



Fingerprinting of the Unsaponifiable Fraction of Vegetable Oils by Using Cryogenically-Modulated Comprehensive Two-Dimensional Gas Chromatography-High Resolution Time-of-Flight Mass Spectrometry

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Abstract

The present research is focused on the development of a cryogenically modulated (CM) comprehensive two-dimensional gas chromatography-high resolution time-of-flight mass spectrometry (GC × GC-HR ToFMS) method for the in-depth profiling of the unsaponifiable fraction of vegetable oils. Specifically, twelve vegetable oils (9 extra-virgin olive, soybean, peanut, and hazelnut oils) were subjected to analysis, with particular attention devoted to the higher molecular weight constituents (sterols, vitamin E, triterpenic alcohols). Peak assignment was carried out by using commercial unit-mass MS databases, accurate mass data, literature and on-line freely-available mass spectral information, as well as an in-lab-constructed HR ToFMS lipid database. The main objective of the work was to develop a method to define a well-characterized extra-virgin olive oil fingerprint, to be potentially exploited for vegetable oil differentiation. An additional objective of the investigation was to evaluate the performance of the HR ToFMS system (operated at a mass resolution of 25,000 FWHM) under the challenging analytical conditions of a CM GC × GC-based experiment. The HR ToFMS accurate-mass lipid database was (previously) constructed by acquiring, for the main part, the mass spectra of vegetable oil constituents, well-separated from both the matrix and column bleed background.

Keywords Vegetable oils · Unsaponifiable fraction · Extra virgin olive oil · Comprehensive two-dimensional gas chromatography · High resolution time-of-flight mass spectrometry

Introduction

Comprehensive two-dimensional gas chromatography (GC × GC) was first reported in 1991, by Liu and Phillips (Liu and Phillips 1991), whereas the first paper reporting the use of GC × GC in combination with mass spectrometry (MS) appeared in 1999 (Frysiner and Gaines 1999). In historical terms, the most popular MS choice, in the GC × GC field, has been low-resolution time-of-flight (ToF) MS, followed rather distantly by single quadrupole mass spectrometry (QMS). With regard to other forms of MS instrumentation, there have been a much lower number of descriptions involving triple-quadrupole and high-resolution (HR) ToFMS (Tranchida et al. 2018). For more general details on GC × GC technology, the reader is directed to the literature (Marriott et al. 2012).

In terms of mass spectrometry, the present contribution is focused on HR ToFMS; this powerful form of MS can be exploited in both untargeted and targeted GC-based experiments (Hernández et al. 2011). Qualitative information is attained through MS database matching and the study of

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accurate-mass ions (the molecular ion if present, along with the fragments). Pre-targeted analysis is performed by using highly selective accurate-mass extracted-ion chromatograms; the spectrum data can also be investigated at a later stage to pinpoint previously unsearched analytes (post-targeted analysis).

Recently, GC × GC experiments focused on the use of an HR ToFMS instrument, with a 200-Hz spectral generation capability at a mass resolution of 25,000 (FWHM), have been reported (Tranchida et al. 2015; Byer et al. 2016), with both directed to the analysis of S-containing compounds in petrochemical samples. The same type of HR ToFMS instrument has been used for the qualitative analysis of the unsaponifiable fraction of milk lipids (Tranchida et al. 2013a); specifically, GC-HR ToFMS was used to support the identification of several lipids, previously subjected to analysis by using GC × GC-QMS. Such a process was performed by a cross-comparison of the one- and two-dimensional chromatograms (the stationary phase used in the first GC × GC dimension was the same as that used in the GC experiment).

Instead of deriving detailed lipid information from the mutual use of GC × GC-QMS and GC-HR ToFMS, in the present study, it is attained directly from the GC × GC-HR ToFMS system. It is noteworthy that GC × GC with dual detection [flame ionization detection (FID) and QMS] has been used to study the entire unsaponifiable fraction of extra-virgin olive (8 samples), sunflower, and peanut oils (Tranchida et al. 2013b). Pre-fractionation of the unsaponifiable fraction was not performed due to the enhanced GC × GC separation space.

A main objective of the present investigation was to develop a method enabling a detailed characterization of the unsaponifiable fraction of vegetable oils [more in particular of extra-virgin olive oils, within the context of a national research project (see Acknowledgments)]. In such a respect, the samples subjected to study were nine extra-virgin olive oils, as well as a soybean, peanut, and hazelnut oil. With respect to the previous GC × GC-FID/QMS study, the analysis time was reduced by 43%, with more focus on the higher boiling point compounds; moreover, approx. 20–25% amounts of the reagents/solvents were used to isolate the unsaponifiable fraction as previously described (Tranchida et al. 2014). Finally, the performance of the HR ToFMS system (operated at a mass resolution of 25,000 FWHM) was evaluated in different aspects under the challenging analytical conditions of a cryogenic-modulation (CM) GC × GC-based experiment.

Material and Methods

Samples, Standard Compounds and Reagents

Nine genuine extra-virgin olive oils (EVOO1–9) were provided by participants of a national project on olive oil. The

remaining three oils (soybean, peanut, hazelnut) were part of an in-lab collection of vegetable oils.

The BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] + 1% TMCS (trimethylchlorosilane) kit was supplied by Merck Life Science (Merck KGaA, Darmstadt, Germany). Powdered anhydrous sodium sulfate, pyridine (anhydrous 99.8%), potassium hydroxide (KOH), diethyl ether (99.0%) and ethanol (LC grade) were supplied by Merck Life Science. Water was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

Sample Preparation

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

After, extraction was performed three times: the first with 20 mL of diethyl ether, and the other two with 15 mL of the same solvent. The extracts were combined and washed with 10 mL of distilled water (washing was performed about 15 times *per* sample). The washed solution was dried with anhydrous sodium sulfate, and the solvent was evaporated under low-pressure conditions at 37 °C.

The unsaponifiable fraction isolated was dissolved in 500 µL of chloroform, and then treated with the derivatization mixture [200 µL of BSTFA (1% TMCS) and 200 µL of pyridine], and then heated at 70 °C for 20 min. The derivatized sample was then ready for GC injection.

Instrumental Conditions

All CM GC × GC-HR ToFMS applications were carried out on a Pegasus GC-HRT 4D system (LECO, Mönchengladbach, Germany). The first column was of low polarity, specifically an SLB-5ms [equivalent in polarity to poly(5%diphenyl/95%dimethyl siloxane)], with dimensions 30 m × 0.25 mm ID × 0.25 µm d_f ; the second column was of intermediate polarity, namely, an SLB-35ms, with dimensions 1.9 m × 0.10 mm ID × 0.10 µm d_f [equivalent in polarity to poly(35%diphenyl/65%dimethyl siloxane)]. Both analytical columns were from Merck Life Science. The column connection was made by using a SilTite mini union (Trajan, Ringwood, Victoria, Australia).

In order to focus attention on the higher boiling point compounds, the following method was developed: temperature program, 90–280 °C at 15 °C min⁻¹ and 280–360 °C at 2.5 °C min⁻¹; modulation period was 4 s, with the hot jet held for 1.4 s and the cold jet for 0.6 s; injector temperature, 360 °C; injection mode and volume, split (10:1), and 3 µL; He flow (constant), 2.4 mL min⁻¹; transfer line temperature, 360 °C; modulation heating and secondary oven temperatures, + 15 °C offset with respect to the temperature of the main GC oven; modulation cooling temperature, – 60 °C.

The HR ToFMS analyses were performed through electron ionization (70 eV), at a source temperature of 250 °C. Masses were analyzed over a range of m/z 45–600, at a spectral production frequency of 120 Hz, using the high resolution mode (25,000 FWHM). The mass spectral databases used were NIST 11, Lipids (Wiley), and an in-lab-constructed high resolution MS lipid database.

Results and Discussion

The combination of CM GC \times GC and HR ToFMS generates a very powerful analytical platform, benefiting from the high sensitivity, selectivity, and resolving power, of both the GC and MS sides. Hence, GC \times GC-HR ToFMS has the capability to perform in-depth investigations, and generate detailed fingerprints, of complex mixtures of volatile compounds.

A GC \times GC-HR ToFMS method was developed, it being characterized by a 44.7-min duration and a variable temperature gradient, namely, rapid in the first part (up until 280 °C) and slower after (to 360 °C at 2.5 °C min⁻¹). The method provided a detailed view of the heavier molecular weight (MW) compounds, such as the sterols. Modulation period was 4 s, with cold and hot jet durations of 0.6 and 1.4 s, respectively. A total ion current (TIC) two-dimensional (2D) chromatogram of the EVOO1 fingerprint is illustrated in Fig. 1, highlighting its compositional complexity (acquisition started at 12 min). A spectral production frequency of 120 Hz was applied, it being sufficient both for the purposes of effective deconvolution and reliable quantification.

In general, peak identification was achieved by exploiting commercial unit-mass MS databases, accurate mass data, literature and on-line freely-available mass spectral information. Moreover, an in-lab HR MS lipid database (containing mainly

derivatized sterol spectra) was exploited; such a database was in-the-past constructed, for the main part, through the analysis of a series of vegetable oils through CM GC \times GC-HR ToFMS.

An MS database search was launched, with only the matches characterized by a minimum spectral similarity of 800 considered: 8 linear saturated hydrocarbons and 2 linear alcohols, practically all eluted along a diagonal (Fig. 1), and were tentatively identified with good spectral similarities (Table 1). Compound numbering is related to elution order, and to the overall number of peaks assigned in all the vegetable oils. Considering the moderate degree of polarity of the second dimension (²D), the elution pattern can be related to the ²D separation temperatures and to the fact that the alcohols are analyzed as trimethylsilyl ethers (polarity is greatly reduced): for example, heptacosane (peak 6—C₂₇H₅₆) is subjected to lower ²D temperatures compared with tetracosan-1-ol (peak 7—C₂₇H₅₈SiO), leading to very similar retention times in the second column. The accurate mass data was not investigated for the linear hydrocarbons and alcohols due to the satisfactory spectral similarities and organized elution pattern; instead, the accurate mass data was helpful to support the identification of higher MW compounds, in particular sterols.

As was expected, the largest peak of the 2D fingerprint was that correspondent to squalene (peak 9; similarity = 928). Additionally, its mass spectrum contained a molecular ion with a mass error of 1.7 ppm. With regard to the most information-rich part of the chromatogram (here defined as the “sterol zone”), enclosed in a rectangle in Fig. 1, thirteen acceptable MS database matches (HR ToFMS database) were attained. Valuable MW information was also attained in nine cases, and among these (peaks 28, 31, 32, 33, 35, 40), further identified information was attained from the literature. In three cases (peaks 36, 38, 39) in which only an acceptable database match was attained, the literature was also consulted for MS

Fig. 1 TIC GC \times GC-HR ToFMS chromatogram of sample EVOO1. For peak identification refer to Table 1

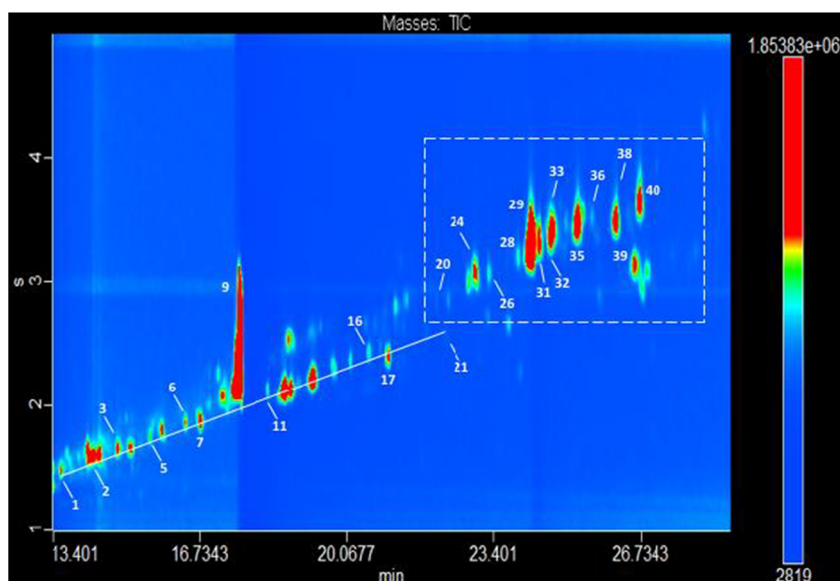


Table 1 Peak assignment, theoretical molecular ion, molecular ion error, and MS database similarity (match; values in bold relate to the HR ToFMS database) for the EVOO1 sample

Peak/compound	[M] ⁺	Molec. ion error (ppm)	Match
1. Tricosane			913
2. Tetracosane			959
3. Pentacosane			923
5. Hexacosane			949
6. Heptacosane			954
7. Tetracosan-1-ol-TMS			868
9. Squalene	410.390703	1.7	928
11. Nonacosane			920
16. Hentriacontane			913
17. Octacosan-1-ol-TMS			918
20. Cholesterol (DesMeSt-TMS)			800
21. Dotriacontane			820
24. Campesterol (DesMeSt-TMS)	472.409494	-2.4	949
26. Stigmasterol (DesMeSt-TMS)	484.409494	-0.7	856
28. Clerosterol (DesMeSt-TMS) ^a	484.409494	5.6	869
29. β -Sitosterol (DesMeSt-TMS)	486.425144	-0.8	920
31. Δ^5 -Avenasterol (DesMeSt-TMS) ^a	484.409494	-3.8	899
32. Parkeol (DiMeSt-TMS) ^b	498.425144	1.2	847
33. β -Amyrin (TriTerpOH-TMS) ^a	498.425144	1.7	911
35. Cycloartenol (DiMeSt-TMS) ^a	498.425144	5.8	892
36. α -Amyrin (TriTerpOH-TMS) ^a			800
38. 24-Methylenecycloartanol (DiMeSt-TMS) ^a			936
39. Erythrodiol (TriTerp2OH-TMS) ^a			915
40. Citrosteradienol (MeSt-TMS) ^a	498.425144	-6.6	931
Aver.		3.0	887

^a Diagnostic ions were consulted in: T.S.C. Li, T.H.J. Beverage, J.C.G. Drover, Phytosterol content of sea buckthorn (*Hippophae rhamnoides* L.) seed oil: Extraction and identification, Food Chem. 101 (2007) 1633–1639

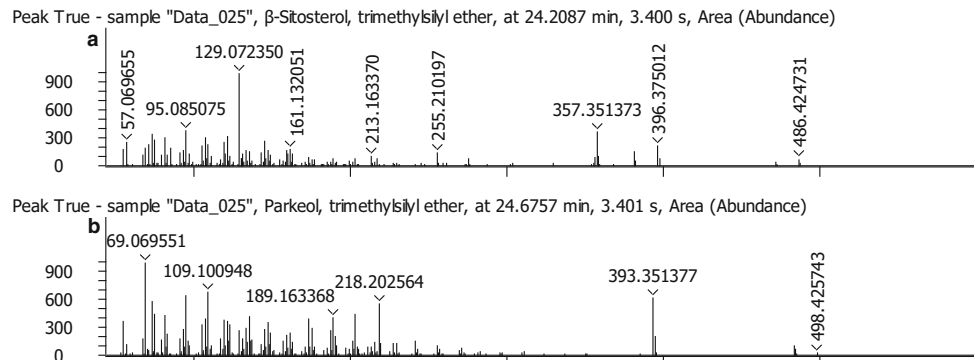
^b The experimental spectrum was visually compared with that located in: Z. Xue, L. Duan, D. Liu, J. Guo, S. Ge, J. Dicks, P. Ó Máille, A. Osbourne, X. Qi, Divergent evolution of oxidosqualene cyclases in plants, New Phytologist 193 (2012) 1022–1038

DesMeSt desmethylsterol; *TriTerpOH* triterpenic alcohol; *DiMeSt* dimethylsterol; *TriTerp2OH* triterpenic dialcohol; *MeSt* methylsterol; *TMS* trimethylsilyl ether

information. For the remaining compound, cholesterol (peak 20), peak assignment of this low-concentration constituent was performed only by using the HR ToFMS database.

Among the thirteen compounds which refer to the sterol zone, there are six desmethylsterols, one methylsterol, three dimethylsterols, two triterpenic alcohols, and one triterpenic

Fig. 2 Accurate-mass spectrum of: (a) β -sitosterol, and (b) parkeol



dialcohol. In general, mass accuracy for all 10 compounds (squalene is also considered) was satisfactory: an average value of 3.0 ppm (absolute value) was calculated. With regard to the HR ToFMS database similarity values, these were acceptable with an average calculated value of 887. The high-resolution mass spectrum of β -sitosterol (peak 29), the most abundant sterol in EVOO, is illustrated in Fig. 2a. The HR ToFMS database match for this compound (920) was satisfactory and an accurate-mass molecular ion (-0.8 ppm) is evident. A high-resolution mass spectrum of parkeol (peak 32), a dimethylsterol, is shown in Fig. 2b. In this instance, a spectral similarity of 847 and a mass accuracy of 1.2 ppm were obtained.

Considering the sterol zone in the other 8 EVOO samples, an additional two compounds were detected: campestanol (a desmethylsterol) and uvaol (a triterpenic dialcohol). All fifteen compounds have been previously related to olive oil (Boskou et al. 2006; Tranchida et al. 2013b). Mass spectral information for the other 8 EVOO samples can be found in

Supplementary Material (Tables S1–8); for samples EVOO2 (Table S1) and EVOO3 (Table S2), the information is related to the entire 2D fingerprint, while for the other EVOO samples, the information relates to the sterol zone. Average mass accuracies for the 8 samples were in the range 2.1–4.9 ppm (absolute values), while the average spectral similarities varied in the range 868–903.

A chromatogram expansion showing the sterol zone of hazelnut oil (HO) is illustrated in Fig. 3a, compared with the EVOO samples the sterol zone fingerprint was entirely different. In such a respect, the sterol zone of sample EVOO9 is illustrated in Fig. 3b, aligned below that of HO. In the latter, a dehydration product of β -sitosterol, namely, stigmasta-3,5-diene (peak 19), was identified exploiting MW accurate-mass information and the HR ToFMS database. Stigmasta-3,5-diene is a stigmastadiene, a class of compounds formed through refining processes (e.g., deodorizing) (Amelio et al. 1998). The circled zone in Fig. 3a (containing peak 19), in itself a smaller fingerprint inside a larger one, is totally absent in

Fig. 3 TIC GC \times GC-HR ToFMS chromatogram expansions of the HO (a) and EVOO9 (b) samples. For peak identification refer to Tables 2 and S8

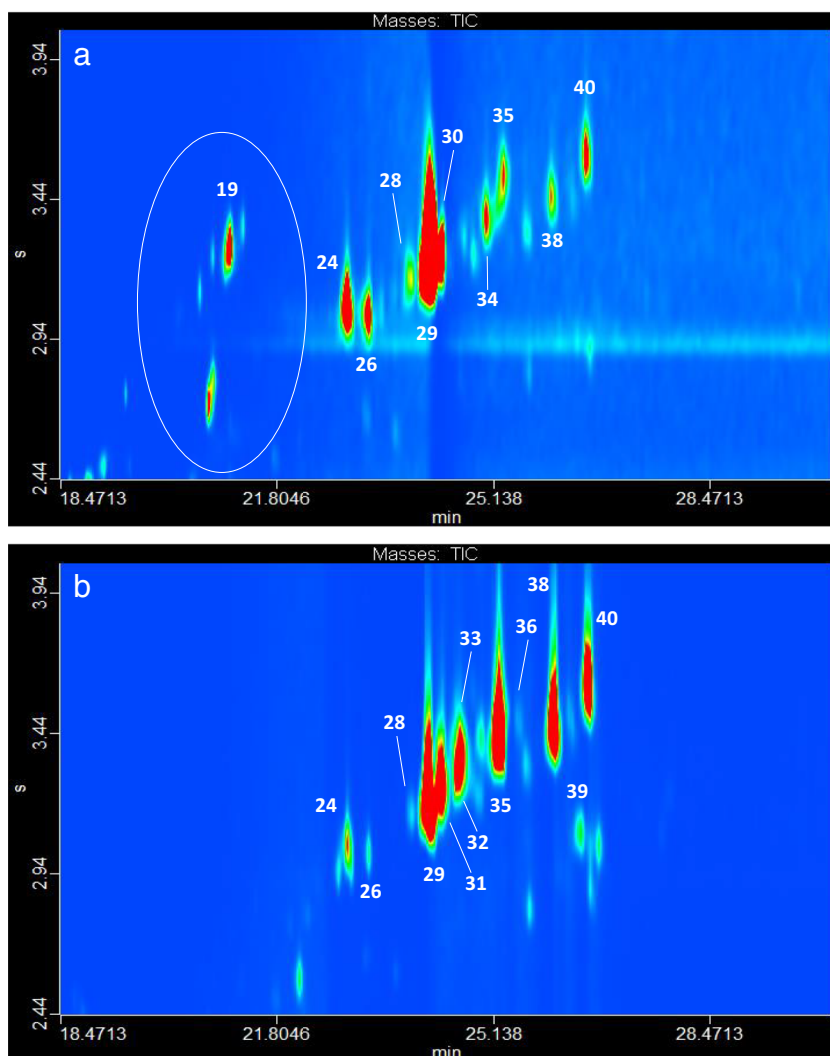


Fig. 3b. Furthermore, sitostanol (a desmethylsterol—peak 30) was found in the HO but not in the EVOO samples. It is noteworthy that the presence of sitostanol in olive oil has been reported (Boskou et al. 2006). Again, and for HO, mass accuracy and spectral similarity values were satisfactory; average values of 3.0 ppm (absolute value) and 882, respectively, were calculated (Table 2).

Compared to EVOO1, only cholesterol was not found in EVOO9. It is noteworthy that cholesterol must not be present in percentages exceeding 0.5% (with respect to the other sterols). Adulterations with palm oil or with a lipid of animal origin would lead to higher percentage values (Tranchida et al. 2013b). Finally, stigmasterol (peak 26), which was one of the sterols with the lowest abundance in Fig. 3b, was reconstructed with 34 data points meaning that it possessed a peak width at the base of circa 280 ms. Such a number of data points is more than sufficient for the purposes of effective deconvolution and reliable quantification (if performed). It is worthy of note that stigmasterol must be present in lower concentrations compared with campesterol, with this being in percentages not exceeding 4%. If, on the other hand, the % of stigmasterol is higher than that of campesterol, then there would be the possible suspected presence of soybean oil (Tranchida et al. 2013b).

With regard to the peanut oil (PO) and soybean oil (SO), information related to the entire 2D fingerprint is reported in

Tables S9 and S10, respectively. Apart from stigmasta-3,5-diene, found in both oils, additional peaks assigned in the sterol zone, and not found in samples EVOO1–9, were as follows: Δ^7 -stigmastenol (a desmethylsterol) in both PO and SO, and brassicasterol (a desmethylsterol), 24-methylenecholesterol (a desmethylsterol), Δ^7 -campesterol (a desmethylsterol), and Δ^7 -avenasterol (a desmethylsterol) only in the SO. Even though not detected in samples EVOO1–9, the presence of Δ^7 -stigmastenol and brassicasterol is regulated in extra-virgin olive oils, with maximum % values of 0.5% and 0.1%, respectively. Both were found in the previous GC \times GC-FID/QMS study on EVOO samples (Tranchida et al. 2013b). In such a respect, the total number of assigned peaks in the sterol zone in the previous and present study reached 24 and 22, respectively.

Conclusions

A CM GC \times GC-HR ToFMS approach for the detailed qualitative profiling of vegetable oils has been developed. The method, a relatively-rapid one, will be exploited for the analysis of 100 s of samples of EVOO for the creation of a sample-specific fingerprint, within the context of an EVOO research project.

Apart from method development and application, a further objective of the present study consisted in the evaluation of the

Table 2 Peak assignment, theoretical molecular ion, molecular ion error, and MS database similarity (values in bold relate to the HR ToFMS database) for the hazelnut oil sample

Peak/compound	[M] ⁺	Molec. ion error (ppm)	Match
7. Tetracosan-1-ol-TMS			954
8. Octacosane			852
9. Squalene	410.390703	0.2	916
11. Nonacosane			868
12. δ -Tocopherol-TMS			858
13. Hexacosan-1-ol-TMS			812
17. Octacosan-1-ol-TMS			832
18. α -Tocopherol-TMS			863
19. Stigmasta-3,5-diene	396.375053	2.2	886
24. Campesterol	472.409494	4.7	909
26. Stigmasterol	484.409494	2.1	886
28. Clerosterol ^a	484.409494	2.2	885
29. β -Sitosterol	486.425144	3.7	952
30. Sitostanol (DesMeSt-TMS) ^a	488.440794	3.5	880
34. Δ^7 -Stigmastenol ^b	486.425144	−1.3	864
35. Cycloartenol ^a	498.425144	7.4	800
38. 24-Methylenecycloartanol ^a			883
40. Citrosteradienol ^a			895
	Aver.	3.0	882

^a Diagnostic ions were consulted in: T.S.C. Li, T.H.J. Beverage, J.C.G. Drover, Phytosterol content of sea buckthorn (*Hippophae rhamnoides* L.) seed oil: Extraction and identification, Food Chem. 101 (2007) 1633–1639

^b Diagnostic ions were consulted in: B. Xu, L. Zhang, H. Wang, D. Luo, P. Li, Characterization and authentication of four important edible oils using free phytosterol profiles established by GC-GC-ToF/MS, Anal. Methods, 6 (2014), 6860–6870

performance of the HR ToFMS system. Such instrumentation was found to be certainly suitable for the challenging circumstances of CM GC × GC analyses. In general, mass accuracy was acceptable, with poorer values observed for the less-abundant compounds.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent is not applicable for this study.

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