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Technical Report

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

GC×GC analysis of the unsaponifiable fraction of milk lipids

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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 lipids belonging to various milk-types. The GC×GC column set used consisted of a low-polarity first dimension, and a medium-polarity secondary one, both characterized by a high thermal stability. The use of dual detection enabled the simultaneous attainment of both qualitative (mass spectral information) and quantitative data (%). The complexity of the fingerprint generated fully justified the employment of the two-dimensional GC technology, whose major valuable benefits were both sensitivity enhancement and the formation of group-type patterns.

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

1. Introduction

Milk is a highly heterogeneous mixture, composed of various chemical classes, among which, lipids. The latter are contained in globules surrounded by a membrane and forms an emulsion with the milk serum. Considering, for instance, cow milk, triacylglycerols are always predominant, and they account for 95–96% of the entire lipid fraction. Other minor components are: diacylglycerols, monoacylglycerols, keto acid glycerides, hydroxy acid glycerides, free fatty acids, phospholipids, and sterols.

The aim of the present work is the investigation of the lipid unsaponifiable fraction of various milks. With regards to milk lipids, the sterol class has been the principal object of studies in dairy products, for reasons related to: the nutritional/health implications of cholesterol (the most abundant component), the presence of oxidation and degradation products, and the detection of vegetable lipids and the quantification of specific phytosterols added to cream and butter as tracers.

A GC×GC method, with dual FID/MS detection, was developed for the generation of a qualitative and quantitative two-dimensional fingerprint of the entire unsaponifiable fraction of lipids belonging to butter (cow milk) and milks (buffalo, goat, ewe), without previous isolation of the naturally-occurring classes of compounds through a TLC process.

The advantages of the developed GC×GC–MS/FID method, over one-dimensional GC, were: I) increased sensitivity (detection of minor constituents); II) greater peak capacity (separation of many more compounds); III) enhanced identification potential, due to the formation of highly organized analyte 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 AppliChem (Milan, Italy). The C_7–C_{40} alkane series, eicosanol, docosanol, tetracosanol, cholesterol, were supplied by Sigma-Aldrich.

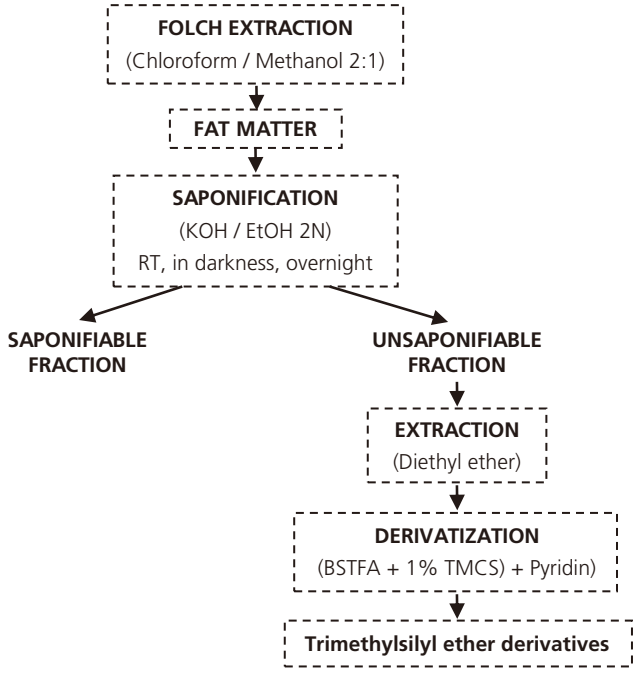

Fig. 1 Scheme of the sample preparation.

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2-2. Sample preparation

The lipid fraction from milk was extracted according to the Folch method, by adding a mixture of chloroform/methanol (2:1), in an ice bath, under magnetic stirring, for 30 min. The content was then agitated, to allow the breakage of the globules. The following centrifugation step enabled the generation of three phases: an upper aqueous one (subjected to extraction for another two times), a lower organic phase (containing the lipid fraction) and an intermediate solid protein phase. The combined organic extracts were dried with anhydrous Na_2SO_4, filtered, then distilled with a rotating evaporator; the lipid matter was then weighed.

The fat, from the cow butter, was extracted using diethyl ether.

For all sample-types, the lipid matter was subjected to cold saponification: 10 mL of a KOH/EtOH 2 N solution was added to the fat and kept overnight, at room temperature and in darkness, under continuous magnetic stirring. After, the unsaponifiable fraction was extracted with diethyl ether, for three times. The combined ether extracts were washed with distilled water until a neutral pH was reached, dried with anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue, namely the unsaponifiable fraction, was subjected to a derivatization process (trimethylsilyl derivatives were formed) as follows: the sample was dissolved in chloroform, added with the BSTFA (1% TMCS) reagent (100 µL), in a pyridine environment (100 µL); heating was at 70 °C, for 30 min.

The scheme of the sample preparation process is illustrated in Fig. 1.

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. 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 (0.25 m × 0.10 mm ID)
MS branch	: uncoated column (0.4 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	: 145 kPa (constant linear velocity mode)
Injection	: 2 µL, split 1:10.
Modulation	: 5 seconds.
Hot jet	: 370 °C (450 msec duration).

2-5. Software

- GCMSsolution version 4.0.

2-6. 2D Software

- ChromSquare version 2.0.

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

The scope of the present research was the generation of a GC×GC fingerprint of the entire unsaponifiable fraction of butter and of various milk lipids, without performing a TLC fractionation to pre-isolate specific chemical classes. An apolar-medium polarity column set of high thermal stability was chosen, because of the high boiling points of many constituents. The positive offset (50 °C) set between the two ovens allowed to avoid an excessive retention of the polar compounds on the 50% phenyl second dimension stationary phase.

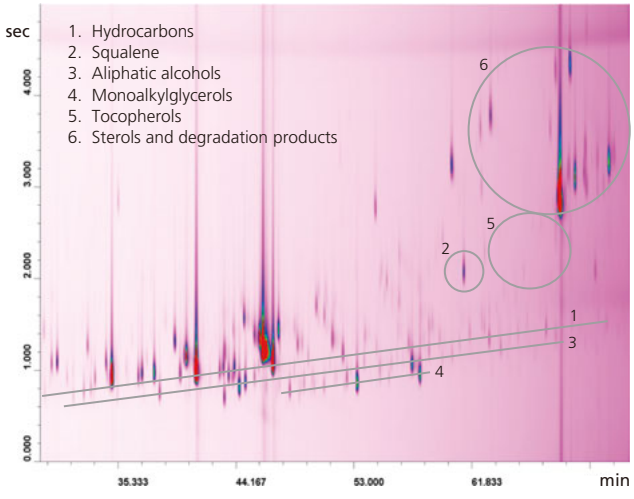

Fig. 2 GC×GC–qMS chromatogram of the entire unsaponifiable fraction of buffalo milk.

Peak assignment was performed through: I) the use of pure standard compounds (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 on fragmentation behaviour; V) chemical class 2D plane locations. Whenever possible, molecular ion information was given the highest importance; in its absence, significant fragment ions were considered. Percentage peak areas were calculated considering the specific chemical group as a whole (e.*g.,* sterols), and hence giving a 100% value to the sum of the constituents of that group.

The entire GC×GC–FID chromatogram relative to the buffalo milk is illustrated in Fig. 2. As can been seen, a series of chemical classes were identified, also on the basis of the specific chromatogram location. Starting from hydrocarbons, linear alkanes (C_{17}–C_{33} range) are nicely aligned along a diagonal, while squalene is the most abundant compound. With regards to the fatty alcohols, eight even-numbered ones, from C_{14} to C_{28}, are situated along a line just below the linear alkanes. Presumably, they underwent wrap-around. Five saturated monoalkylglycerols were identified in the C_{14}–C_{18} range, again positioned along a diagonal, slightly below the alcohol line, with $C_{16:0}$ and $C_{18:0}$ being the most abundant. Monoalkylglycerols are naturally-occurring endogenous components of human and animal tissues, possessing many demonstrated biological activities such as antitumoral effects, haematopoiesis stimulation, fertility improvement.

Fig. 3 illustrates an expansion of the GC×GC–FID chromatogram of the unsaponifiable fraction of buffalo milk, showing the vitamin and sterol components. A single vitamin (E), namely α-tocopherol, was identified through MS database searching. Overall, 10 sterols were identified and quantified (relative % data). A good MS database match was attained for some sterols, while for the others an on-line lipid database and literature information were considered. Cholesterol is by far the most abundant constituent of the entire zone, while coprostanol, the product of bio-reduction of cholesterol, was the lowest-amount one. Six cholesterol precursors (lathosterol, desmosterol, dihydrolanosterol, 14-desmethyllanosterol, 7-dehydrocholesterol, lanosterol) were tentatively identified. Moreover, 7-dehydrocholesterol is a vitamin D_3 precursor. A phytosterol, namely campesterol, was also identified, and it presumably came from the diet animal. Finally, a product of dehydration of cholesterol, namely cholesta-3,5-diene, was tentatively identified through MS database research, with a 91% spectral similarity.

Considering the other milk-types, no great qualitative differences were found. In the butter sample, two additional vitamins were identified, namely vitamin A and γ-tocopherol. Besides, no sterol degradation compounds were pinpointed. With respect to buffalo milk, again an additional vitamin, specifically γ-tocopherol, was identified in the goat sample (Fig. 4). Among sterols, coprostanol was not detected, and ergosterol, also known as provitamin D_2, was identified with a good MS database similarity (88%). Finally, no tocopherols were found in the ewe milk, while the same type of sterols were present compared to buffalo milk.

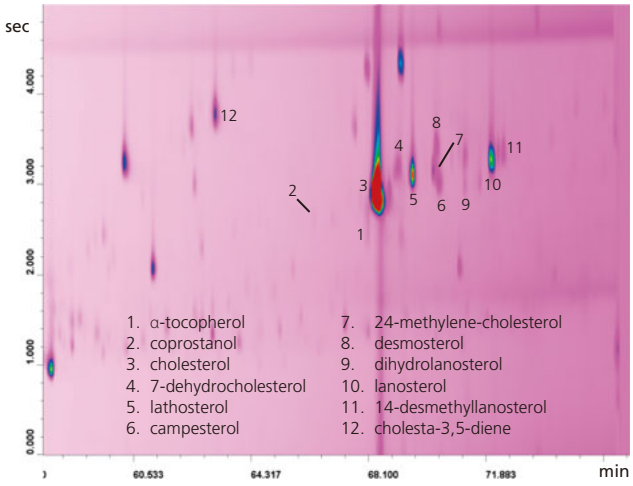

Fig. 3 Expansion of the GC×GC–FID chromatogram of the unsaponifiable fraction of buffalo milk.

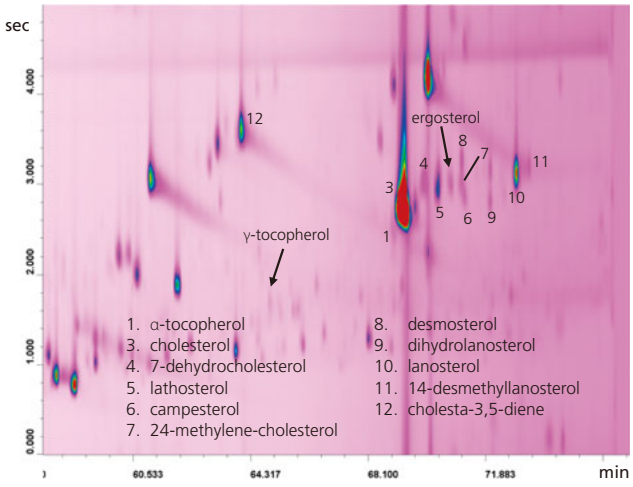

Fig. 4 Vitamin and sterol zone relative to the GC×GC–FID chromatogram of goat milk.

Table 1 reports quantitative % data (resulted from two consecutive applications) relative to the sterol fraction of butter and the milk samples. Cholesterol is the predominant constituent of the entire fraction, and it reached the lowest value of 90.5%, in buffalo milk, and the highest one in butter and ewe milk (95.2 and 95.9%). Lanosterol and lathosterol were the following major compounds. With regards to the other minor sterols, it can be affirmed that, overall, they are present in higher % amounts in buffalo milk, with respect to the other types (Table 1). Finally, cholesta-3,5-diene was not quantified, because it was not considered part of the naturally-occurring sterols.

Table 1 Sterol % composition, derived from the GC×GC–FID analysis of butter, buffalo, goat and ewe milks (n = 2).

Sterol	% buffalo	% butter	% goat	% ewe
coprostanol	0.06	0.08	nd*	0.01
cholesterol	90.56	95.22	94.03	95.91
7-dehydrocholesterol	0.97	0.49	0.59	0.45
lathosterol	2.41	1.12	0.94	0.73
ergosterol	nd	nd	0.37	nd
campesterol	0.62	0.40	0.23	0.35
24-methylene-cholesterol	0.69	0.15	0.30	0.45
desmosterol	0.90	0.23	0.39	0.16
dihydrolanosterol	0.32	0.29	0.28	0.19
lanosterol	2.96	1.95	2.49	1.57
14-desmethyllanosterol	0.51	0.07	0.38	0.17

*: not detected.

4. Conclusions

The results reported in the present technical report lead to the following concluding considerations:

- the GC×GC–MS/FID method herein proposed is potentially a valuable tool for the the assessment of quality (*e.g.*, presence or absence of degradation products) and genuineness (*e.g.*, presence or absence of phytosterols derived from vegetable lipids) in dairy products.
- the sensitivity and the formation of group-type patterns were the GC×GC characteristics most exploited in the specific applications reported.

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