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Research Article

On the Combined Application of Iatroscan TLC-FID and GC-FID to Identify Total, Neutral, and Polar Lipids and Their Fatty Acids Extracted from Foods

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An efficient separation and quantification of the individual neutral and polar lipid classes and their constituent fatty acids was achieved by the combination of two different detection techniques: Iatroscan TLC-FID and GC-FID. The solvent composition and ratio of development system, the sample size, the fidelity, and precision were tested in order to estimate the effectiveness of separation of individual neutral and polar lipid classes and the quantitative reproducibility of the Iatroscan TLC-FID technique. GC-FID method, with a high-quality capillary column, allowed sensitive and reproducible fatty acid qualitative and quantitative analyses, separation of fatty acid structural isomers (e.g., n-C16:0, iso-C16:0 and anteiso-C16:0), positional isomers (e.g., C18:1ω-9 and C18:1ω-7), geometrical isomers (*cis-trans*), and homologues (e.g., C16:0, C17:0, C18:0, etc.) in standards and complex lipid samples. Seventeen (17) lipid classes and fifty-two (52) saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids were identified and quantified, respectively, in samples of standard lipid and fatty acid mixtures, simulating the composition of natural lipids and their fatty acid methyl esters in common foods. The wide number of applications establishes this combination of Iatroscan TLC-FID and GC-FID methods as a powerful tool for lipid class and fatty acid analysis of any fat origin.

1. Introduction

Fat and fatty acids especially polyunsaturated ones contribute to important aspects of fish, meat, and plant products' quality and are critical for their nutritional and sensory value. Therefore, it is required to improve methods for the separation of total lipids into their neutral and polar fractions and for the analysis of their fatty acids (FA). Neutral lipid FA composition of animal and marine fat is strongly associated with their diet, whereas polar lipids regulate the function of membrane cells.

Iatroscan is an instrument that combines thin-layer chromatography (TLC) resolution efficacy with the capacity of quantification by flame ionization detection (FID) [1, 2]. Neutral and polar lipids (e.g., triglycerides, sterols, phospholipids) cannot be separated by gas chromatography (GC) or high-performance liquid chromatography (HPLC) without fractionation, saponification, derivatization, or other pretreatment methods [3] and HPLC also requires availability of suitable columns for analysis of different polarity lipid. On the other hand, TLC/FID is adaptable and flexible as the separation can be achieved by adjusting polarity of solvent system

without changing the stationary phase. Besides, Iatroscan is able to analyze 30 samples in 2-3 hr, a rate that cannot be matched by GC or HPLC [4].

The purposes of this work were (a) to study the suitability of the Iatroscan TLC-FID analysis for rapid and complete separation and quantitation of neutral lipids (NL) into individual NL classes as well as polar lipids (PL) into individual phospholipid (PhL) classes; (b) to improve the separation capacity of GC-FID analysis of saturated and unsaturated fatty acid methyl esters (FAME) due to the nutritional and health benefits of fatty acids, especially the ω-3 and ω-6 ones. Finally this study was designed to improve the suitability of the combined use of Iatroscan TLC-FID and GC-FID for lipid analysis. Moreover, this combination is examined and proposed as a method of choice for complete qualitative and quantitative analysis of food lipids and their constituents' fatty acids.

2. Materials and Methods

2.1. Standards and Solvents. The lipid standards used were cholesteryl oleate, cholesterol, octadecyl hexadecanoate, squalene, tristearoyl-glycerol, stearic acid, oleic acid, 1,3-distearoyl-glycerol, 1,2-distearoyl-glycerol, 1-monostearoyl-rac-glycerol, phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyeline standards of the Sigma Chemical Co (Sigma-Aldrich Company, Dorset, Great Britain and St. Louis, MO, USA) as well as egg yolk and mollusc muscle lipids available by our laboratory (Instrumental Food Analysis Laboratory, Department of Food Technology).

Fatty acid methyl esters standards with chain lengths from C4 to C24 from two sources were used: (a) authentic standard mixtures commercially available (Supelco 37 Component FAME Mix C4–C24, 100 mg Neat. Catalog No.: 18919-1AMP and Supelco PUFA No. 1, Marine Source, 100 mg Neat. Catalog No. 47033. Fatty acid methyl ester standards (5 mg of each) from Sigma Chemical Co: palmitic acid M-E; stearic acid M-E, oleic acid (*cis*-9) M-E, linoleic acid (cis-9,12) M-E and cis-5,8,11,14,17-eicosapentaenoic acid M-E (MIDI Inc.; Supelco; Lordano; Sigma-Aldrich), and (b) FAME gained from egg yolk, mollusc muscle, and lamb meat lipids available by our laboratory. All solvents used for sample preparation were of analytical grade and the solvents used for GC and Iatroscan TLC-FID analyses were of HPLC grade from Merck (Darmstadt, Germany). Double-distilled water was used throughout this work. All reagents used were of analytical grade and were purchased from Mallinckrodt Chemical Works (St. Louis, MO, USA) and from Sigma Chemical Co (Sigma-Aldrich Company, UK).

2.2. Iatroscan Analysis of Neutral and Polar Lipids. Lipid classes were separated on silicic acid-coated quartz rods, chromarods (Type SIII) (5 mm silica gel-coated quartz rod, Iatron Labs, Tokyo, Japan), and afterwards they were quantified using a thin layer chromatography-flame ionisation detection system. TLC-FID analysis was performed by an Iatroscan thin-layer chromatograph (Model MK-6 TLC/FID-FPD Analyser Iatron Laboratories, Tokyo, Japan) equipped with a flame ionization detector and connected to a personal computer for collecting the chromatograms. Operation conditions for the Iatroscan were 160 mL min^{-1} hydrogen flow, 2 L min^{-1} air flow, and 30 s/chromarod scan speed [5]. Chromarods were activated by passing them through the FID scanner immediately before sample spotting and 1 μL of the sample solution was applied on each rod. A 1 μL Hamilton syringe (Hamilton Co., Reno, NV, USA) was used to spot standards and samples. The rods were developed 10 cm from the origin in a lined chromatography tank with a one- or two-solvent system. To obtain a good vapour saturation of the development tank, a filter paper was erected along one side of the bath and wetted with solvent system. Each bath was prepared 30 min before development. The temperature of the room is maintained at 22°C. After developing, the chromarod holder (10 chromarods) was dried in a desiccator for a few minutes and then immediately each chromarod was scanned with the FID to detect and quantify the compounds separated on silica. Chromstar 6.3 Software was used to calculate peak areas and retention times.

2.3. Gas Chromatography Analysis of Fatty Acid Methyl Esters. Fatty acid methyl esters (FAME) of total lipids were prepared according to the procedure described by Sinanoglou and Miniadis-Meimaroglou [6]. Both quantitative and qualitative analyses were performed on an Agilent 6890 Series Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector. DB-23 capillary column (60 m × 0.25 mm i.d. 0.15 μm film) [50%-Cyanopropyl-methylpolysiloxane] (Agilent Technologies. Catalogue No.: 122-2361) was used. The analysis was split injection. Helium was used as a carrier gas. The injector and detector temperatures were 250°C and 260°C, respectively. The temperature was programmed at 100°C for 0 min, raised from 100 to 150°C by a rate of 10°C min^{-1}, and held constant at 150°C for 0 min. Then it raised from 150 to 195°C by a rate of 2°C min^{-1} and held constant at 195°C for 5 min. Then it raised from 195 to 210°C by a rate of 1°C min^{-1} and held constant at 210°C for 0 min and finally raised from 210 to 240°C by a rate of 10°C min^{-1} and held constant at 240°C for 5 min. The duration of the analysis was altogether 55.50 min. The injection volumes were 1.0 μL. Hewlett-Packard Chem Station Software was used to calculate peak areas and retention times.

2.4. Statistical Analysis. All measurements were obtained (at least) in triplicate and values were averaged and reported along with the standard deviation (S.D). All data concerning lipid and fatty acid composition were analyzed with One-Way ANOVA Post Hoc Tests and pairwise multiple comparisons were conducted with the Tukey's honestly significant difference test. Possibilities less than 0.05 were considered statistically significant ($P < 0.05$). All statistical calculations were performed with the SPSS (IBM SPSS Statistics, version 19) statistical software for Windows.

3. Results and Discussion

3.1. Separation of Neutral and Polar Lipids by Iatroscan. The present study focuses on introducing a procedure for the efficient separation and quantification of the individual neutral and polar lipid classes. Several solvent systems consisting of n-hexane, diethyl ether, petroleum ether, chloroform, methanol, acetone, formic acid, acetic acid, ammonia, and water were tested with lipid standards and egg yolk lipids in order to achieve their efficient separation. Solvent systems consisting of n-hexane-diethyl ether gave better separation of neutral from polar lipid classes than those of n-hexane-petroleum ether. Chloroform-methanol combination gave better phospholipids separation than chloroform-acetone. The presence of acetone causes the development of carotenoids associated with the phospholipids. Formic and acetic acid addition in neutral solvent systems gave better separation of triglycerides and free fatty acids than ammonia. Formic acid was preferable than acetic acid since it was more volatile and thus easily removed from the rods prior to scanning. Thus, the neutral and polar solvent systems chosen were hexane-diethyl ether with or without formic acid system combinations (HDF) and chloroform : methanol : water : with or without formic acid (CMWF) solvent system combinations, respectively.

The neutral solvent systems further examined consisted of n-hexane-diethyl ether-formic acid (n-H : DE : FA) [42 : 28 : 0.3, 55 : 20 : 1.5, 60 : 15 : 1.5, 62.6 : 6.6 : 0.8, 65 : 5 : 0.15, 66.5 : 3.5 : 1.7, and 54.9 : 3.7 : 1.4] (by vol.) as well as of n-hexane-diethyl ether (n-H : DE) [72 : 0.8 by vol.]. The presence of formic acid was found necessary in order to avoid broadening peaks. Wax ester appeared as a small peak just before sterol ester but it was incompletely separated, when present in large amount, in all the solvent system tested. The most effective separation of TG from FFA as well as of diacylglycerol isomers 1,2 and 1,3 from free sterols was achieved when a solvent system of n-H : DE : FA was used, with the ratio of n-H : DE exceeding 4 : 1. Thus, n-H : DE : FA (60 : 15 : 1.5 by vol.) (solvent A) was selected as the most appropriate solvent system for individual neutral lipid separation. A typical separation of neutral lipid standards using the Iatroscan and the above-mentioned solvent system designed to separate neutral lipids is shown in Figure 1(a). The retention times of the separated standards are shown in Table 1.

Different ratios in chloroform : methanol : water solvent system, conventionally used in TLC for the separation of polar lipids, were tested. The examined polar solvent systems consisted of chloroform : methanol : water (C : M : W) [45.2 : 22.6 : 2.2; 50 : 25 : 2.5; 45 : 20 : 2 (2 times); 45 : 20 : 2 (1 time); 48 : 22 : 1; 50 : 20 : 2.5; 50 : 20 : 2; 50 : 20 : 2.5 (5 cm) and 60 : 10 : 1 (10 cm)] (by vol.) as well as of chloroform : methanol : water : formic acid (C : M : W : FA) [45 : 25 : 2.5 : 1 and 45 : 20 : 2 : 1] (by vol). The best separations for phospholipids were obtained with C : M : W (50 : 20 : 2, 45 : 20 : 2 and 48 : 22 : 1, by vol.), while the best separation between PI and PS was obtained with C : M : W (50 : 20 : 2, by vol.). L-PE appeared as a small peak just after PS but it was incompletely separated, when present in large amounts.

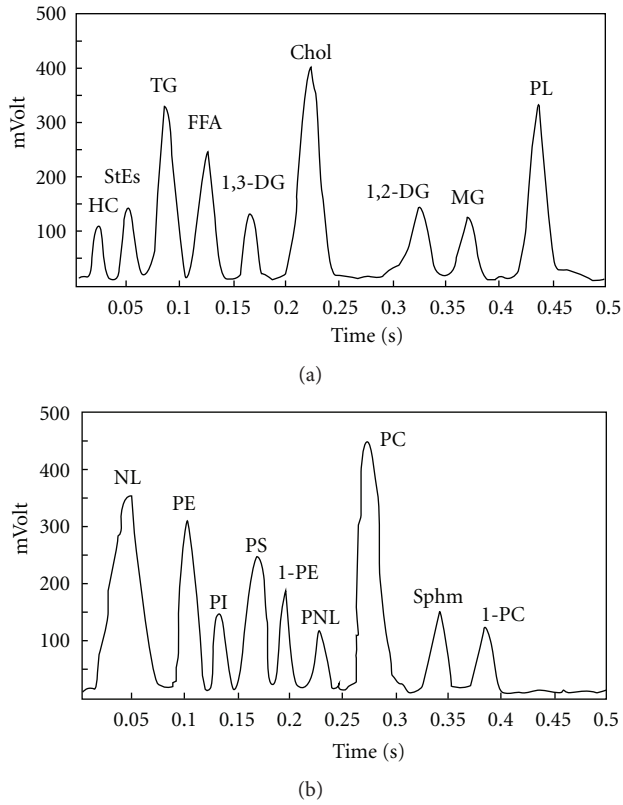

Figure 1: Chromatograms of (a) standard neutral lipids developed with n-hexane-diethyl ether-formic acid (60 : 15 : 1.5 by vol.), (b) standard phospholipids developed with chloroform : methanol : water (50 : 20 : 2 by vol.).

As natural lipid samples contain trace amount of l-PE this peak could be easily separated from PS peak. In the same solvent system ceramide aminoethylphosphonic acid (phosphonolipid) appeared as a small peak between PS and PC when mollusc muscle lipid sample was applied in chromarods, in accordance to the findings of Sinanoglou and Miniadis-Meimaroglou [7]. Therefore, the solvent system selected for the separation of phospholipid standards was C : M : W (50 : 20 : 2, by vol.) (solvent B). The chromatogram of standard phospholipids developed by the above-mentioned solvent system is shown in Figure 1(b). The retention times of the separated standards are tabulated in Table 1.

Various multiple development solvent systems were examined, consisting of C : M : W (first development) and n-H : DE : FA (second development) [50 : 20 : 2.5 (5 cm) and 65 : 5 : 0.15 (10 cm) by vol], [50 : 20 : 2.5 (6 cm) and 65 : 5 : 0.15 (10 cm) by vol], [50 : 20 : 2.5 (7 cm) and 65 : 5 : 0.15 (10 cm) by vol], [50 : 20 : 2 (5 cm) and 65 : 5 : 0.15 (10 cm) by vol], [50 : 20 : 2 (5 cm) and 60 : 15 : 0.15 (10 cm) by vol], and [45 : 20 : 2 (5 cm) and 60 : 15 : 0.15 (10 cm) by vol]. The overlap of MG and PE peaks did not allow the quantification of these two classes of compounds when their amount was higher than 0.2 μg on the chromarod. In order to achieve better separation, the polarity of the first elution

Table 1: Retention time, calibration data, and reproducibility for neutral and polar lipid standards.

Solvent system	Lipid standards	Retention time	Regression equation	Correlation coefficients (r^2)	CV% of intraday variability	CV% of interday variability
Solvent A (n-H : DE : FA) (60 : 15 : 1.5 by vol.)	HC	0.02–0.03	$y = 124.3x + 10.2$	0.9994	1.28	1.69
	Sterol esters (wax esters)	0.04–0.06	$y = 178.4x - 2.7$	1	1.34	1.76
	TG	0.07–0.10	$y = 253.45x + 17.7$	0.9984	2.33	1.83
	FFA	0.11–0.12	$y = 157.7x + 68$	0.9926	1.57	2.21
	1,3-DG	0.16–0.18	$y = 136.5x + 6.1$	0.9987	1.62	2.49
	Sterol	0.20–0.24	$y = 301.3x - 5$	1	2.28	3.46
	1,2-DG	0.31–0.34	$y = 149.2x + 4.4$	1	1.29	1.87
	MG	0.36–0.39	$y = 126.9x - 3.2$	0.9993	1.56	2.17
	PL	0.42–0.45	—	—	—	—
Solvent B (C : M : W) (50 : 20 : 2, by vol.)	NL	0.02–0.08	—	—	—	—
	PE	0.09–0.11	$y = 201.35x + 6.3$	0.9987	1.74	2.86
	PI	0.11–0.14	$y = 205.11x - 5.6$	0.9997	2.64	3.35
	PS	0.15–0.18	$y = 136.8x + 3.9$	0.9997	2.47	3.68
	l-PE	0.19–0.20	$y = 158.2x + 4.1$	0.9995	1.92	3.04
	PnL	0.21–0.23	—	—	—	—
	PC	0.24–0.30	$y = 277.4x + 14$	0.9997	1.40	2.48
	Sphm	0.33–0.35	$y = 190.5x + 1$	0.9994	1.76	2.55
	l-PC	0.37–0.40	$y = 137.23x + 3.4$	0.9998	1.63	2.26

Development distance/times of development: 10 cm (1 time).

system was increased, by decreasing the ratio of chloroform. Thus, C : M : W (first development) and n-H : DE : FA (second development) [45 : 20 : 2 (5 cm) and 60 : 15 : 0.15 (10 cm) by vol] were selected as the most appropriate solvent systems for NL and PL separation in the same Iatroscan chromatogram.

Based on the results obtained, the quantification of individual neutral and polar lipid classes was achieved using calibration curves obtained for each authentic standard by plotting peak area against lipid concentration (different concentrations: 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 20.0 μg μL^{-1} per standard lipid) under the same chromatogram and development conditions (solvent A and B, resp.). The FTD response of neutral and polar fractions versus the lipid amount spotted could be expressed as linear or nonlinear regression. Different models were tested and, finally, the linear curve $y = a + bx$ was chosen for both neutral and polar fractions, where y is the peak area and x is the lipid amount (μg) spotted on the rod. Linear regression curve offers the advantages of good fitting and simplicity for further calculations in order to determine the quantity of lipid components. All correlation coefficients for standard lipids calibration curves were higher than 0.9926 (r^2 = 0.9926 to 1.0000) (Table 1). Four concentrations of 0.01, 0.02, 0.05, and 0.1 μg μL^{-1} for each lipid standard were prepared and spotted to chromarods. The detection limit (DL) were calculated based on signal-to-noise (S/N) ≤3 and quantitation limit (QL) based on S/N ≤10. DL was found 0.02 μg μL^{-1} and QL 0.05 μg μL^{-1} per lipid standard, respectively.

The reproducibility of the measurement was performed from the analysis of two pure standard mixtures of neutral and polar lipids in appropriate ratios simulating the lipid composition of most food samples. Therefore, a composite standard for neutral lipids using squalene, cholesteryl oleate, tristearoyl-glycerol, cholesterol, oleic acid, 1,3-distearoyl-glycerol 1,2-distearoyl-glycerol, and 1-monostearoyl-rac-glycerol, in the ratios 1/1/5/3/1/1/1/1 by wt, as well as for the polar lipids using phosphatidylcholine, phosphatidylethanolamine, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyeline in the ratios 4/2/1/1/1/l/1 by wt, was prepared. Reproducibility was tested by spotting the same standard mixture on 10 chromarods and measuring the standard deviation (SD) and the coefficient of variation (CV%). The neutral solvent system consisted of n-H : DE : FA (60 : 15 : 1.5 by vol.) while the polar solvent system consisted of C : M : W (50 : 20 : 2. by vol.). Four series of ten rods were analysed over a three-day period per mixture selecting a total lipid concentration ≤20.0 μg μL^{-1}. The results of coefficient of variation (CV%) of the intra-day variability and the inter-day variability for the lipid standards (Table 1) proved that the reproducibility of the method was satisfactory for analytical validation purposes [8].

The above separation of NL and PL by Iatroscan TLC-FID offers several advantages over TLC. Higher sensitivity and better recovery were obtained, particularly for lipids present in very small amounts. TLC-FID has the additional advantage to run mixture of lipid standards in separate

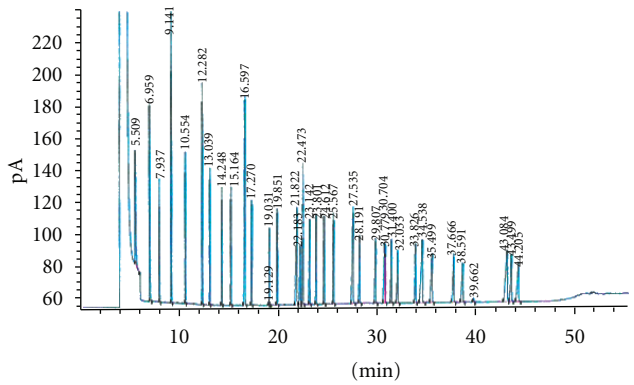

Figure 2: Typical chromatogram showing the separation of FAME standards Supelco 37 Component FAME Mix C4–C24, (Merck, Darmstadt, Germany) on a DB-23 capillary column (60 m × 0.25 mm i.d. 0.15 μm film). Chromatographic conditions: split injection (split ratio 1 : 2).

chromarod to check separation in each analysis. The neutral and polar lipid separation in individual NL and PhL classes is completed in a reasonable time of approximately 1 and 0.5 h, respectively, per 10 analyses. Complex lipid samples do not require pretreatment and even a small sample size (few μg) is sufficient for analysis. Seventeen lipid classes (HC, TG, FFA, Sterols, Wax esters, Sterol Esters, 1,2-DG, 1,3-DG, MG, PE, l-PE, PI, PS, PnL, PC, l-PC and Sphm) were identified in samples of standard lipid mixtures.

3.2. Identification and Quantification of Fatty Acid Methyl Esters by Modified GC-FID. The procedure for fatty acid methyl esters (FAME) analysis using the GC conditions as modified in this study and described in the Material and Methods paragraph, was applied to commercially FAME standards. Figure 2 presents a GC chromatogram of the reference standard mixture. Fatty acids were identified by comparing the retention times of FAME with the Supelco 37 Component FAME Mix, Supelco PUFA No.1, Marine Source (Table 2). FAMEs not included in these standards were compared with the peaks of well-recognized samples, that is, egg yolk, mollusc, and crustacean total lipid fatty acids from previous studies realised by the same research team and under similar conditions [9–12]. According to the results of Table 2, FAME retention time (RT) is influenced by stationary phase polarity, column length, temperature program, fatty acid molecule chain length, unsaturation degree, and isomerisation and chromatographic conditions, as expected. Fifty-two (52) saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were identified and quantified. The temperature program selected was a tradeoff between the resolution of fatty acid structural isomers (e.g., n-C16:0, iso-C16:0, and anteiso-C16:0) or positional isomers (e.g., C18:1ω-9 and C18:1ω-7) or geometrical isomers (*cis-trans*) or homologues (e.g., C16:0, C17:0, and C18:0) and the maximum elution time. Therefore, the temperature program used, although long, was considered appropriate for FAME separation and quantification even for complex fat samples.

Table 2: FAME retention times and response factors.

A/A	FAME	Rt (min)	Rf (split ratio1 : 2)
1	C4:0	4.200–4.405	0.37369 ± 0.00613
2	C6:0	4.580–4.760	0.37436 ± 0.00628
3	C8:0	5.438–5.808	0.38637 ± 0.00043
4	C10:0	6.897–7.158	0.35074 ± 0.00134
5	C10:1	7.325–7.579	0.35074 ± 0.00134
6	C11:0	7.877–8.088	0.34422 ± 0.00189
7	C12:0	9.064–9.251	0.33555 ± 0.00149
8	C13:0	10.484–10.641	0.32852 ± 0.00054
9	C14:0	12.168–12.398	0.32561 ± 0.00068
10	C14:1	12.954–13.131	0.32998 ± 0.00015
11	Iso-C15:0	13.556–13.685	0.33183 ± 0.00018
12	Anteiso-C15:0	13.782–13.956	0.33183 ± 0.00018
13	C15:0	14.148–14.354	0.33183 ± 0.00018
14	C15:1 ω-5	15.064–15.274	0.32497 ± 0.00054
15	C16:0	16.400–16.791	0.32226 ± 0.00096
16	Iso-C16:0	16.978–17.162	0.32226 ± 0.00096
17	C16:1 ω-9 (cis)	17.212–17.394	0.32717 ± 0.00102
18	Iso-C17:0	18.465–18.588	0.41283 ± 0.00352
19	Anteiso-C17:0	18.612–18.688	0.41283 ± 0.00352
20	Cyclo-C17:0	18.705–18.855	0.41283 ± 0.00352
21	C17:0	18.948–19.100	0.41283 ± 0.00352
22	C17:1 ω-7	19.728–19.994	0.31718 ± 0.00197
23	C18:0	21,573–22,000	0.33312 ± 0.00352
24	C18:1 ω-9 trans	22.000–22.285	0.32846 ± 0.00342
25	C18:1 ω-9 cis	22.285–22.668	0.32299 ± 0.00323
26	C18:1 ω-7 cis	22.749–22.909	0.32299 ± 0.00323
27	C18:2 ω-6 cla	23.011–23.301	0.32597 ± 0.00337
28	C18:2 ω-6 t9, t11	23.314–23.301	0.32597 ± 0.00337
29	C18:2 ω-6 cis	23.671–23.951	0.32122 ± 0.00311
30	C18:3 ω-6 cis	24.488–24.764	0.32818 ± 0.00318
31	C18:3 ω-3 cis	25.434–25.721	0.29582 ± 0.00282
32	C18:4 ω-3 cis	26.305–26.515	0.29582 ± 0.00282
33	C19:0	27.336–27.492	0.34611 ± 0.00491
34	C20:0	27.492–27.653	0.34611 ± 0.00491
35	C20:1 ω-9 cis	28.042–28.485	0.33910 ± 0.00490
36	C20:2 ω-6 cis	29.660–29.867	0.32028 ± 0.00444
37	C20:3 ω-6 cis	30.528–30.660	0.33346 ± 0.00356
38	C20:4 ω-6 cis	31.235–31.508	0.34295 ± 0.00620
39	C20:3 ω-3 cis	31.888–32.037	0.33598 ± 0.00527
40	C21:0	32.964–33.282	0.37696 ± 0.00544
41	C20:5 ω-3 cis	33.675–34.151	0.33962 ± 0.00476
42	C22:0	34.329–34.598	0.36969 ± 0.00904
43	C22:1 ω-9 cis	36.285–36.361	0.34866 ± 0.00589
44	C22:1 ω-11 cis	36.534–36.892	0.34866 ± 0.00589
45	C22:2 ω-6 cis	37.633–37.753	0.34738 ± 0.00604
46	C23:0	37.979–38.308	0.38454 ± 0.00781
47	C22:4 ω-6 cis	38.527–38.743	0.34295 ± 0.00620
48	C22:5 ω-6 cis	39.855–40.198	0.34295 ± 0.00620
49	C22:5 ω-3 cis	40.386–40.822	0.34295 ± 0.00620
50	C24:0	42.544–42.703	0.40016 ± 0.00753
51	C22:6 ω-3 cis	43.255–44.162	0.34295 ± 0.00620
52	C24:1 ω-9 cis	44.162–44.392	0.35047 ± 0.00748

Table 3: Summary of applications of the combined Iatroscan TLC-FID and GC-FID methodologies.

	Lipid sample origin		Iatroscan TLC-FID		GC-FID	Reference
			Neutral lipids	Polar lipids	Fatty acids (FA)	
Animal origin	Egg yolk	Ostrich (*Struthio camelus camelus*)	TG, FFA, Chol, 1,3-DG, MG	PE, PI, PC, Sphm, l-PC	30 FA	Sinanoglou et al. [9]
		Turkey (*Meleagridis gallopavo*)			33 FA	
		Quail (*Coturnix coturnix japonica*)			33 FA	
		Duck (*Anas platyrhynchos*)			30 FA	
		Goose (*Anser anser*)			30 FA	
	Medium-growth broilers	Intramuscular fat	TG, FFA, Chol, 1,3-DG, MG	PE, PS, PC, Sphm, l-PC	35 FA	Sinanoglou et al. [15]
		Abdominal fat			26 FA	
	Lamb/kid	Intramuscular fat	TG, FFA, Chol, DG, MG	PE, PS, PC, Sphm, l-PC	33–36 FA	Sinanoglou et al. [16]
		Perirenal fat	TG, FFA, Chol, DG, MG	PE, PC	30–35 FA	
	Lamb milk/creme		TG, Chol, MG		35–40 FA	Unpublished data
Marine origin	Langoustine (*N. norvegicus*) Lobster (*P. vulgaris*)	Muscle—cephalothorax	Waxes, sterol esters, TG, FFA, Chol, 1,3-DG, MG		13–15 FA 24–30 FA	Tsape et al. [17]
	Shrimp (*P. kerathurus*)	Muscle—cephalothorax	Waxes, sterol esters, TG, FFA, Chol, 1,3-DG, MG	PE, PI, PS, PnL, PC, Sphm, l-PC	23–33 FA	Tsape et al. [17] Miniadis-Meimaroglou et al. [18]
	Red porgy wild and cultured (*P. pagrus*)	Muscle				
	Greater weever (*T. draco*)			Cardiolipine, PE, PI, PS, PC, Sphm, l-PC	29 FA	Loukas et al. [10]
	Piper gurnard (*T. lyra*)					
	Picarel (*Spicara smaris*)		TG, FFA, Chol, 1,3-DG, MG	PE, PI, PS, PC, Sphm, l-PC	38 FA	Zervou et al. [19]
Plant origin	Sesame seeds (*Sesamum indicum L.*)		TG, FFA, Chol, 1,3-DG, MG	PA, PE, PI, PS, PC, Sphm, l-PC	18 FA	Zoumpoulakis et al. [20]
Mushrooms	*P. ostreatus*	Fruit body			28 FA	Papaspyridi et al. [21]
		Mycelium			22 FA	
	G. australe	Fruit body			29 FA	
		Mycelium			19 FA	

In order to validate the method in terms of linearity, standard solutions containing different concentrations of 37 Component FAME Mix were prepared and subjected to GC analysis. Injection of solutions containing ten different concentrations of 37 Component FAME Mix in n-hexane (ranging from 0.5 to 5.0, from 5.0 to 20.0, and from 10.0 to 30.0 μg μL^{-1} for split ratios 1 : 2, 1 : 20, and 1 : 50, resp.) served to calibrate the system with regard to the different response obtained with each FAME. Chromatographs showed that discrimination ability and analytical sensitivity decreased with the increase of split ratio. Higher split ratio improved peak shape but it had the risk of the loss of ingredients in trace amounts. Since the total lipid content is determined before the analysis, low split ratios were selected. The standard curves for individual FAME were obtained by plotting concentration ratio against area ratio and the correlation coefficient (r^2) was calculated with the linear equations used for quantitation. FAME response factors (Rfs) were then determined according to the FAME standards chromatograms and calculated by the following equation:

$$\mathrm{Rf}_{\mathrm{FAME}} = \frac{m \text{ FAME } (\mu\mathrm{g})}{\text{FAME peak area } (\mathrm{cm}^2)}. \quad (1)$$

The amounts of FAME in the studied samples were then calculated via the individual FAME peak area and the Rf.

FAME response factors (Rfs) for split ratio 1 : 2 are presented in Table 2.

Dodds et al. [13] demonstrated that FID response factors for different FAME are not equal, a fact confirmed by the analysis of the standards. For FAME identified from known fat samples without corresponding standards, Rf was obtained from their structural or positional isomer or homologue with equal or similar chain length. Individual FAME Rfs remained constant regardless of split ratio since the same integrator events were selected.

The detection limit (DL) and quantitation limit (QL) were obtained for a signal-to-noise (S/N) ratio of 3 and 10, respectively. Four concentrations of 0.001, 0.002, 0.005, and 0.01 μg μL^{-1} in n-hexane for palmitic acid M-E, stearic acid M-E, oleic acid (cis-9) M-E, linoleic acid (cis-9,12) M-E, and cis-5,8,11,14,17-eicosapentaenoic acid M-E were prepared and subjected to GC analysis. Standard deviations were ≤0.01 for all of the analytes. DL was found to be 0.001 μg μL^{-1} and QL 0.002 μg μL^{-1} per fatty acid methylester, respectively.

Three different concentrations of 37 Component FAME Mix (10.0, 15.0, and 20.0 μg μL^{-1} in n-hexane) were used to assess precision. Each sample was run three times within a single day to evaluate repeatability. Reproducibility was evaluated by repeating these analyses on three different days. From the results obtained standard deviations was ≤0.01% and the coefficient of variation (CV%) of the intra-day variability and the inter-day variability for the fatty acid methyl ester standards were ≤1.0% in all cases. The instrumental parameters on the integrator were appropriate to the peak widths at various times during analysis.

The above separation of FAME by GC-FID offers several advantages, such as high repeatability and reproducibility of retention times and high precision in quantitation based on peak area measurements. Several research studies confirmed the stationary phase polarity and column length impact to the resolution of fatty acids isomers [14]. The results of the study showed that the selection of a capillary column with appropriate length resulted in the best separation of FAME isomers.

3.3. Application. The combination of Iatroscan TLC-FID and GC-FID methodologies has been applied to several food samples to identify neutral and polar lipid profiles, composition and fatty acid content of total fat extracted. A summary of these applications is presented in Table 3. The advantages of Iatroscan TLC-FID and GC-FID implementation in lipid and fatty acid analysis were important. Specifically for Iatroscan TLC-FID analysis, the solvent systems and the chromatographic conditions proposed gave satisfactory separation for the lipid classes regardless of the fat origin and the fatty acid constituents of glycerolipids. Diacylglycerol isomers 1,2 and 1,3 were separated from each other, as well as, from free sterols in standards and food samples. Total lipids development in the neutral solvent system A (n-H : DE : FA 60 : 15 : 1.5 by vol.), when scanned by the Iatroscan FID, provided polar and neutral lipid proportions. The reproducibility of the method was satisfactory for all standards and fat samples examined.

Besides, for GC-FID analysis, the efficiency of the selected column and temperature programme for separation of the C18 fatty acids and their *cis/trans* isomers was satisfactory. The method was very useful for the analysis of a wide variety of saturated and unsaturated fatty acid profiles in complex fat mixtures with different compositions. Finally the method provided the profile analysis of individual FAME, expressed as percent of total FAME and the quantitative analysis of individual FAME expressed as w/w of total lipids of food, a factor which is of particular importance when determining the nutritional value of fatty foods.

Conflict of Interests

The authors declare no conflict of interests.

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