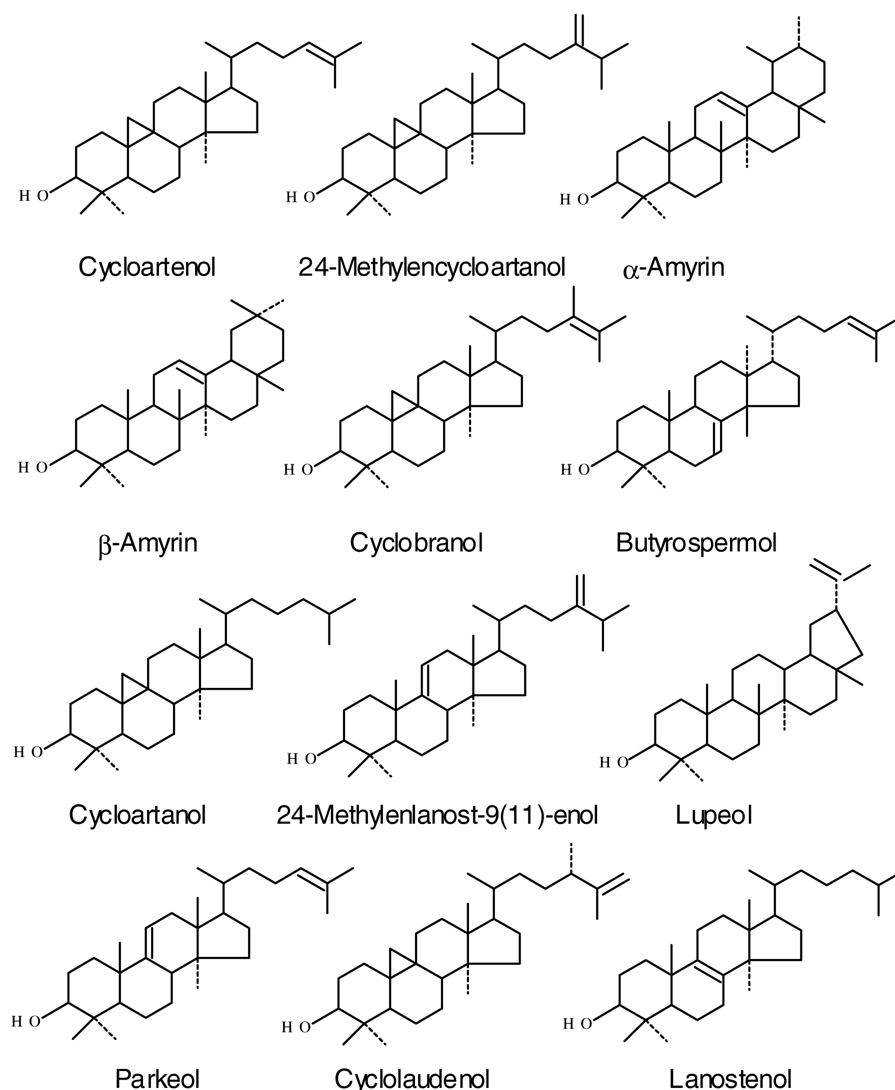

Fig. 8. Chemical structure of main linear and triterpenic alcohols (4,4′-dimethylsterols).

13) [70,86–96] found in food matrices is relatively low [28] as compared to that of other components of the unsaponifiable matter. Regarding the silica TLC development of methylsterols, this fraction elutes between the linear (triterpenic) alcohols band and that of the sterols [18,20,28,73–77].

Fig. 14 shows the cGC traces of the TMS derivatives of methylsterols obtained from corn oil before and after hydrogenation [84]. Similarly to the triterpenic alcohols (Figs. 10 and 11), isomerization of methylsterols after refining or hydrogenation [80,84] evinces illegal addition of refined oils to virgin olive oils.

9. Sterols (4-desmethylsterols)

The most studied fraction of the unsaponifiable matter is the sterol one (see Table 8 and Fig. 15) [70,90,97–116], which has been analyzed by using GC packed columns [4–27], as well as short [28] (Fig. 16) and long capillary columns [28,30–39].

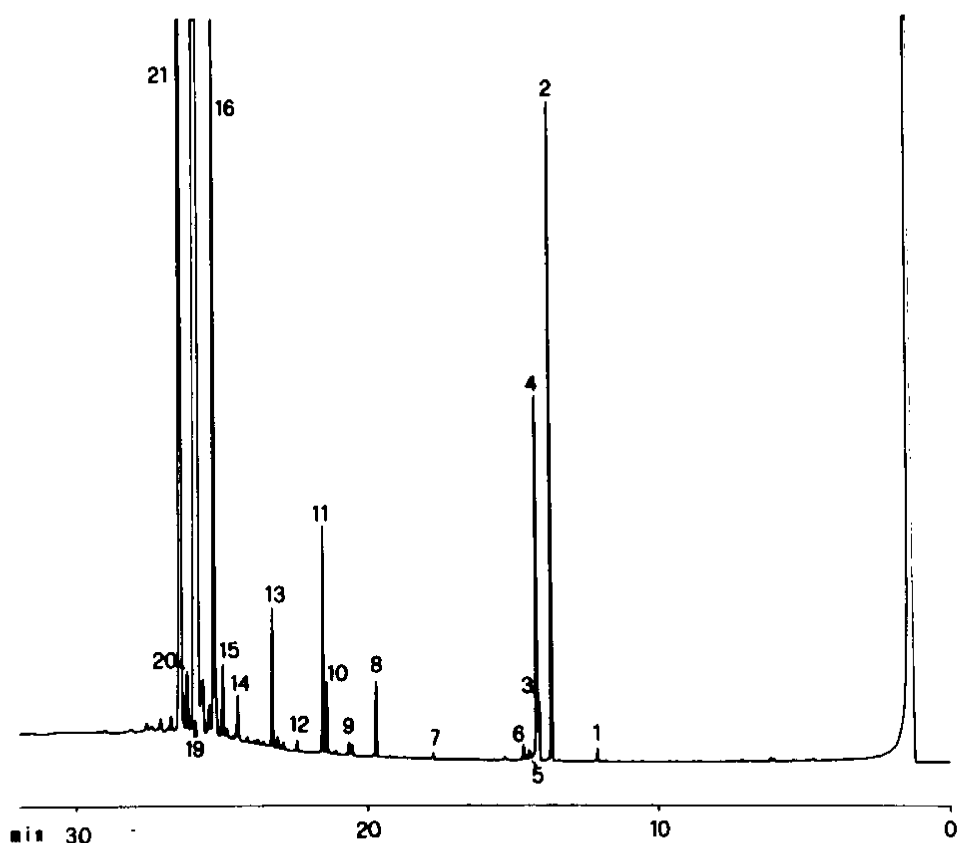

Fig. 9. cGC trace of the linear and triterpenic alcohols (4,4′-dimethylsterols) obtained from a linseed oil. Peak identification: (2) phytol; (4) geranyl–geraniol; (7,8,9,11,12,13,15) linear hydrocarbons with 22, 24, 25, 26, 27, 28, and 30 total carbon atoms, respectively; (16) β-amyrin; (19) cycloartenol; (21) 24-methylenecycloartanol; the other components were not identified. The alcohols fraction was injected into a SE-52 glass capillary column (25 m×0.32 mm I.D., 0.1–0.15 μm film thickness) with a temperature program from 150 to 300°C at 8°C/min and a helium flow-rate of 2.5 ml/min. (From Ref. [30], with permission).

Table 7
Trivial and IUPAC names of main 4-methylsterols

Trivial name	IUPAC name	Conf.[a]	MS Ref.[b]
Obtusifoliol	4α,14α-Dimethyl-24-methylene-9β,19-cyclo-5α-cholest-8-ene 3β-ol		[71,91]
Gramisterol	4α-Methyl-24-methylene-5α-cholest-7-ene 3β-ol		[95]
Citrostadienol	4α-Methyl-24-ethylidene-5α-cholest-7-ene 3β-ol	24Z	[95]
Cycloeucalenol	4α,14α-Dimethyl-9β,19-cyclo-24-methylene-5α-cholestane 3β-ol		[92–94]
Lophenol	4α-Methyl-5α-cholest-7-ene 3β-ol		[73,90]
31-Norcycloartenol	4α,14α-Dimethyl-9β,19-cyclo-5α-cholest-24-ene 3β-ol		[86]
4α-Methylzymostenol	4α-Methyl-5α-cholest-8-ene 3β-ol		[73]
4α-Methylzymosterol	4α-Methyl-5α-cholesta-8,24-diene 3β-ol		[89]
31-Norlanostenol	4α,14α-Dimethyl-5α-cholest-8-ene 3β-ol		[94]
31-Norlanosterol	4α,14α-Dimethyl-5α-cholesta-8,24-diene 3β-ol		[87,88]

[a] Conf. = Configuration.
[b] Reference that includes the mass spectra identification.

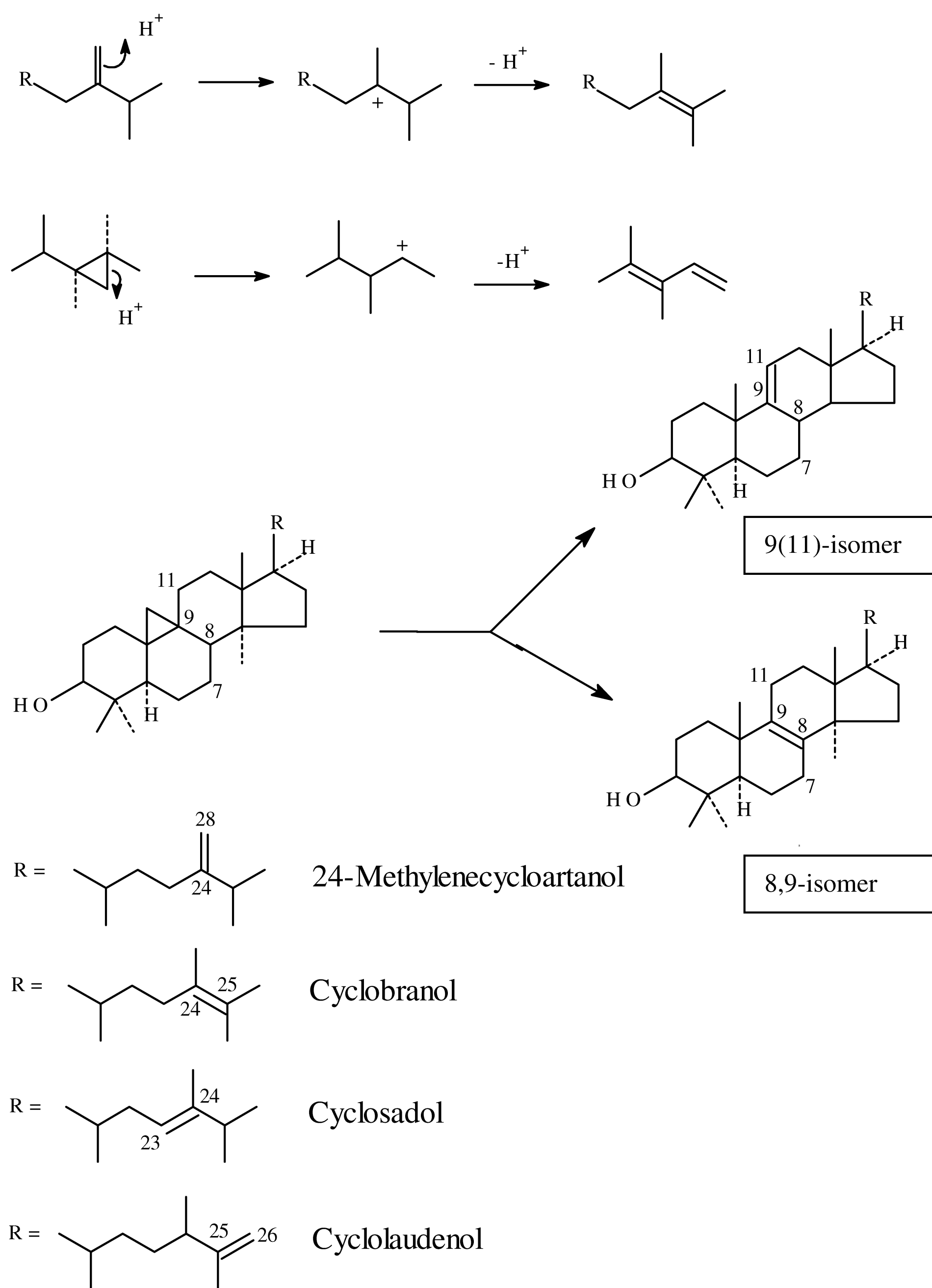

Fig. 10. Isomerization mechanisms of side chain and alycyclic structures of 4,4′-dimethylsterols.

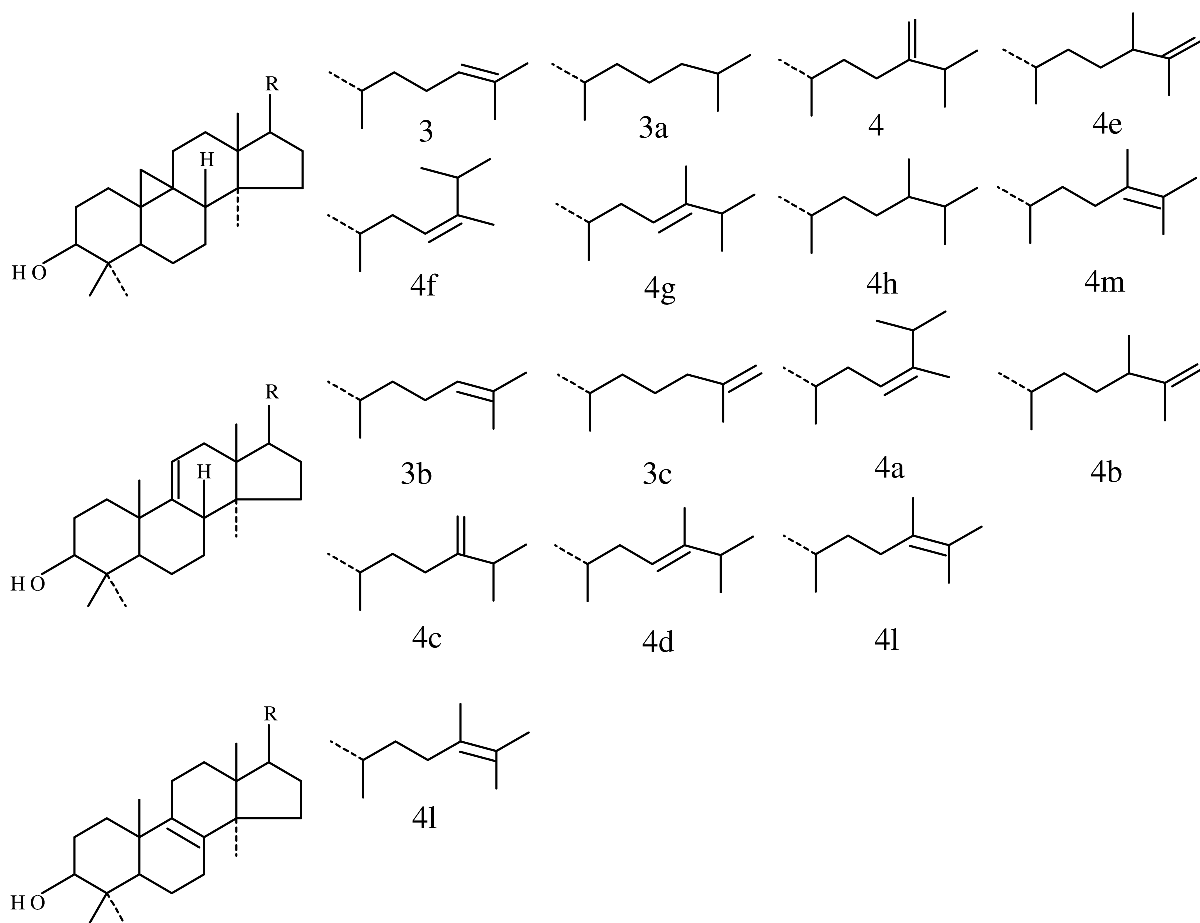

Fig. 11. Side chain isomers of 4,4′-dimethylsterols. Each isomer is coded with a number, which corresponds to peaks of the GC trace shown in Fig. 12.

The determination of the total sterols and that of the sterols in their free form, allow the quantitation of the esterified ones. Several preparative methods have been suggested for the separation of the two types of sterols [117–123] and they report different recoveries [123].

On the other hand, it has been observed that sterols are so efficiently separated on a polar cGC column (65% phenyl–35% dimethylpolysiloxane) that it is possible to detect a series of substances [124] (Fig. 17) that cannot be separated by other cGC columns.

During the silica TLC fractionation of the unsaponifiable matter, it is possible to separate the 7-sterols from the 5-ones, which are quantitatively predominant. Fig. 18 reports the cGC traces of the two separately analyzed TLC bands, together with the total cGC trace of the sterols [31].

The fraction of sterols, probably due to their relative abundance, is frequently used for tracking commercial frauds [4–6,36,37,39,43,57,77,79,123, 125–133]. Positional isomers of the double bond in the sterols ring have been recently detected, which can be used as fraud tracers in virgin olive oils [134].

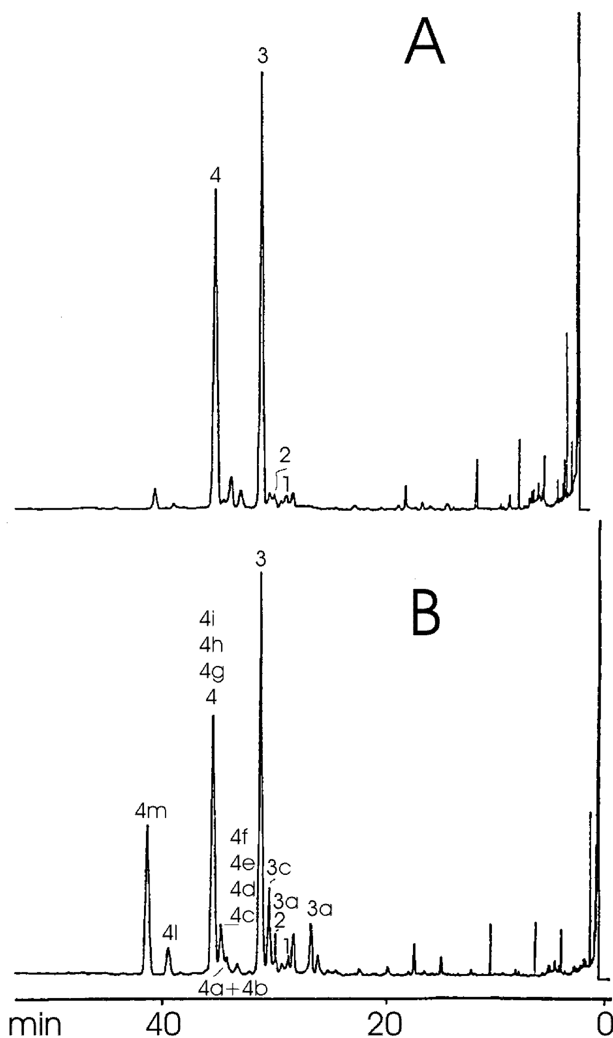

Fig. 12. cGC traces of the triterpenic alcohols (4,4′-dimethylsterols) obtained from a corn oil, before (A) and after industrial hydrogenation (B). Peak identification: (1) β-amyrin; (2) α-amyrin; (3) cycloartenol; (4) 24-methylenecycloartanol; (3a–c), products generated by cycloartenol isomerization; (4a–m) 24-methylenecycloartanol isomers (see Fig. 11). The triterpenic alcohols were injected into a SE-52 glass capillary column (30 m×0.32 mm I.D., 0.2 μm film thickness) at 260°C and a helium flow-rate of 1 ml/min. (From Ref. [85], with permission).

Refining and, in particular, acidity can modify the sterol composition [135–145], leading to isomerization of fucosterol and 5-avenasterol [145].

On the other hand, some changes have been observed in the olive oil composition during the drupe ripening [146,147].

10. Sterol oxidation products

The analysis of the sterol oxidation products is mostly focused on cholesterol, since the toxicity of some of these components has been asserted [148].

To perform the analysis of the total sterol oxida-

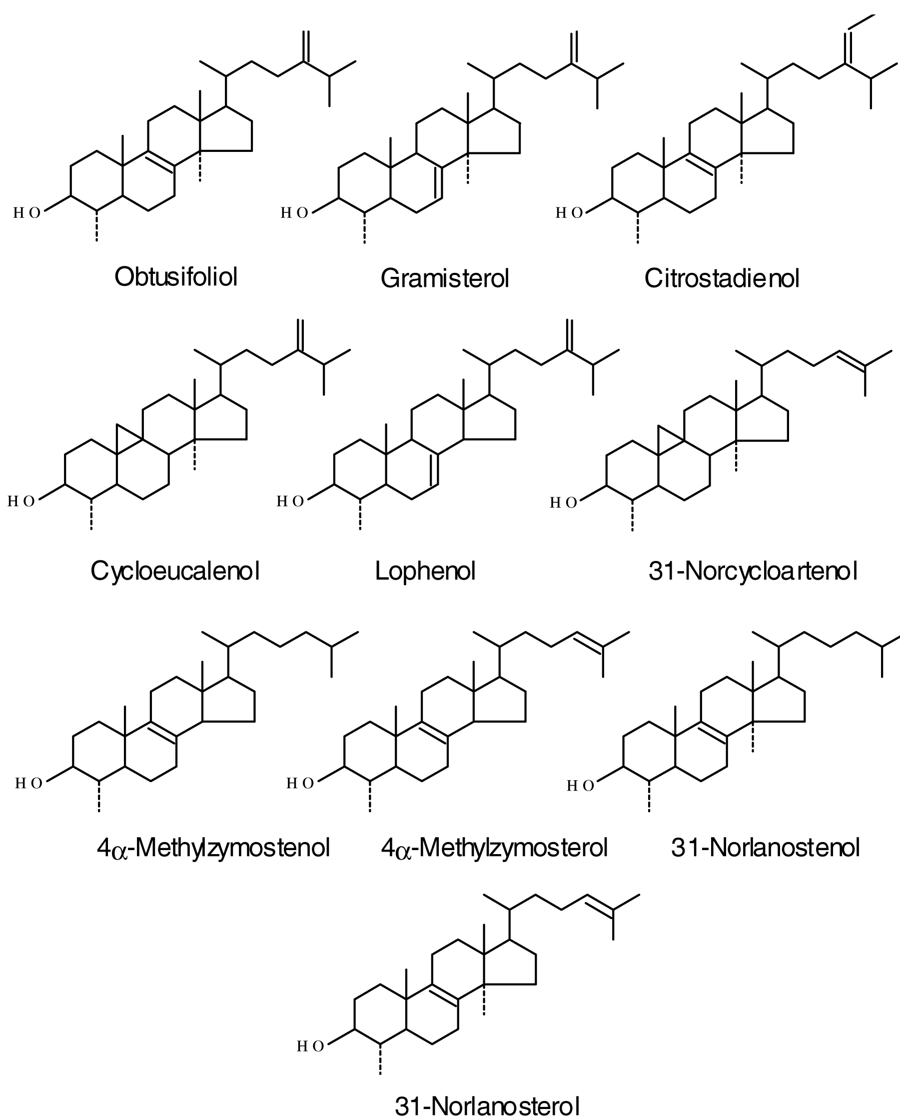

Fig. 13. Chemical structures of main 4-methylsterols.

tion products (i.e., the free plus the esterified ones), the oil or extracted lipids are subjected to cold saponification [149] and enrichment by either silica or aminopropyl solid-phase extraction (SPE) [150,151]. It is advisable to use cold saponification instead of hot saponification, in order to avoid artifact formation [152,153]. In addition, precautions should be taken so as to protect the sample against light, oxygen and high-temperature exposure, which are well-known promoters of lipid oxidation.

Several HPLC [154] and cGC [149,155] methods have been set up for the analysis of cholesterol oxidation products (COPs). cGC has proven to give better results than HPLC, as shown in Fig. 19.

Table 8
Trivial and IUPAC names of some desmethylsterols (sterols) and triterpenic dialcohols

Trivial name	IUPAC name	Conf.[a]	MS Ref.[b]
Cholesterol	5α-Cholest-5-ene 3β-ol		[97–99]
Cholestanol	5α-Cholestane 3β-ol		[146]
Brassicasterol	Ergosta-5,22-diene 3β-ol	22 E	[101–103]
24-Methylencholesterol	Ergosta-5,24(28)-diene 3β-ol		[13,70,97,98]
Campesterol	Ergost-5-ene 3β-ol	24 E	[104]
Ergostanol	Ergostane 3β-ol		
Stigmasterol	5α-Stigmasta-5,22-diene 3β-ol		[104]
22-Stigmastenol	5α-Stimast-22-ene 3β-ol	22 E	[146]
7-Campesterol	Ergost-7-ene 3β-ol		
Clerosterol	5α-Stigmasta-5,25-diene 3β-ol	24 S	[112,113,146]
Sitosterol	5α-Stigmast-5-ene 3β-ol	24 R	[104]
Fucosterol	5α-Stigmasta-5,24(28)-diene 3β-ol	24 E	[98,105,106,109,110]
Stigmastanol	5α-Stigmastane 3β-olo		[146]
Isofucosterol (5-avenasterol)	5α-Stigmasta-5,24(28)-diene 3β-ol	24 Z	[13,70,107–109]
7-Stigmastenol	5α-Stigmast-7-ene 3β-ol		[17,70,90,114,115]
7-Avenasterol	5α-Stigmasta-7,24(28)-diene 3β-ol	24 Z	[110,111]
α-Spinasterol	5α-Stigmasta-7,22-diene 3β-ol		[17,70]
Zymostenol	5α-Cholest-8-ene 3β-ol		
Zymosterol	5α-Cholesta-8,24-diene 3β-ol		[100]
22,23-Dihydrobrassicasterol	Ergost-5-ene 3β-ol		
Erythrodiol	5α-Olean-12-ene-3β,28-diol		
Uvaol	5α-Urs-12-ene-3β,28-diol		

[a] Conf. = Configuration.
[b] Reference that includes the mass spectra identification.

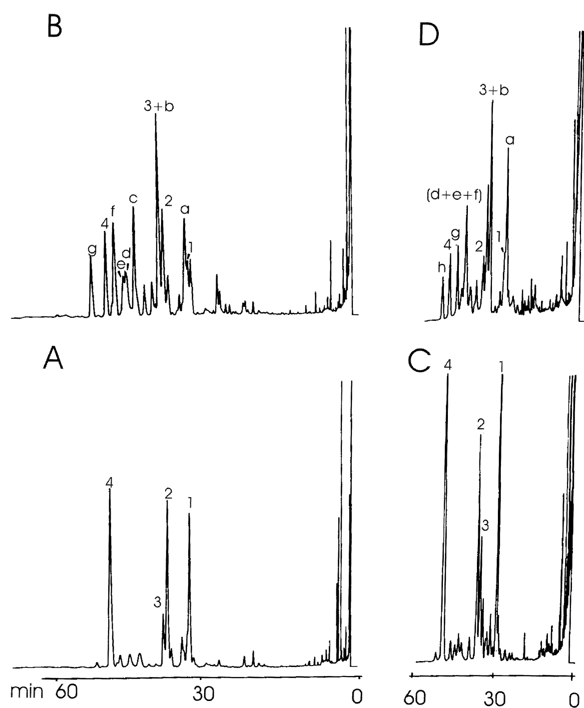

Fig. 14. cGC traces of the 4-methylsterols obtained from corn oil before (A and C) and after hydrogenation (B and D). Samples that correspond to traces (A) and (B) were injected into a SE-52 capillary glass column (30 m×0.32 mm I.D., 0.32 μm film thickness) at 260°C and a helium flow-rate of 1 ml/min. Samples that correspond to traces (C) and (D) were injected into a free fatty acid phase (FFAP) column (20 m×0.32 mm I.D., 0.2 μm film thickness) at 220°C and a helium flow rate of 1.5 ml/min. Peak identification: (1) obtusifoliol; (2) gramisterol; (3) cycloeucalenol; (4) citrostadienol; (a, b and e) products totally or partially generated by obtusifoliol isomerization (b) also derives from a gramisterol isomer; (c) gramisterol isomer; (f and g) citrostadienol isomers. (From Ref. [85], with permission).

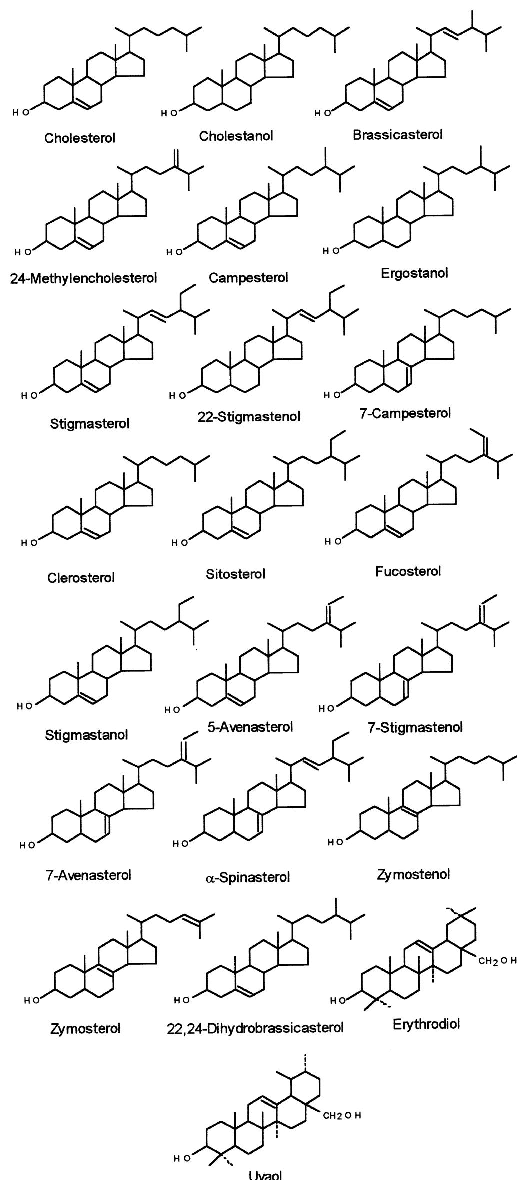

Fig. 15. Chemical structures of main desmethylsterols (sterols) and triterpenic alcohols.

11. Polar minor components

This class of components is extracted with very polar solvents (methanol or water–methanol mixture) and is usually present in virgin olive oils and grapeseed oils. Among these substances, polyphenols are of great interest due to their antioxidant properties [156–158]. Fig. 20 [159] displays the HPLC traces of two olive oil extracts obtained with a polar solvent. The oils were produced from the same batch of olives, but the contact time between the oil and the olive paste was different (0 and 70 min). Dissimilar polar minor component (PMC) profiles were observed, which seems to explain somehow the variations in resistance with respect to accelerated oxidation; apparently, not all polyphenols exhibit antioxidant properties and this is the reason why the concentration of total polyphenols cannot be directly correlated to the opposition to accelerated oxidation that has been detected in many olive oils.

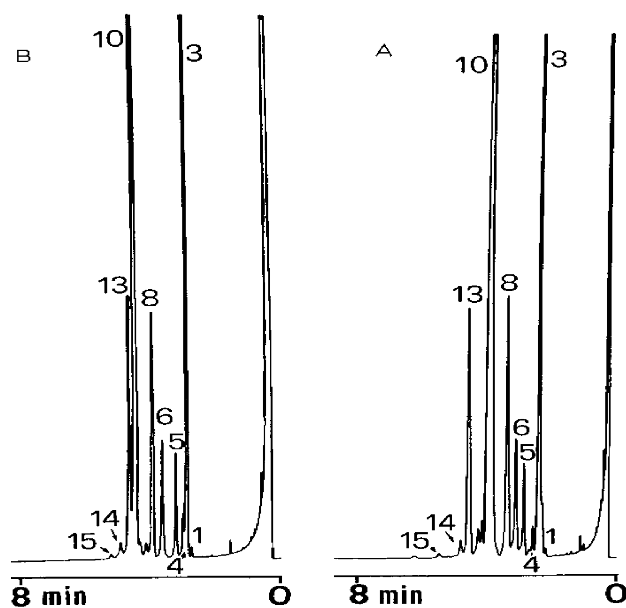

Fig. 16. cGC traces of the TMS derivatives of the total sterols of tomato seeds. Peak identification: (1) dehydrocholesterol (tentative); (3) cholesterol; (4) 24-methylenecholesterol; (5) brassicasterol; (6) campesterol; (8) stigmasterol; (10) sitosterol; (13) 5-avenasterol; (15) 7-stigmastenol. For trace (16A), a FFAP column (9 m×0.32 mm I.D., 0.1–0.15 μm film thickness) was used, whereas a SE-30 column (10 m×0.32 mm I.D., 0.1–0.15 μm film thickness) was utilized for trace (16B). The analysis were run at 205°C with a helium flow-rate of 2.5 ml/min. (From Ref. [29], with permission).

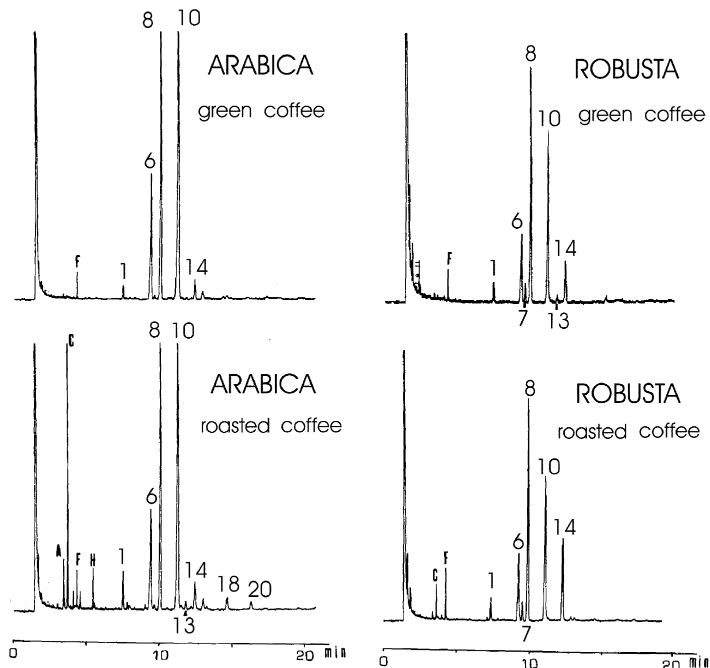

Fig. 17. cGC traces of the TMS derivatives of the free sterols before and after roasting of the Arabic (left) and Robusta (right) coffee varieties. Peak identification: (1) cholesterol; (6) campesterol (the small sterol ahead of campesterol has been identified as 22, 23-dihydrobrassicasterol by other authors [34]); (8) stigmasterol; (10) sitosterol; (14) 5-avenasterol. Samples were injected into a thermostable polar cGC column (65% phenyl–35% dimethylpolysiloxane) (25 m×0.25 mm I.D., 0.1 μm film thickness) at 240°C and 1 ml/min of helium flow-rate. (From Ref. [124], with permission).

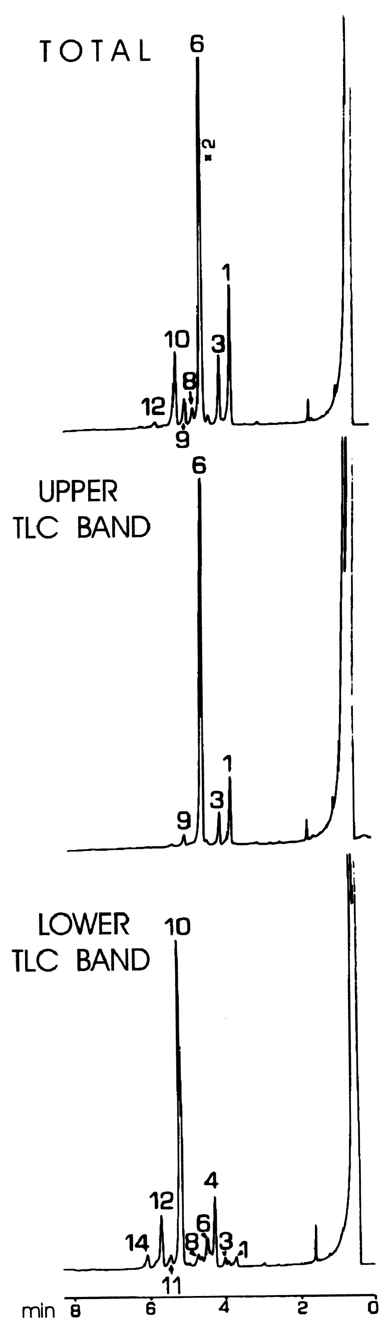

Fig. 18. cGC traces of the TMS derivatives of sterols obtained from safflower seed oil. The upper trace corresponds to the total mixture, whereas the middle and the lower traces correspond to the top and the bottom TLC bands, respectively. Fractions were injected into a SE-30 column (10 m×0.32 mm I.D., 0.1–0.15 μm film thickness) at 205°C and 2.5 ml/min of helium flow-rate. (From Ref. [30], with permission).

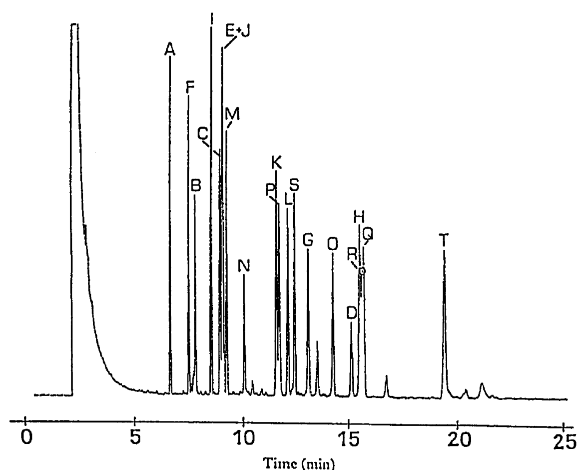

Fig. 19. cGC trace of the TMS derivatives of a standard mixture of "oxysterols" (cholesterol oxidation products or COPs). The mixture was injected into a thermostable polar cGC column (65% phenyl–35% dimethylpolysiloxane) (25 m×0.25 mm I.D., 0.1 μm film thickness), with a temperature program from 245 to 265°C at 3.5°C/min and then taken to 290°C at 2.5°C/min. Helium flow-rate was 0.7 ml/min. Peak identification: (A) 5α-chlolestane; (B) cholesta-3,5-diene; (C) dihydrochlolesterol; (D) cholesta-3,5-diene-7-one; (E) cholesterol; (F) 7α-OH; (G) 4-cholesten-3-one; (H) 5-cholesten-3-one; (I) 19-OH; (J) 7β-OH; (K) 5β,6β-epoxy; (L) 5α,6α-epoxy; (M) 4β-OH; (N) 20α-OH; (O) 5α-cholestan-3β-ol-7-one; (P) triol; (Q) 5α-cholestan-3gb-ol-6-one; (R) 7-k; (S) 25-OH; (T) cholestan-3β,5α-diol-6-one. (From Ref. [155], with permission).

Due to the complexity of the PMC fraction, it has still not been possible to identify several of the components, even though many research groups have dealt with this subject [160–162].

12. Conclusions

Several GC methods are nowadays utilized for the quality control of lipids. Although some of these analytical determinations provide very specific information, it should be underlined that one of the main advantages of GC is that it is a low-cost technique and that it can be really helpful for elucidating several problems related to the raw material used for the elaboration of food products, processing and storage conditions. The choice of the method to be employed will depend on the scope of the analytical control, the amount of information that

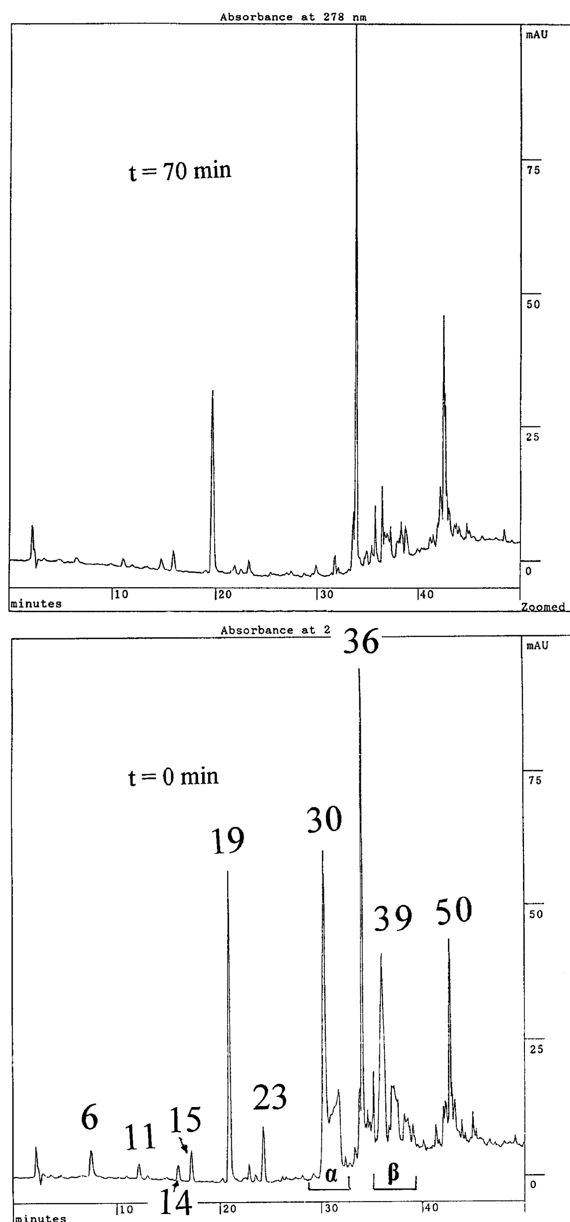

Fig. 20. Reversed-phase HPLC trace of the minor polar components extracted from extravirgin olive oils obtained from the same olive paste batch at the beginning ($t=0$ min) and after 70 min of kneading. Part of the components here indicated had already been identified as polyphenolic substances [156]. Samples were injected into a Spherex 3 ODS (150 mm×4.6 mm I.D.) using a mixture of 2% acetic acid solution (v/v, in water) and methanol as the mobile phase; the mobile phase program started with 2% acetic acid–methanol (95:5; v/v) up to a 0:100 ratio (v/v) after a 50-min run. (From Ref. [159], with permission).

can be acquired and the cost of the overall analytical operation.

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