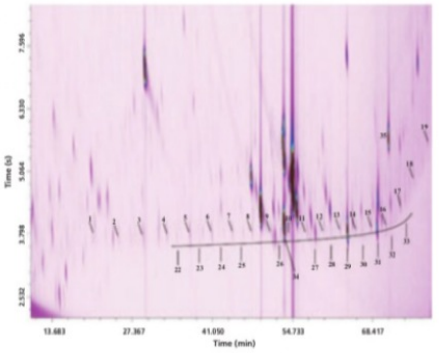
Flow-Modulated Comprehensive 2D Gas Chromatography–Triple Quadrupole MS Elucidation of the Fatty Acids and Unsaponifiable Constituents of Oil Derived from Lemon Seeds, A Food-Industry Waste Product



This article is focused on the detailed qualitative analysis of the fatty acids and the unsaponifiable constituents of a vegetable oil derived from a food-industry waste product, namely lemon seeds. The seed oil was subjected to two sample preparation processes, the first enabling the formation of fatty acid methyl esters (FAMEs), and the other the isolation of the constituents of the entire unsaponifiable fraction (sterols, hydrocarbons, vitamins, etc.). Both sets of compounds were subjected to flow-modulation (FM) comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC–MS), with identification performed through full-scan data. Relative percentage data, relative to FAMEs and sterols, were derived through gas chromatography–flame ionization (GC–FID). Finally, a sterol identified through a clear signal as cholesterol, was subjected to absolute quantification using multiple reaction monitoring (MRM).

Peter Q. Tranchida*, Flavio A. Franchina*, Simona Salivo*, Marina Russo*, Paola Dugo*,†,‡, and Luigi Mondello*,†,‡

**Dipartimento di Scienze del Farmaco e Prodotti per la Salute, Università degli Studi di Messina, Messina, Italy*

†Centro Integrato di Ricerca (C.I.R.), Università Campus Bio-Medico, Roma, Italy

‡ Chromaleont s.r.l. A start-up of the University of Messina, c/o Dipartimento di Scienze del Farmaco e Prodotti per la Salute, University of Messina, Messina, Italy

The worldwide production of *Citrus* fruits is enormous, reaching 91 million tons in the period 1999–2000. A high percentage of this fruit (≈ 40%) is involved in industrial processing, with essential oils and juices the main end-products. Considering single *Citrus* species, the orange represents approximately 67% of the fruit produced, while the lemon makes up about 6% (1).

It is clear that the industrial processing of such a large amount of fruit generates a great amount of waste, in terms of peel, pulp, and seeds. Studies on the use of lemon seeds as a source of vegetable oil have been reported previously. For example, Malacrida et al. reported that the main fatty acids (FAs) in lemon seed oil (approximately 35% of the seed mass) were $C_{16:0}$ (≈ 21.0%), $C_{18:1\omega9}$ (≈ 20.8%), $C_{18:2\omega6}$ (≈ 44.3%), and $C_{18:3\omega3}$ (≈ 9.0%), while minor FAs were $C_{18:0}$ (≈ 3.7%), $C_{16:1\omega7}$ (≈ 0.7%), $C_{17:0}$ (traces), $C_{20:0}$ (≈ 0.3%), $C_{22:0}$ (≈ 0.1%), and $C_{24:0}$ (≈ 0.2%) (2). Furthermore, a tocopherol content of 125 mg/kg was present in the oil, with the α-isomer present in by far the highest amounts (102.5 mg/kg). Reda et al. studied the FAs in Sicilian lemon seed oil (approximately 38% of the seed mass), reporting the following composition: $C_{8:0}$ (1.0%), $C_{14:0}$ (0.1%), $C_{16:0}$ (19.6%), $C_{18:0}$ (3.0%), $C_{18:1\omega9}$ (28.6%), $C_{18:2\omega6}$ (34.4%), $C_{18:3\omega3}$ (10.0%), and $C_{20:0}$ (0.2%) (3). Saïdani et al. performed research on Tunisian lemon seed oil, reporting a surprisingly high oil yield, namely 79% (4). The FA composition was reported to be: $C_{8:0}$ (0.04%), $C_{10:0}$

Table I: Lemon seed oil FAMEs, MS database similarity results and percentage values (%). Abbreviations: a = anteiso; i = iso

FAME	Similarity	%
$C_{14:0}$	90	
$C_{15:0}$	84	
a-$C_{16:0}$	87	
$C_{16:0}$	90	18.4
$C_{17:0}$	90	
i-$C_{18:0}$	88	
$C_{18:0}$	90	3.4
a-$C_{19:0}$	82	
i-$C_{20:0}$	75	
$C_{20:0}$	90	
$C_{22:0}$	90	
$C_{23:0}$	83	
$C_{24:0}$	76	
$C_{16:1\omega5}$	90	
$C_{17:1\omega7}$	86	
$C_{18:1\omega9}$	90	27.9
$C_{20:1\omega7}$	91	
$C_{18:2\omega6}$	92	36.6
$C_{18:3\omega3}$	85	12.2
$C_{20:4\omega3}$	78	

(0.05%), $C_{12:0}$ (0.04%), $C_{14:0}$ (0.10%), $C_{14:1}$ (0.10%), $C_{16:0}$ (21.40%), $C_{18:0}$ (2.3%), $C_{18:1\omega9}$ (36.6%), $C_{18:2\omega6}$ (31.4%), and $C_{18:3\omega3}$ (6.90%). Compared to the research performed by Malacrida et al. and Reda et al., an inverted relationship between oleic and linoleic acid was reported.

The objective of the current research is focused on the qualitative analysis of the fatty acids and of the unsaponifiable constituents of lemon seed oil. Consequently, two distinct analyses were performed using a flow-modulation (FM) comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC–MS) method. The MS system used was a "rapid" triple quadrupole one, fast enough for GC×GC requirements, in both the full-scan and targeted (for example, multiple reaction monitoring [MRM)]) analyses. Gas chromatography–flame ionization detection (GC–FID) was exploited to quantify a series of FAs and sterols in percentages.

Experimental

Samples, Reagents, and Pure Standards

The lemon seed oil was produced in the laboratory, through a laboratory cold-pressing device.

The boron trifluoride–methanol complex was purchased from Merck. The BSTFA (N,O-bis[trimethylsilyl] trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) kit, *n*-hexane, diethyl ether, ethanol, anhydrous Na_2SO_4, pyridine, KOH, $NaOCH_3$, and NaCl solution were supplied by Sigma-Aldrich. The C_7–C_{30} alkane series, eicosanol, docosanol, tetracosanol, cholesterol and β-sitosterol, were kindly supplied by Sigma-Aldrich.

Sample Preparation

Fatty Acid Methyl Esters (FAMEs)

The FAMEs were prepared in the following way: A 100 µL sample of oil was saponified with 1 mL of methanolic sodium methoxide (0.5% w/v) at 100 °C in a closed Pyrex tube for 15 min. The subsequent methyl esterification was performed with 1 mL boron trifluoride-methanol reagent at 100 °C for 15 min. The FAMEs were extracted by adding 1 mL of *n*-hexane and 4 mL of a saturated NaCl solution to the mixture, and agitating manually for 2 min, before a 5-min centrifugation (3000 rpm). Finally, the 1 mL *n*-hexane layer was transferred to a GC injector vial.

Unsaponifiable Fraction (TMSE – Trimethyl Silyl Ethers)

An amount equivalent to 1 g of vegetable oil was added to 10 mL of a 2N KOH/EtOH solution and was heated at 80 °C, under reflux, and magnetic stirring (for about 20 min after solution clarification). Extraction was performed three times with 15 mL of diethyl ether. The combined extracts were washed with 10 mL of distilled water until neutralization was reached. The washed diethyl ether solution was dried with anhydrous sodium sulfate, and the solvent was distilled leaving a few microliters. The latter was transferred into a vial, previously weighed, and the solvent was evaporated under a gentle nitrogen flow at room temperature. The dried residue, namely the unsaponifiable fraction, was weighed.

The unsaponifiable fraction was dissolved in 1 mL of chloroform and was treated with 200 µL of BSTFA (1% TMCS) and 200 µL of pyridine, and heated at 70 °C for 20 min. The derivatized sample was then ready for GC injection.

FM GC×GC–MS Conditions

All FM comprehensive two-dimensional GC–MS applications were performed on a system consisting of two independent Shimadzu GC2010 gas chromatographs (GC1 and GC2), and a TQ-8030 triple quadrupole mass spectrometer. Data were acquired using the GCMSsolution software (Shimadzu). Bidimensional chromatograms in all applications were generated using the ChromSquare software v. 2.0 (Shimadzu Corporation). The two GC ovens were linked through a heated transfer line. The primary GC (GC1) was equipped with an AOC-20i auto-injector and a split-splitless injector (310 °C).

The primary column (situated in GC1), an 20 m × 0.18 mm, 0.18-µm d_f SLB-5ms (Supelco) (silphenylene polymer,

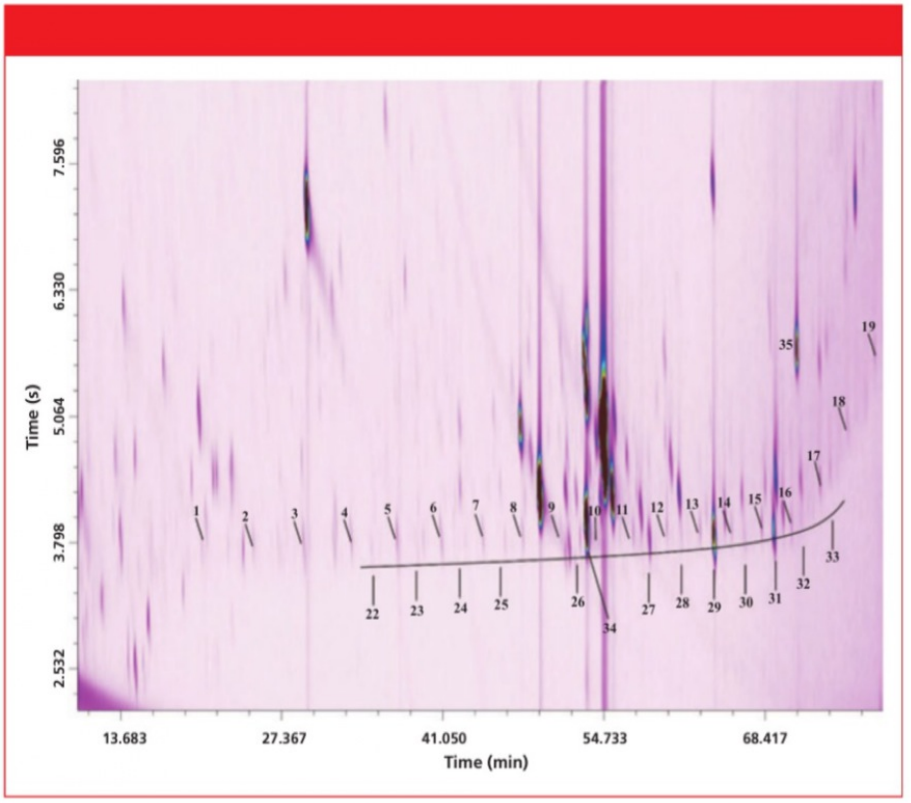


Figure 1: Total-ion-current GC×GC–MS chromatogram expansion I, relative to the unsaponifiable fraction of lemon seed oil (for peak identification refer to Table II).

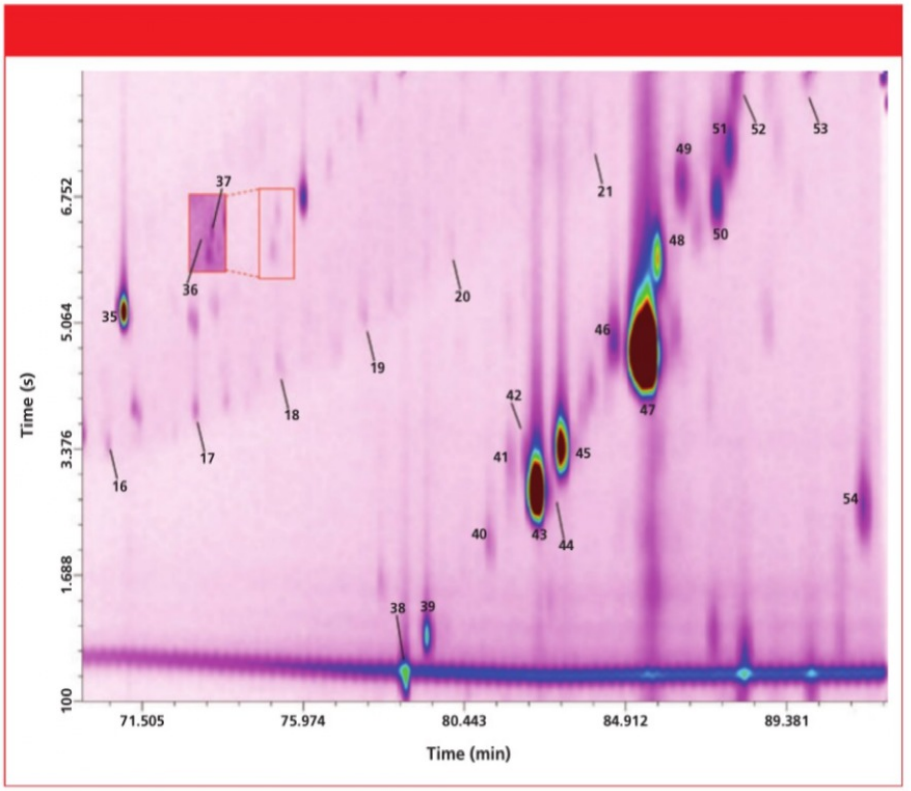


Figure 2: Total-ion-current GC×GC–MS chromatogram expansion II, relative to the unsaponifiable fraction of lemon seed oil (for peak identification refer to Table II).

practically equivalent in polarity to poly[5% diphenyl/95% methylsiloxane]), was connected to position 1 of the wafer-type interface (SGE), after passing through the heated transfer line. A 10 m × 0.32 mm, 0.20-µm d_f SPB-50 capillary segment (poly[50% diphenyl/50% dimethyl] siloxane) column (Supelco) was connected to position six of the interface. An external loop in stainless steel was used. A description of the modulator can be found in a previous article (5).

GC1 and GC2 temperature programmes (FAMEs): 80–310 °C at 3 °C/min. GC1 temperature programme (unsaponifiable fraction): 80–310 °C at 3 °C/min (a +20 °C temperature offset was used in GC2). Initial He head pressure (constant linear velocity): 106.5 kPa. Initial auxiliary (advanced pressure control [APC]) He pressure (constant linear velocity): 60 kPa. Injection volume: 0.5 µL; split ratio: 1:200 (FAMEs); and 1:10 (unsaponifiable fraction). Modulation period: 8.5 s (accumulation period 7.95 s/injection period 0.55 s). Triple quadrupole MS conditions: ionization mode: electron ionization (70 eV). Interface and ion source temperatures: 280 °C and 250 °C. Collision gas and pressure: Ar (200 kPa). In the full-scan mode, a mass range of *m/z* 50–510 and 25 Hz spectral aquisition frequency were used. TMS-cholesterol MRM transitions: *m/z* 368>228 and 329>97 [10 eV collision energy]; acquisition frequency: 20 Hz (during the simultaneous scan/MRM mode). Mass spectral library matching was carried out by using the FAMEs library (Wiley), and NIST08 and Wiley MS databases.

GC–FID Conditions

The GC–FID analyses were performed on a GC2010 Plus instrument (Shimdazu). Data were acquired and processed by the GCsolution software ver. 2.41 (Shimadzu). The gas chromatograph was equipped with an AOC-20i auto-injector and a split-splitless injector (310 °C).

Seed oil FAMEs were separated on a 30 m × 0.25 mm, 0.25-µm d_f Supelcowax-10 column (100% polyethylene glycol) (Supelco), using an oven temperature programme of 50 °C to 280 °C (3 min), at 3 °C/min. The unsaponifiable fraction was separated on a 30 m × 0.25 mm, 0.25-µm d_f SLB-5ms column (Supelco), using an oven temperature program of 90 °C to 320 °C (2 min) at 3 °C/min. Initial He head pressure (constant linear velocity): 99.5 kPa (FAMEs) and 107.7 kPa (unsaponifiable fraction). Injection volume: 1.0 µL; split ratio: 1:100 (FAMEs) and 1:10 (unsaponifiable fraction). The FID was operated at 320 °C.

Results and Discussion

In recent research, the entire unsaponifiable fraction (no thin-layer chromatography pre-separation was performed) of

Table II: Components found in the unsaponifiable fraction of lemon seed oil, type of identification process, and percentage (%) of various constituents of the sterol fraction

Peak	Compound	Identification	%
1	C13:0	*a*	
2	C14:0	*a*	
3	C15:0	*a*	
4	C16:0	*a*	
5	C17:0	*a*	
6	C18:0	*a*	
7	C19:0	*a*	
8	C20:0	*a*	
9	C21:0	*a*	
10	C22:0	*a*	
11	C23:0	*a*	
12	C24:0	*a*	
13	C25:0	*a*	
14	C26:0	*a*	
15	C27:0	*a*	
16	C28:0	*a*	
17	C29:0	*a*	
18	C30:0	*a*	
19	C31:0	*a*	
20	C32:0	*a*	
21	C34:0	*a*	
22	C13:0-ol	*b*	
23	C14:0-ol	*b*	
24	C15:0-ol	*b*	
25	C16:0-ol	*b*	
26	C18:0-ol	*b*	
27	C20:0-ol	*a, b*	
28	C21:0-ol	*b*	
29	C22:0-ol	*a, b*	
30	C23:0-ol	*b*	
31	C24:0-ol	*a, b*	
32	C25:0-ol	*b*	
33	C26:0-ol	*b*	
34	phytol	*b*	
35	squalene	*b*	
36	β-tocopherol	*b*	
37	γ-tocopherol	*b*	
38	α-tocopherol	*b*	
39	cholesterol	*a, b*	0.9
40	lathosterol	*c*	
41	ergosterol	*b, c*	
42	24-methylene-cholesterol	*c*	
43	campesterol	*b*	13.6
44	campestanol	*c*	
45	stigmasterol	*b*	5.2
46	clerosterol	*c*	0.9
47	β-sitosterol	*a, b*	74.7
48	Δ^5-avenasterol	*c*	3.1
49	β-amyrin	*c*	
50	Δ^7-stigmasterol	*c*	1.6
51	cycloartenol	*b, c*	
52	Δ^7-avenasterol	*c*	
53	24-methylene-cycloartanol	*b, c*	
54	citrostadienol	*c*	

a: standard injection; *b*: MS database; *c*: on-line database and literature data (7,8).

a series of vegetable oils (mainly extra-virgin olive oils) was subjected to cryogenic GC×GC analysis, after TMSE derivatization (6). A dual-detector (rapid-scanning quadrupole MS and FID) configuration was used, with the FID data exploited to determine relative quantitative information for the sterols. In this study, two FM GC×GC–MS methods were used to extrapolate detailed information on the lemon seed oil composition in terms of fatty acids and unsaponifiable constituents. Two GC–FID approaches were used to extrapolate percentage data.

Fatty Acid Methyl Esters

The fatty acids were analyzed as FAMEs. Identification was performed through: i) MS database matching (similarity values lower than 75% were not considered); ii) the support of linear retention index (LRI) data; iii) specific 2D chromatogram analyte locations. Considering the latter, the presence of group-type patterns has been previously observed in GC×GC FAME analysis, and is related to carbon number and double-bond number or position (5). Even though no pure standards were used, peak assignment can be considered as "positive". Altogether 20 FAMEs were identified (Table I), 13 of which were "saturates".

Relative quantification FID data were derived from two consecutive applications (Table I), and is reported only for the five main FAs, the summed percentage values of which reached 98.5% (all other FAs were present in percentage values ≤ 0.3%). A 30 m × 0.25 mm polyethylene glycol column was used, with no problem encountered in the separation and identification (based on elution order and peak intensity) of the five major peaks.

In general, the C16 and C18 groups were the most predominant ones, in agreement with previous results (2–4). Palmitic acid was the most abundant (18.4%) "saturated" species, followed by stearic acid, present at the 3.4% level. Such data are in agreement with those reported previously ($C_{16:0}$ ≈ 19–21%; $C_{18:0}$ ≈ 2–4%) (2–4). Other linear saturated fatty acids found were $C_{14:0}$, $C_{15:0}$, $C_{17:0}$, $C_{20:0}$, $C_{22:0}$, $C_{23:0}$, and $C_{24:0}$. Among these, arachidate ($C_{20:0}$) methyl ester was slightly more abundant (0.3%). Moreover, four iso-/anteiso- species were identified.

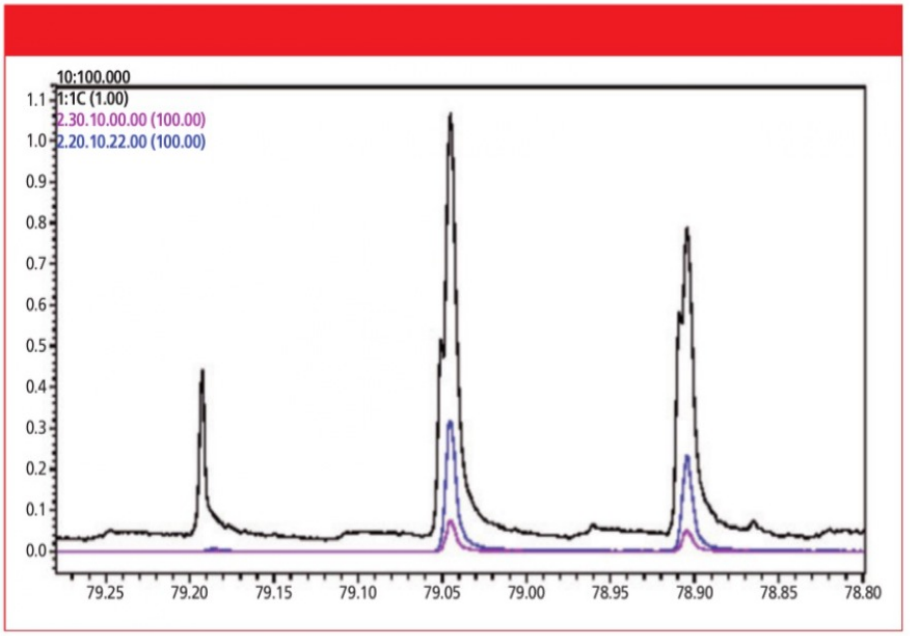


Figure 3: Untransformed simultaneous full-scan/MRM traces (three modulations), relative to the analysis of cholesterol.

Linoleic and oleic acid were by far the most abundant of the unsaturated species, reaching levels of 36.6% and 27.9%, respectively. Such percentages were similar to those reported by Reda et al. (3). Linolenic acid ($C_{18:3\omega3}$) was found at the 12.2% level, slightly higher than values previously reported (2–4). The total percentage of oleic, linoleic, and linolenic acid reached 76.7%. Finally, three minor monounsaturated fatty acids were identified, namely $C_{16:1\omega5}$, $C_{17:1\omega7}$, and $C_{20:1\omega7}$, along with a polyunsaturated FA, specifically $C_{20:4\omega3}$.

Unsaponifiable Fraction

The constituents of the unsaponifiable fraction (calculated to be approximately 1.5% of the oil, slightly higher than in olive oil [6]) were initially analyzed with the QqQ MS operated in the full scan mode. The unsaponifiable fraction of lemon seed oil is illustrated in two GC×GC–MS chromatogram expansions in Figures 1 and 2. Compound identification (Table II) was performed through: i) the comparison of database mass spectra with experimental ones (database matches with a spectral similarity lower than 70% were not considered); ii) the visual comparison of freely-available mass spectra (7) with experimental values; iii) the use of literature information (8,9); iv) chemical class 2D plane locations. When available, pure standard compounds (C_7–C_{30} alkane series, eicosanol, docosanol, tetracosanol, cholesterol, and β-sitosterol) were also injected. A discussion on the constituents of the unsaponifiable fraction will now follow.

Hydrocarbons: squalene (peak 35 — triterpene hydrocarbon with six double bonds [$C_{30}H_{50}$]) and linear alkanes (peaks 1–21 — C_{13}–C_{34} range) are present.

Three tocopherols were found (β, γ, α — peaks 36–38), with the "alpha" compound the most abundant on the 2D plane (it co-elutes with the primary column bleed), confirming the results of previous research (2).

Derivatized aliphatic alcohols from C_{13} to C_{16} (peaks 22–25), and from C_{18} to C_{26} (peaks 26–33), were identified. The alcohols C17 and C19 were not found, while the most intense peak was C_{22} (peak 29). The alcohol diagonal was positioned below the hydrocarbons on the 2D plane (Figure 1). Because of the medium-polarity nature of TMS ethers, their location was expected to be higher (along the *y*-axis) in the chromatogram. The most probable explanation for this is wrap-around, which is when an analyte is characterized by a second-dimension retention time exceeding the modulation period.

Overall, 16 sterols, located in a specific zone of the GC×GC–MS chromatogram (Figure 2), were given a name, and could be sorted into three groups: 4,4-dimethyl-, 4-methyl-, and 4,4-desmethyl-sterols.

Three 4,4-dimethyl-sterols were tentatively identified, namely β-amyrin (peak 49), cycloartenol (peak 51), and 24-methylene-cyclolartanol (peak 53); in addition, a single 4-methyl-sterol was found, specifically citrostadienol (peak 54). Cycloartenol and 24-methylene-cycloartanol were identified through MS database matching, even though low similarity values were attained (<80%); information found in the literature, related to significant ions, was therefore very useful to support peak assignment (8,9). Ions corresponding to the loss of a methyl-, trimethylsilyl hydroxy-, and methyl- + trimethylsilyl hydroxy- groups were considered. Beta-amyrin and citrostadienol were tentatively identified using literature data (8), and through acquired knowledge of the elution sequence (6).

Twelve 4,4-desmethylsterols were identified (10 tentatively), with those of interest from a regulation viewpoint (olive oil is herein considered as reference) accompanied by percentage information (10) derived from two replicates (Table II). The same stationary phase used in the GC×GC first dimension was again used and so the elution order was maintained. Beta-sitosterol (positive identification) and campesterol (peaks 47 and 43) were the predominant compounds, reaching 74.7% and 13.6%, respectively. The percentage of campesterol should be noted as it was considerably higher than that of olive oil (<4%) (10). Cholesterol (positive identification) was found at the 0.9% level, higher than in olive oil (maximum 0.5%) (10).

With regards to the other nine 4,4-desmethyl-sterols, an acceptable MS database match was attained only for stigmasterol (peak 45) and ergosterol (peak 41); for other sterols, namely lathosterol, 24-methylene-cholesterol, clerosterol, Δ^5-avenasterol, Δ^7-stigmasterol, and Δ^7-avenasterol (peaks 40, 42, 46, 48, 50, and 52, respectively), the elution order (6) and the visual comparison of freely-available mass spectra, along with fragmentation pattern information (8,9), were the tools used for tentative peak assignment. Finally, a stanol, namely campestanol (peak 44), was identified on the basis of the elution order (6) and the visual comparison of freely-available mass spectra. Among the remaining nine 4,4-desmethyl-sterols,

Δ^5-avenasterol and Δ^7-stigmasterol were the most abundant, with percentage levels equal to 3.1% and 1.6%, respectively (Table II). It should be noted that the percentage level of Δ^7-stigmasterol is regulated in olive oils, with values which must be lower than 0.5% (10). A further important regulation is the total sum of β-sitosterol, clerosterol, sitostanol (not found in the present research), and Δ^5-avenasterol, which must exceed 93% in olive oil; in the present research the total percentage reached 78.7%.

Finally, the MRM capability of the QqQ MS system was exploited for the absolute quantification of cholesterol, present in unusually high percentages for a vegetable oil. A 3-point calibration curve was constructed (10–50–100 mg/L), using standard cholesterol (dissolved in chloroform) and was subjected to derivatization. Two product ions were selected, a quantifier (*m/z* 228) and qualifier (*m/z* 97), characterized by an ion ratio of 24.5%. The untransformed simultaneous full-scan/MRM traces, relative to three modulations, are shown in Figure 3. Partial overlapping of cholesterol with column bleed is evident (upper trace), as well as the high selectivity of MRM analysis. Derivatized cholesterol was quantified at a concentration of 104 mg/L in the unsaponifiable fraction solution (ready for GC analysis), corresponding to an absolute quantity of 146 µg/g in lemon seed oil. Such a value is an approximation because cholesterol is present in the free form, in the unsaponifiable fraction

Conclusions

The results described here give a detailed view on the lipid profile of lemon seed oil. The FA results confirm the data present in the literature, even though a higher number of low-amount FAs were found. With regards to the information on the unsaponifiable fraction, to the best of the authors' knowledge, no such studies have been previously reported. The flow modulator described here is being used in a routine manner, and can be considered as a valid, flexible, low-costing alternative to cryogenic modulation. In addition, the QqQ MS used, characterized by rapid simultaneous scan/MRM capabilities, has confirmed its usefulness for both untargeted and targeted GC×GC applications. Future FM GC×GC–QqQ MS research will focus on the elucidation of the aroma of lemon seed oil.

Acknowledgments

The project was funded by the "Italian Ministry for the University and Research (MIUR)" within the National Operative Project "Hi-Life Health Products from the industry of foods". Project ID: PON01 01499.

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Peter Q. Tranchida *is an associate professor in Food Chemistry in the Department of Pharmaceutical Sciences and Health Products at the University of Messina (Italy). His main research activities are focused on the use of fast and very-fast gas chromatographic techniques. Furthermore, he is actively engaged in the development and application of classical multidimensional GC, comprehensive two-dimensional GC (GC×GC), and LC–GC (LC×GC) methods, combined with various forms of mass spectrometry.*

Flavio A. Franchina *is a post-doc. student in the Department of Pharmaceutical Sciences and Health Products at the University of Messina (Italy). His main research activities are focused on the development and application of flow-modulated comprehensive 2D GC methods, combined with single-quadrupole and triple-quadrupole mass spectrometry.*

Simona Salivo *is a PhD student in the Department of Pharmaceutical Sciences and Health Products at the University of Messina (Italy). Her principle research activities are focused on the development and application of cryogenically-modulated comprehensive 2D GC methods, combined with rapid-scanning single-quadrupole mass spectrometry. She is also actively engaged in the use of one-dimensional GC, in combination with high-resolution mass spectrometry.*

Marina Russo *is a post-doc. student in the Department of Pharmaceutical Sciences and Health Products at the University of Messina (Italy). Her main research activities are focused on the development and application of monodimensional HPLC analytical methods, combined with photodiode array and single-quadrupole MS detection, for the characterization of bioactive molecules in foodstuffs (for example, Citrus-base products and olive oil), she is also actively engaged in the isolation of such molecules by using multidimensional preparative HPLC.*

Paola Dugo *is a full professor in Food Chemistry in the Department of Pharmaceutical Sciences and Health Products at the University of Messina (Italy) and at the "Campus Bio-Medico" in Rome. Her research interests include the development of comprehensive chromatography and high-throughput separation methods, to the study of Citrus products (essential oils and juices), compounds with possible biological activity in natural samples (carotenoids, anthocyanins, coumarins), plant essential oils, the aromatic fraction of wine and other alcoholic beverages, and triglycerides in food lipids.*

Luigi Mondello *is a full professor in Analytical Chemistry in the Department of Pharmaceutical Sciences and Health Products at the University of Messina (Italy) and at the University "Campus Bio-Medico" in Rome. His research interests are focused on the development of one-dimensional chromatography techniques (HRGC, HPLC, GC–MS, HPLC–MS), as well as multidimensional (heart-cutting and comprehensive) chromatography ones, such as LC–GC, GC–GC GC×GC–MS,LC×LC–MS, LC×GC–MS, and their use in the study of complex samples. Please direct correspondence to: lmondello@unime.it*