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Technical Report

Comprehensive 2D GC with Dual Mass Spectrometry / Flame Ionization Detection for the Analysis of the Unsaponifiable Fraction of Vegetable Oils

GC×GC analysis of the unsaponifiable fraction of vegetable oils

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Abstract:

A comprehensive two-dimensional GC (GC×GC) method, with dual FID/MS detection was developed for the qualitative and quantitative analysis of the entire unsaponifiable fraction of vegetable oils. The analysis of the fatty acid (FA) composition of a vegetable oil can provide valuable information on both its quality and genuineness. However, because many oils have a similar FA profile, the investigation of a minor but highly specific group of compounds, namely the unsaponifiable fraction, is helpful for the assessment of a possible adulteration.

Keywords: comprehensive 2D gas chromatography, vegetable oil, unsaponifiable fraction, food analysis

1. Introduction

Vegetable oils can be considered as a "100%" lipid food, whose predominant part consists of acyl lipids (mainly triacylglycerols), along with minor amounts of mono-/diacylglycerols, waxes, sterol esters, phospholipids, etc. The GC analysis of the saponifiable (fatty acids) fraction of vegetable oils is a classical application, and its qualitative and quantitative elucidation can provide specific information regarding the quality, genuineness and origin of the oil.

However, because many oils are characterized by a similar FA profile (*i.e.*, olive and hazelnut oil), it becomes very difficult to pinpoint an adulteration when low amounts of a cheap oil are used. For such a reason, the analysis of a minor group of compounds (< 3%) of a vegetable oil, namely the unsaponifiable fraction, can overcome the above mentioned lack of specificity, because its composition is highly specific.

This technical report describes the development of a GC×GC method, with dual FID/MS detection for the generation of a two-dimensional fingerprint of the entire unsaponifiable fraction of vegetable oils, without a previous isolation of the naturally-occurring classes of compounds through a TLC process.

The use of dual detection enabled the attainment of both quantitative (% values) and qualitative data (mass spectral information). However, besides the increased separation power, more emphasis was devoted to the enhanced sensitivity and to the formation of group-type patterns.

2. Experimental

2-1. Reagents and materials

The BSTFA [N,O-bis(trimethylsilyl) trifluoroacetamide] + 1% TMCS (trimethylchlorosilane) kit was supplied by Sigma-Aldrich (Milan, Italy). Powdered anhydrous sodium sulphate was purchased from ApliChem (Milan, Italy). The C_7–C_{30} alkane series, eicosanol, docosanol, tetracosanol, cholesterol, erythodiol, uvaol, and β-sitosterol, were supplied by Sigma-Aldrich.

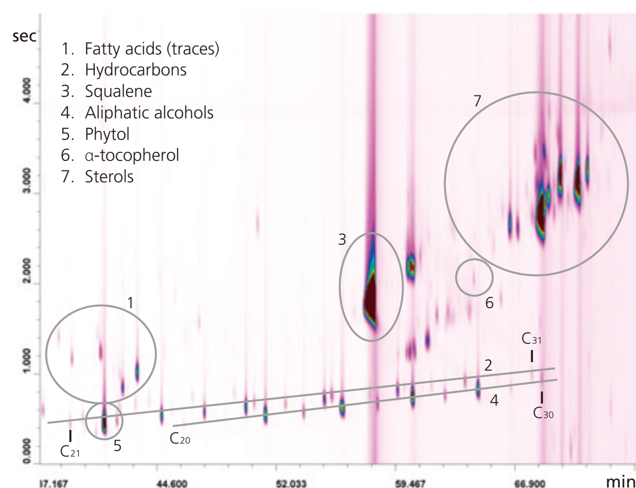

Fig. 1 GC×GC–qMS chromatogram of the entire unsaponifiable fraction of an extra-virgin olive oil.

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2-2. Sample preparation

An amount equivalent to 1 g of vegetable oil was added to 10 mL of a KOH/EtOH 2N solution, heated at 80 °C, under reflux, and magnetic stirring (for about 20 minutes after solution clarification).

Extraction of the unsaponifiable fraction was carried out three times with 15 mL of diethyl ether. The combined extracts were washed with distilled water until neutralization was reached. The washed diethyl ether solution was dried with anhydrous sodium sulphate, and the solvent was distilled leaving a few microliters. The latter were transferred into a vial, previously weighed, and the solvent was evaporated under a gentle nitrogen flow, at room temperature. The dried residue obtained, namely the unsaponifiable fraction, was weighed.

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

2-3. Instrumentation (Shimadzu)

- AOC-20i split-splitless auto-injector.
- GC-2010 gas chromatograph (GC2).
- Zoex dual-stage loop-type cryogenic modulator with closed cycle refrigeration.
- GCMS-QP2010 Ultra.

2-4. Software

- GCMSsolution version 4.0.

2-5. 2D Software

- ChromSquare version 2.0.

2-6. Chromatographic method

D1 column	: SLB-5ms 30 m × 0.25 mm ID × 0.25 µm d_f column [silphenylene polymer virtually equivalent in polarity to poly (5% diphenyl/95% methylsiloxane)] (Supelco, Milan, Italy)
Delay loop	: uncoated column (1 m × 0.25 mm ID)
D2 column	: Rxi-17Sil MS 2 m × 0.25 mm ID × 0.25 µm d_f (silarylene phase, similar to 50% phenyl/50% dimethyl polysiloxane) (Restek, Bellefonte, USA)
FID branch	: uncoated column (0.3 m × 0.10 mm ID)
MS branch	: uncoated column (0.5 m × 0.10 mm ID)
GC1 oven	: from 90 °C to 325 °C at 3 °C/min
GC2 oven	: from 140 °C to 360 °C (5 min) at 3 °C/min
Carrier gas	: Helium
Inlet pressure	: 178 kPa (constant linear velocity mode)
Injection	: 3 µL, split 1:10.
Modulation	: 5 seconds.
Hot jet	: 370 °C (450 msec duration).

2-7. Detection

MS parameters	
MS ionization mode	: electron ionization
Scan speed	: 20,000 amu/sec
Mass range	: 40–600 *m/z*
Acquisition frequency	: 25 Hz
Ion source temperature	: 200 °C
Interface temperature	: 280 °C

FID parameters	
Acquisition frequency	: 50 Hz
Temperature	: 360 °C
Gasses	: make-up (He): 40 mL/min; H_2: 40 mL/min; air: 400 mL/min

3. Results and discussion

Considering a typical GC application on the unsaponifiable fraction of vegetable oils, this is generally subjected to a TLC fractionation process, with the following elution order from the top of the TLC plate: 1) squalene + hydrocarbons, 2) tocopherols, 3) triterpene alcohols (methylsterols) and aliphatic ones, 4) desmethylsterols and triterpenic dialcohols (*e.g.*, erythodiol and uvaol), from here onwards defined as the "sterol" fraction, and 5) the retained free fatty acids. Considering sterol analysis, sample preparation is rather laborious, because first the saponifiable group must be separated from the unsaponifiable one; then, the corresponding band is isolated from the other unsaponifiable constituents through the TLC process, prior to derivatization as trimethylsilyl ethers.

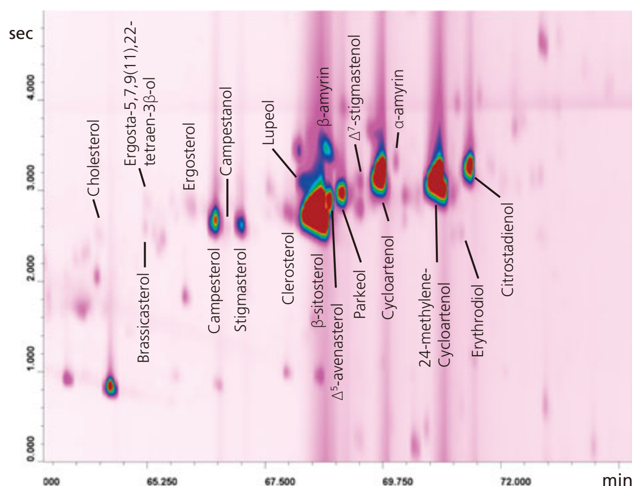

Fig. 2 Sterol zone relative to extra-virgin olive oil.

The scope of the present research was the generation of a GC×GC fingerprint of the entire unsaponifiable fraction of vegetable oils, for the purpose of assessment of quality and genuineness (no TLC fractionation process was performed). Consequently, the time required for sample preparation was shortened.

Identification of compounds was performed through: I) the use of pure standards (when available), II) spectral research in MS databases (NIST08 and Wiley); III) the visual comparison of freely-available on-line mass spectra; IV) the use of literature information; V) chemical class 2D plane locations.

Relative % peak areas were calculated considering the specific chemical group as a whole, and hence giving a 100% value to the sum of the constituents of that group.

The GC×GC–FID chromatogram of a genuine extra-virgin olive oil (EVOO) is illustrated in Fig. 1. A series of chemical classes are nicely spread out in the 2D space. Considering hydrocarbons, linear alkanes are present in the C_{21}–C_{33} range, while squalene is the most abundant of the entire fraction. A single tocopherol, namely the "alpha" type, is present on the 2D plane. The aliphatic alcohols ranged from C_{20} to C_{30}, and are aligned below the hydrocarbons. Their somewhat unexpected location in the chromatogram (along the y-axis) can probably be related to wrap-around.

Fig. 2 illustrates an expansion of the GC×GC–FID chromatogram with the 4-methyl-, 4,4-dimethyl-, 4,4-desmethyl-sterols, and triterpenic dialcohols. Among methylsterols, cyclolartenol and 24-methylene-cycloartenol are the most abundant (both are 4,4-dimethylsterols). Considering 4,4-desmethylsterols, β-sitosterol is, by far, the most abundant compound of the entire zone. As is well know, β-sitosterol is an important marker in olive oil analysis. The triterpenic dialcohol erythrodiol was also positively identified.

With regards to other vegetable oils, the GC×GC–FID chromatogram of the unsaponifiable fraction of sunflower oil is illustrated in Fig. 3. The main differences, with respect to the EVOO, are as follows: I) the amount of squalene is much lower; II) β-tocopherol, apart from α-tocopherol, is present; III) the aliphatic alcohols ranged from C_{20} to C_{32}; IV) the alkanes ranged from C_{23} to C_{33}. The "sterol" zone, relative to the sunflower oil experiment, is highlighted in Fig. 4. A single compound, namely ergosta-7-en-3β-ol, was not found in EVOO, thus it can probably be considered as a potential marker. It is worthy of note that compounds such as Δ^7-sitosterol and Δ^7-avenasterol were not identified in EVOO, but co-elution with the large cycloartenol peak cannot be excluded. Finally, uvaol was not found in EVOO.

Table 1 reports quantification data ($n = 3$) for eight extra-virgin olive oils. In terms of regulation, Table 1 also reports the limits for specific sterols in extra-virgin olive oil. For instance, cholesterol must not be present in % not higher than 0.5 (to avoid adulterations with a lipid of animal origin). All the oils subjected to analysis respected such a limit. The 0.1% limit for brassicasterol is to avoid the addition of oils from the Brassicaceae family (*e.g.*, rapeseed). Brassicasterol was found only in sample EVOO1. With regards to stigmasterol and campesterol, the former was always present in higher amounts. The apparent β-sitosterol content corresponds to a sum of sterols (Table 1), and must reach 93%.

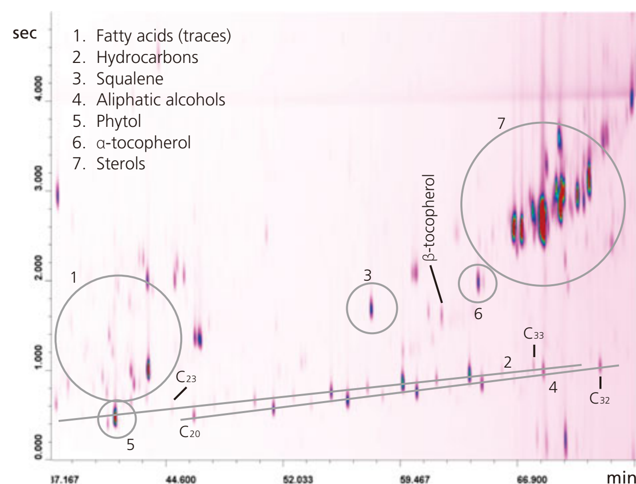

Fig. 3 GC×GC–FID chromatogram of sunflower oil.

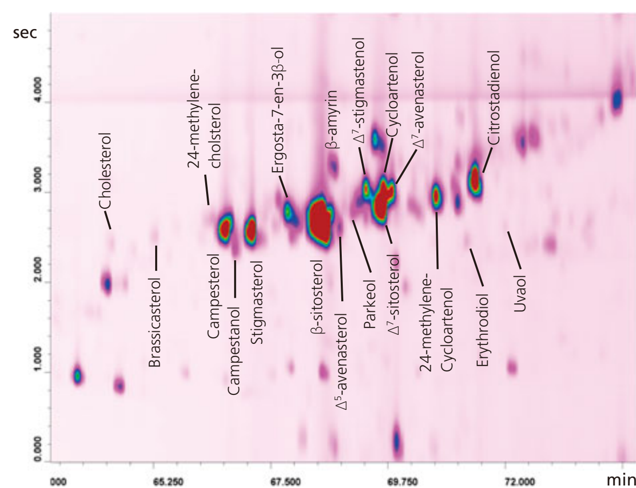

Fig. 4 Sterol zone relative to sunflower oil.

In the present research, four samples were slightly below such a value. A possible explanation of the slightly "low" % concentration values could be related to the higher resolving power of the GC×GC technique, enabling the full separation of β-sitosterol from other compounds. In fact, three samples (EVOO6–8) were subjected to one-dimensional experiments ($n = 3$), in order to compare the quantification data generated by the mono- and bi-dimensional peak areas. As expected, the apparent β-sitosterol content, in the one-dimensional applications, was slightly higher due to compound overlapping (*e.g.*, lupeol and β-amyrin).

Table 1 Legislation relative % values for sterols in olive oil, and the % values (n = 3) found in the eight samples of virgin olive oil. Abbreviations: Leg. = legislation; nd = not detected.

Sterol	Leg.	EVOO1	EVOO2	EVOO3	EVOO4	EVOO5	EVOO6	EVOO7	EVOO8
Cholesterol	≤ 0.5	0.17	0.55	0.05	nd	nd	0.12 (nd)	0.04 (nd)	nd
Brassicasterol	≤ 0.1	0.06	nd	nd	nd	nd	nd	nd	nd
Campesterol	≤ 4.0	4.44	4.67	4.78	3.71	4.23	4.46 (4.12)	4.67 (4.52)	3.99 (3.73)
Stigmasterol	≤ Camp.	2.01	0.77	0.87	1.46	0.78	1.03 (0.95)	0.90 (1.03)	1.24 (1.23)
β-sitosterol	≥ 93.0 (1)	91.92	90.90	91.28	93.01	93.85	92.46 (93.10)	90.86 (91.18)	93.49 (93.70)
Δ^7-stigmastenol	≤ 0.5	1.03	1.00	0.27	0.76	1.14	1.10 (0.94)	0.85 (0.77)	0.90 (1.08)
Erythrodiol		0.31	1.23	2.75	1.03	nd	0.83 (0.89)	2.53 (2.50)	0.39 (0.26)
Uvaol		nd	0.26	nd	0.03	nd	nd	0.13 (nd)	nd
Erythrodiol + Uvaol	≤ 4.5	0.31	2.10	2.75	1.06	nd	0.83 (0.89)	2.67 (2.56)	0.39 (0.26)

(1) Sum of: Clerosterol + β-Sitosterol + sitostanol + Δ^5-avenasterol.

4. Conclusions

A GC×GC–FID/MS method has been developed and applied for the qualitative and quantitative analysis of the unsaponifiable fraction of different vegetable oils.

The instrumental method proposed proved to be a valuable tool, in this specific analytical field. In fact, the benefits of GC×GC have proved to be very useful for the acquisition of a more-in-depth analysis of the lipid unsaponifiable fraction.

In this application, the enhanced sensitivity and resolving power, and the formation of group-type patterns, represented clear and important advantages over one-dimensional GC technologies.

First Edition: February, 2015

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Printed in Japan 3655-06408-20ANS