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A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples

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ABSTRACT

Isolation is the main bottleneck in the analysis of fatty acids in biological samples and foods. In the last few decades some methods described direct derivatization procedures bypassing these steps. They involve the utilization of methanolic HCL or BF3 as catalysts, but several evidences from previous works suggest these reagents are unstable, lead to the formation of artifacts and alter the distribution of specific compounds as hydroxy fatty acids or CLA. However, the main issue is that they are excellent esterification reagents but poor in transterification, being not suitable for the analysis of all lipid classes and leading to erroneous composition quantitations. The present research work is a comprehensive comparison of six general methylation protocols using base, acid or base/acid catalysts plus a proposed method in the analysis of total fatty acids in lipid standards mixtures, foodstuff and biological samples. The addition of aprotic solvents to the reaction mixture to avoid alterations was also tested. Results confirmed that procedures solely involving acid catalyst resulted in incomplete derivatizations and alteration of the fatty acid profile, partially corrected by addition of the aprotic solvent. The proposed method combining sodium methoxyde and sulfuric acid showed absence of alteration of the FAME profile and the best values for response factors (short chain fatty acids to PUFA), accuracy in the determination of total cholesterol and derivatization performance, thus showing a high reliability in the determination of the total fatty acid composition in biological samples and foods.

1. Introduction

In the recent years analysis of lipids has gained much attention as it was demonstrated that specific fatty acids (FA) may exert an important role in the prevention of human diseases [1]. Conjugated linoleic acid isomers (CLA), mainly found in dairy products, are described as a potent anticarcinogenic agent [2]. Polyunsaturated fatty acids (PUFAs) have been associated with prevention of sudden cardiac death and arrhythmia [3], participation in development of newborn's nervous system [4] and positive effects on depression or Alzheimer's disease [5,6]. FA compounds are routinely analyzed by gas liquid chromatography as methyl esters after isolation of lipids, which is time-consuming and cumbersome. One-step direct derivatization (DT) procedures bypass these extraction steps. Transesterification of glycerolipids is catalyzed by alkali in methanol (potassium hydroxide, sodium methoxide at room temperature, less than 5 min) [7]. On the other hand acids (BF_3, HCl or H_2SO_4, 50–100 °C, 5–60 min) are suitable for the esterification of free fatty acids (FFA) and transmethylation of sphingomyelins [8]. However, some issues arise from the use of acid reagents: first, they lead to protonation of alcohols, which react with CLA and hydroxy FA to form artifacts and *trans* isomers of FA, resulting in erroneous quantitative and qualitative compositions [9]. Aprotic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO) added to the reaction mixture can avoid these alterations, preventing the addition of protonated methanol to the double bonds [10].

While methanolic hydrochloric acid can be prepared bubbling hydrogen chloride into dry methanol or from acetyl chloride, HCL reacts violently with methanol resulting in chloromethane, water and acidic gas, leading to the loss of 50% of the tritratable acid in six weeks at room temperature [11]. Moreover commercial BF_3 in methanol (14%) is one of the most common reagents; several authors have reported great instability and formation of artifacts [12,13]. Finally, although methanol in the presence of sulfuric acid can form dimethyl sulfate it can be prepared daily adding acid to cooled alcohol showing a good stability. Then the selection of the acid catalyst arises as an important question as to assess total FA composition in large studies of foods and biological samples a high-throughput procedure has to be rapid, accurate and complete. This latter requirement is very important for the calculation of the real concentration of each compound as well as to avoid degradation of the capillary column. In general, derivatization

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methods involve one single reagent; then a basic/acid method seems to be the most suitable approach as that of Bondia-Pons et al. [14] using sodium methoxide/BF_3. Even more, it would be interesting to test if H_2SO_4/MeOH solutions, according to their ease of preparation, can be used after the transesterification reagent.

The aim of the present work is to compare the most commonly used DT procedures plus one proposed method involving both base catalyst and sulfuric acid to select the most appropriate procedure for the analysis of total FA in samples from clinical trials or food quality control.

2. Materials and methods

2.1. Chemicals and standards

Hexane, dichloromethane, N,N-dimethylformamide, 2-propanol (HPLC grade) and sulfuric acid (98%) were purchased from LABSCAN (Dublin, Ireland) while toluene (Analytic grade) was from Carlo Erba Reagents-SdS (Sabadell, Spain). Potassium hydroxide (85%), anhydrous hydrogen sulfate (97%), sodium chloride (99%) and sodium bicarbonate (99.9%) were from Panreac (Barcelona, Spain). Supelco 37 fame mix, sodium methoxide, acetyl chloride (puriss, 99%), boron trifluoride (14% in methanol), cholesterol (99%; Ch), cholesteryl palmitate (98%; CE-C16), dilinolein (98%; DG-C18:2), methyl nonadecanoate (99.5%; FAME-C19) and methyl tricosanoate (99%; FAME-C23) were obtained from Sigma (St. Louis, Missouri, USA). Glyceryl tritridecanoate (99%; TG-C13), monolinolenin (99%; MG-C18:3) and margaric acid (99%; FFA-C17) were purchased from Nu-Chek Prep, Inc. (Elysian, Minnesota, USA). Skim milk was purchased from Scharlau (Sentmenat, Barcelona, Spain) while reference butterfat CRM-164 (EU Commission; Brussels, Belgium) was from Fedelco Inc. (Madrid, Spain). Commercial CLA-rich oil (Tonalin® TG-80; 80% g CLA/100 oil) was kindly donated by Cognis (Düsseldorf, Germany). As plasma samples, an animal-based control serum was used (seronorm lipid, SERO AS, Stasjonsveien, Norway).

2.2. Lipid standard solutions and mixtures

Stock solutions of TG-C13, DG-C18:2, MG-C18:3, Ch, CE-C16, FFA-C17 and FAME-C19 were prepared in dichloromethane (25 mg/mL). Serial dilutions for each compound (1.5, 1, 0.75, 0.50, 0.25 and 0 mg/mL) were used to obtain the corresponding calibration curves. A solution of TG-C13, DG-C18:2, MG-C18:3, Ch, CE-C16 and FFA-C17 (5 mg compound/mL) was prepared in duplicate and labeled as LM1 and LM2. Tritridecanoin and methyl tricosanoate were accurately weighed to obtain a mixture of 1.40 mg/mL for each compound (EM).

2.3. Derivatization methods

In the present study six general methylation protocols using base, acid or base/acid catalysts were compared: potassium hydroxide (KOH) [15], sodium methylate/boron trifluoride in methanol (MBF) [14], boron trifluoride in methanol (BF3) [16], acetyl chloride in methanol (ACL) [17] and sulfuric acid (H_2SO_4) assayed according to Christie et al. [18] as follows: samples were incubated (details about temperature and time are given in the subsection*Experiment 1*) with 3 mL 1 M H_2SO_4 in methanol *then cooled in an ice bath* followed by pipetting 1 mL hexane, 1 min vortex (waiting 5 min for the separation of layers), addition of 7.5 mL 6% w/v Na_2CO_3 and finally 3500 rpm, 10 min. Upper layer was collected and transferred into vials.

Two proposed basic/acid methods were assayed. The first (MHS) involved a sodium methoxide (MetNa) transesterification (2.5 mL 0.5 M, 80 °C, 10 min) and the second (KHS) was with potassium hydroxide (0.250 mL 2 N plus 1 mL hexane at room temperature 5 min) and both followed by 1 M H_2SO_4.

All reactions were accomplished in 16 mL borosilicate glass tubes (16×125 mm^2) with acid/heat resistant cap sealed with a PFTE thread tape (5 cm × 12 mm × 0.10 mm) (Fisher scientific, Madrid, Spain). Tubes were placed into an incubator with shaker function (1250 rpm/min) (TR100-G; JP Selecta, Barcelona, Spain).

Methods involving an acid catalyst were studied with the addition of the aprotic solvents DMF and DMSO in the reaction mixture (one-third of the total volume incorporated prior to reagent) to test, qualitatively and quantitatively, the prevention of alterations in the FA composition with special attention to the CLA isomer profile.

2.4. Experiments

2.4.1. Experiment 1

As part of the development of the proposed method (MHS, KHS), an assay was carried out to select the most suitable temperature and time conditions for acid catalyst: 200 μL of lipid mixtures (LM1, LM2) was placed in separated tubes and dried with a N_2 stream. Mixtures were incubated at 30 °C/60 °C/100 °C during 5/30/60 min according to the H_2SO_4 method. The selected conditions were used in further experiments for MHS and KHS.

2.4.2. Experiment 2

This assay was intended to assess the efficiency of different DT methods MBF, BF3, ACL plus the proposed MHS and KHS in the derivatization of 200 μL of samples LM1 and LM2 (previously evaporated to addition of reagents). To know the suitability of the basic catalyst in MHS and KHS, KOH and MetNa were also tested with the lipid mixtures. The impact of utilization of aprotic solvents when using an acid catalyst was also studied.

2.4.3. Experiment 3

This assay was designed to know the possible alteration of the fatty acid profile focusing in CLA when using BF3, ACL, MBF and MHS and the protective properties of aprotic solvents in the derivatization of CLA-rich oil (Tonalin®; 5 mg). KOH was used as the control method as it is an international standardized protocol using a non-altering reagent.

2.4.4. Experiment 4

A response factor (RF) quantification assay to test the feasibility of the obtained FA composition was carried out using 500 μL of reconstituted skim milk powder (10% w/v) spiked with CRM-164 to a final concentration of 10 mg/mL. As a methylation control, the KOH method was used after lipid extraction [19].

2.4.5. Experiment 5

Finally, methods showing a complete derivatization, which did not alter the composition of Tonalin® oil and with good RF, were used in the FAME analysis of 100, 250 and 500 μL of an animal-based control serum. Samples were spiked with 200 μL of EM solution to test derivatization efficiency.

2.5. Analytical methods

Analysis of lipids and the corresponding FAME after derivatization in Experiments 1 and 2 were accomplished in a CLARUS 400 gas chromatograph (Perkin Elmer, Massachusetts, USA) equipped with a FID detector and a Rtx-65TG column (30 m × 0.25 mm × 0.10 μm; Resteck Corporation, Bellefonte, PA, USA). Temperature program conditions were 120 °C held for 30 s, 10 °C/min to 220 °C held for 30 s, and 6 °C/min to 350 °C held for 30 min. Injector and FID temperatures were 355 °C and 370 °C, respectively. Helium was used

as carrier gas (25 psig; split ratio 4:1) and the injection volume was 0.5 μL.

FAME formation (measure moles × 100)/theoretical moles), disappearance of TG, CE, FFA, DG and MG (100-(final moles × 100/initial moles) and recovery total cholesterol (measure moles (Free+CE) × 100/theoretical moles (Free+CE)) were calculated using concentration values from the corresponding calibration curve.

In experiments 1–3 FAME were analyzed (1 μL; 1:10 split ratio) in a 6890 Agilent GLC (Palo Alto, CA, USA) fitted with an MS detector (Agilent 5973N) operated in the scan mode (50–550 Da) and equipped with 100 m a CPSil-88 capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, the Netherlands). Chromatographic conditions were as in Rodriguez-Alcala and Fontecha [20].

2.6. Statistical analysis

All the experiments were carried out in triplicate. Data were analyzed according to the general linear model (GLM) using Bonferroni's test for multiple comparisons and Pearson correlation of SPSS Statistics software v22.0 for Mac (IBM, Armonk, NY, USA). Level of significance was $p < 0.05$.

3. Results and discussion

3.1. Experiment 1

Table 1 shows the obtained results for FAME formation, lipids disappearance and total cholesterol recovery after derivatization of LM solutions with H_2SO_4 assaying 30/60/100 °C and 5/30/60 min of reaction time. Triglyceride (TG-C13), diglyceride (DG-C18:2) and cholesterol esters (CE-C16) were not completely transesterified although temperature and time increased FAME formation and lipid disappearance ($p < 0.05$). Significant differences were not found in the results using conditions above 60 °C and 30 min. However it was observed that an increment in FAME C18:3 formation was accompanied by higher values for FAME C13 and FAME C18:2 ($p < 0.05$). This effect can be explained attending to the emulsifying properties of MG; in contact with methanol this compound forms micelles emulsifying TG and DG and protecting them from transesterification. Thus, some authors have reported a low derivatization rate for MG [21]. When hexane is added for extraction, micelles locate in the interface. In order to avoid collecting methanol, the organic layer was not completely gathered. As MG are converted into FAME, it increases the availability of TG and DG to react. On the other hand, the rapid derivatization of FFA acids agrees with the fact that they are polar compounds dissolving in methanol. Therefore, kinetics of reaction is influenced by solubility in methanol.

In further experiments, the selected acid derivatization conditions for H_2SO_4 were 60 °C and 30 min.

3.2. Experiment 2

In previous trials carried out by the authors assaying DMSO in DT, a peak coeluted with the same retention time of pentadecanoic acid (C15:0), whereas DMF did not interfere with any FA peak. Therefore only DMF was selected in the tested reaction mixtures of this work. In this experiment the selected acid conditions for H_2SO_4 were tested in basic/acid derivatization of a lipid mixture using sodium methoxyde (MHS) or potassium hydroxide (KHS) and compared versus other DT procedures.

According to the obtained results (Table 2), complete derivatization was only accomplished by the MBF and MHS methods. The obtained results for BF3 and ACL did not agree with the studies where those methods were developed as seen by the fact that diglycerides and free fatty acids were converted into FAME. In the present research work this was only observed for FFA C17 in accordance with the results of experiment 1 and others elsewhere reviewed stating that acid catalysts are suitable for esterification [22].

Although results from the transesterification of TG C13, DG C18:2 and MG C18:3 using KOH were promising, when combined with sulfuric (KHS), FAME formation for the corresponding compounds decreased ($p < 0.05$). Elsewhere it has been reported that hydroxide bases can lead to the hydrolysis of FAME [7]. The low conversion rates of glycerolipids observed for MetNa suggest that emulsifying compounds can interfere with derivatization. This agrees with the increments in reaction performance when DMF was added, for all the methods involving an acid catalyst. Data suggest that DMF may contribute to decrease the surface tension and thus the size of the emulsion droplets. It was observed that using MBF leads to the overestimation of FAME formation rates (C13, C16, C17, C18:2 and C18:3) but addition of this solvent improved the results ($p < 0.05$).

The KHS method was showed to be inaccurate in the analysis of FAME from CE and MG as well as it did not allow the determination of total cholesterol (sum of free and from CE). Therefore, it was discarded for further experiments.

Table 1
TG-C13, CE-C16, FFA-C17, DG-C18:2 and MG-C18:3 disappearance (%), FAME formation (%) and recovery of cholesterol after assaying the H_2SO_4 method at 30, 60 and 100 °C during 5, 30 and 60 min (Experiment 1).

	30 °C			**60** °C			**100** °C		
	5 min	**30 min**	**60 min**	**5 min**	**30 min**	**60 min**	**5 min**	**30 min**	**60 min**
FAME-C13	$n.d^{D}$	4 ± 1^{C}	10 ± 0.1^{B}	5 ± 0.1^{C}	31 ± 1^{A}	40 ± 3^{A}	33 ± 1^{A}	34 ± 1^{A}	33 ± 0.1^{A}
TG-C13	23 ± 7^{C}	31 ± 7^{B}	41 ± 1^{B}	41 ± 5^{B}	67 ± 1^{A}	73 ± 2^{A}	67 ± 0.1^{A}	67 ± 1^{A}	67 ± 5^{A}
FAME-C16	$n.d^{B}$	$n.d^{B}$	$n.d^{B}$	$n.d^{B}$	23 ± 0.1^{A}	30 ± 3^{A}	23 ± 6^{A}	21 ± 1^{A}	25 ± 4^{A}
CE-C16	28 ± 8^{D}	30 ± 9^{C}	30 ± 2^{C}	42 ± 2^{B}	54 ± 7^{AB}	65 ± 3^{A}	55 ± 0.1^{A}	50 ± 3^{AB}	56 ± 1^{A}
FAME-C17	69 ± 7^{C}	100 ± 3^{B}	104 ± 1^{B}	92 ± 8^{B}	104 ± 1^{AB}	100 ± 9^{AB}	107 ± 1^{A}	107 ± 2^{A}	105 ± 2^{A}
FFA-C17	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1
FAME-C18:2	2 ± 1^{D}	8 ± 1^{C}	16 ± 1^{B}	9 ± 0.1^{C}	39 ± 2^{A}	46 ± 2^{A}	42 ± 2^{A}	43 ± 2^{A}	41 ± 0.1^{A}
DG-C18:2	48 ± 4^{C}	61 ± 2^{B}	63 ± 6^{B}	58 ± 3^{B}	80 ± 2^{A}	89 ± 1^{A}	79 ± 3^{A}	79 ± 2^{A}	81 ± 1^{A}
FAME-C18:3	10 ± 1^{D}	20 ± 2^{C}	28 ± 1^{C}	49 ± 0.1^{B}	54 ± 8^{AB}	63 ± 2^{A}	61 ± 3^{A}	62 ± 2^{A}	60 ± 1^{A}
MG-C18:3	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 1	100 ± 1
Total-Ch	45 ± 1^{C}	52 ± 5^{B}	52 ± 2^{B}	48 ± 1^{B}	63 ± 2^{A}	60 ± 1^{A}	59 ± 1^{A}	63 ± 1^{A}	62 ± 2^{A}

Results expressed as mean ± SD ($n=3$). **FAME formation**: $(moles_{EXP} \times 100)/moles_{TH}$. (EXP for measure amount, TH for calculated amount). **Disappearance of TG, CE, FFA, DG and MG**: 100 − (moles $c_F \times 100$/moles c_O) (CF for final concentration, CO for initial). **Recovery of Total Cholesterol:** moles $(Free+CE)_{EXP} \times 100$/ moles $(Free+CE)_{TH}$; **n.d:** not detected. Superscript letters in a row for significant differences by conditions ($p < 0.05$).

Table 2
TG-C13, CE-C16, FFA-C17, DG-C18:2 and MG-C18:3 dissapearance (%), FAME formation (%) and recovery of cholesterol by different methods and the effect of adding DMF when using acid catalyst (Experiment 2).

	FAME C13	TG C13	FAME C16	CE C16	FAME C17	C17 FFA	FAME C18:2	DG C18:2	FAME C18:3	MG C18:3	Total Ch
No DMF											
KOH	104 ± 3^{AB}	100 ± 0.1^{A}	2 ± 0^{h}	56 ± 2^{E}	n.d^{E}	20 ± 4^{B}	105 ± 3^{C}	100 ± 0.1^{A}	78 ± 3^{C}	100 ± 0.1^{A}	45 ± 1^{E}
MetNa	54 ± 10^{D}	100 ± 0.1^{A}	54 ± 10^{E}	100 ± 0.1^{A}	n.d^{E}	100 ± 0.1^{A}	57 ± 10^{F}	100 ± 0.1^{A}	64 ± 10^{DC}	100 ± 0.1^{A}	44 ± 10^{DE}
BF3	53 ± 2^{D}	77 ± 3^{B}	23 ± 2^{F}	40 ± 0.1^{F}	107 ± 7^{B}	100 ± 0.1^{A}	80 ± 1^{E}	100 ± 0.1^{A}	67 ± 20^{DC}	100 ± 0.1^{A}	63 ± 4^{C}
ACL	29 ± 3^{E}	77 ± 2^{B}	20 ± 2^{F}	59 ± 2^{DE}	84 ± 8^{D}	100 ± 0.1^{A}	56 ± 5^{F}	92 ± 1^{B}	52 ± 10	100 ± 0.1^{A}	54 ± 5^{D}
MBF	112 ± 3^{A}	100 ± 0.1^{A}	141 ± 0^{A}	100 ± 0.1^{A}	145 ± 4^{A}	100 ± 0.1^{A}	123 ± 2^{B}	100 ± 0.1^{A}	116 ± 1^{A}	100 ± 0.1^{A}	29 ± 0^{F}
MHS	102 ± 4^{AB}	100 ± 0.1^{A}	104 ± 1^{C}	100 ± 0.1^{A}	110 ± 1^{B}	100 ± 0.1^{A}	101 ± 1^{D}	100 ± 0.1^{A}	83 ± 10^{C}	100 ± 0.1^{A}	88 ± 4^{B}
KHS	88 ± 7^{C}	100 ± 0.1^{A}	16 ± 5^{FG}	86 ± 3^{C}	89 ± 10^{D}	100 ± 0.1^{A}	80 ± 10^{E}	100 ± 0.1^{A}	78 ± 10^{C}	100 ± 0.1^{A}	44 ± 7^{DE}
DMF											
BF3	110 ± 5^{A}	100 ± 0.1^{A}	87 ± 7^{D}	95 ± 0.1^{B}	107 ± 8^{B}	100 ± 0.1^{A}	113 ± 6^{C}	100 ± 0.1^{A}	98 ± 10^{B}	100 ± 0.1^{A}	45 ± 9^{DE}
ACL	79 ± 3^{C}	100 ± 0.1^{A}	77 ± 3^{D}	100 ± 0.1^{A}	79 ± 1^{D}	100 ± 0.1^{A}	83 ± 3^{E}	100 ± 0.1^{A}	60 ± 10^{D}	100 ± 0.1^{A}	51 ± 2^{D}
MBF	100 ± 1^{B}	100 ± 0.1^{A}	128 ± 2^{B}	100 ± 0.1^{A}	116 ± 1^{B}	100 ± 0.1^{A}	137 ± 3^{A}	100 ± 0.1^{A}	105 ± 1^{B}	100 ± 0.1^{A}	59 ± 1^{C}
MHS	108 ± 1^{A}	100 ± 0.1^{A}	102 ± 2^{C}	100 ± 0.1^{A}	100 ± 1^{C}	100 ± 0.1^{A}	105 ± 3^{C}	100 ± 0.1^{A}	101 ± 1^{B}	100 ± 0.1^{A}	99 ± 0.1^{A}
KHS	106 ± 5^{A}	100 ± 0.1^{A}	13 ± 1^{G}	64 ± 2^{D}	101 ± 7^{BC}	100 ± 0.1^{A}	107 ± 6^{C}	100 ± 0.1^{A}	78 ± 10^{C}	100 ± 0.1^{A}	60 ± 3^{CD}

Results expressed as mean ± SD ($n=3$). **FAME formation**: $(\text{moles}_{EXP} \times 100)/\text{moles}_{TH}$. (EXP for measure amount, TH for calculated amount). **Disappearance of TG, CE, FFA, DG and MG**: $100-(\text{moles } c_F \times 100/\text{moles } c_O)$ (CF for final concentration, CO for initial). **Total Cholesterol:** moles $(\text{Free}+\text{CE})_{EXP} \times 100/\text{moles } (\text{Free}+\text{CE})_{TH}$; **n.d:** not detected. Superscript letters in a column for significant differences by method ($p < 0.05$).

Table 3
Fatty acid composition (mg FA/g oil) of CLA-rich oil (Tonalin® TG80) obtained assaying five different procedures based on direct transesterification with or without dimethylformamide (DMF) added to the reaction mixture (Experiment 3).

	RM	No DMF				DMF			
	KOH	BF3	ACL	MBF	MHS	BF3	ACL	MBF	MHS
C16	3 ± 0.1^{C}	3 ± 0.1^{C}	5 ± 0.1^{A}	3 ± 0.1^{C}	2.6 ± 0.1^{D}	3 ± 0.1^{C}	3 ± 0.1^{B}	3 ± 0.1^{C}	3 ± 0.1^{C}
C18	23 ± 0.1^{E}	23 ± 0.1^{E}	89 ± 1^{A}	26 ± 0.1^{D}	28 ± 3^{CD}	30 ± 0.1^{C}	51 ± 0.7^{B}	30 ± 0.1^{C}	32 ± 1^{C}
C18:1 c9	126 ± 1^{C}	134 ± 1^{B}	36 ± 0.8^{E}	138 ± 0.5^{B}	135 ± 9^{B}	151 ± 0.6^{A}	284 ± 1^{A}	151 ± 0.6^{A}	122 ± 1^{C}
18:2 Non-conjugated isomers									
c9, c12	2 ± 0.1^{B}	2 ± 0.1^{B}	1 ± 0.1^{B}	2 ± 0.2^{B}	2 ± 0.1^{B}	2 ± 0.1^{B}	6 ± 0.2^{A}	2 ± 0.1^{B}	2 ± 0.1^{B}
Total t, t	2 ± 0.1^{C}	2 ± 0.1^{B}	0.5 ± 0.1^{E}	2 ± 0.1^{B}	1 ± 0.1^{D}	2 ± 0.1^{B}	5 ± 0.2^{A}	2 ± 0.1^{B}	1 ± 0.3^{C}
Total c, t	2 ± 0.1^{B}	2 ± 0.1^{B}	1 ± 0.1^{D}	2 ± 0.1^{B}	1 ± 0.1^{C}	2 ± 0.1^{B}	4 ± 0.2^{A}	2 ± 0.1^{B}	2 ± 0.4^{B}
18:2 Conjugated isomers									
c9, t11	402 ± 0.6^{A}	368 ± 0.4^{C}	315 ± 10^{D}	395 ± 0.1^{A}	400 ± 3^{A}	390 ± 0.1^{A}	335 ± 10^{D}	390 ± 0.1^{A}	412 ± 8^{A}
t10, c12	422 ± 2^{A}	386 ± 0.1^{D}	263 ± 7^{E}	407 ± 0.6^{B}	407 ± 1^{B}	400 ± 0.7^{CD}	242 ± 5^{E}	400 ± 0.7^{CD}	409 ± 8^{AB}
Total c, c	10 ± 0.1^{C}	11 ± 0.1^{C}	14 ± 0.2^{B}	10 ± 0.1^{C}	13 ± 2^{B}	10 ± 0.1^{C}	23 ± 0.1^{A}	10 ± 0.1^{C}	10 ± 0.9^{C}
Total t, t	10 ± 0.1^{D}	17 ± 0.2^{B}	17 ± 0.6^{B}	11 ± 0.1^{C}	9 ± 0.5^{D}	10 ± 0.1^{D}	39 ± 0.5^{A}	10 ± 0.1^{D}	10 ± 0.7^{D}
Total CLA	844 ± 1^{A}	782 ± 0.1^{D}	698 ± 5^{E}	824 ± 0.4^{A}	829 ± 10^{A}	809 ± 0.8^{C}	640 ± 9^{E}	809 ± 0.8^{C}	840 ± 2^{A}
Artifact	n.d^{D}	52 ± 1^{B}	168 ± 7^{A}	3 ± 0.1^{C}	1 ± 0.1^{D}	n.d^{D}	n.d^{D}	n.d^{D}	n.d^{D}

RM: Reference method; **DMF**: N,N dimethylformamide; **FA**: fatty acid; ***c***: cis double bond; ***t***: trans double bond. Superscript letters in a row for significant differences by methods ($p < 0.0.5$). $n=3$ in all tests.

In general, results obtained with MHS were better than with the rest of the methods. Total cholesterol measure was only possible with this method.

3.3. Experiment 3

In this next experiment (Table 3) the possible alteration of the FA profile of a CLA-rich oil (Tonalin® TG80) was studied when assaying BF3, ACL, MBF and the proposed MHS. KOH was used as the reference method since reaction is carried out by potassium hydroxide and therefore does not alter the FAME profile. The protective effect of DMF was also tested.

The results from ACL showed higher concentrations of C16 and C18 than in KOH ($p < 0.05$). On the other hand contents for C18:1 c9 and the non-conjugated C18:2 isomers were significantly lower than in the reference method. The utilization of DMF led to overestimation of those compounds. DT with ACL is an exothermic reaction that may alter the sample, yielding higher levels of palmitic (C16) and oleic acid (C18:1 *c*9), except when the reaction is carried out overnight at room temperature [23]. In addition, a recent study confronted the utilization of NaOH followed by $NaHSO_4$ or BF_3 in the FA analysis of phospholipids from reindeer and fish muscle and found that the latter reagent gave higher concentrations of C18, C18:1 *t*, C18:2 *c*9, *c*12, C18:3 *c*9, *c*12, *c*15, C20:2 n6 and C20:3 n3 [24]. Similar variations were found for C18 and C18:1 c9 using MBF with and without DMF. When using the proposed method MHS, the aprotic solvent resulted in values similar to that in KOH except for C18, which was slightly higher ($p < 0.05$).

Formation of artifacts was associated with increments in the concentration of *trans* isomers of CLA (CLA *t*, *t*) and therefore decreases of the major isomers of CLA present in Tonalin® oil such as C18:2 *c*9, *t*11 and C18:2 *t*10, *c*12. It is stated that at low pH, pronated alcohols interact with hydroxy fatty acids and double bonds of CLA, thus forming methoxy compounds and CLA *t*, *t* isomers [10].

Although these side reactions are well known, many investigations are carried out without considering these points. Massod et al. [25] and recently Glaser et al. [26] reported the FA composition in human plasma using methanolic hydrochloric acid, respectively, without the addition of DMF or DMSO. Their analysis did not show the presence of CLA isomers but the results from the present study suggest that concentrations of palmitic, stearic and oleic acid could be overestimated if the aprotic solvent is not

Table 4
Fatty acid composition of commercial animal-based control serum (SERNORM lipid) using MHS and MBS methods at three levels of the sample amount (Experiment 5).

Fatty acid	MHS						MBF					
	100 μL		250 μL		500 μL		100 μL		250 μL		500 μL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C12	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.03	0.1	0.01
C14 DMA	0.1^{D}	0.01	0.1^{CD}	0.02	0.1^{CD}	0.01	0.2^{B}	0.01	0.3^{B}	0.03	0.4^{A}	0.01
C15i DMA	0.2^{C}	0.01	0.2^{C}	0.01	0.2^{C}	0.01	0.3^{B}	0.01	0.3^{B}	0.03	0.5^{A}	0.02
C15ai DMA	0.4^{C}	0.01	0.4^{C}	0.02	0.4^{C}	0.01	0.6^{B}	0.02	0.7^{B}	0.04	1^{A}	0.02
C14	2	0.01	2	0.03	2	0.06	2	0.05	2	0.10	2	0.03
C15ai	0.2	0.01	0.3	0.02	0.3	0.01	0.2	0.04	0.3	0.01	0.3	0.02
C15i	0.4^{B}	0.01	0.6^{AB}	0.02	0.5^{AB}	0.01	0.5^{B}	0.05	0.5^{AB}	0.04	0.6^{A}	0.02
C14:1 c9	0.1^{B}	0.01	0.1^{AB}	0.01	0.1^{A}	0.01	$n.d^{C}$	n.a	0.1^{AB}	0.01	0.1^{AB}	0.01
C15	0.4^{AB}	0.01	0.4^{A}	0.01	0.4^{A}	0.01	0.3^{B}	0.03	0.4^{AB}	0.01	0.4^{A}	0.01
C16 DMA	0.4^{D}	0.01	0.5^{D}	0.02	0.5^{D}	0.01	0.7^{C}	0.01	0.9^{B}	0.01	1^{A}	0.10
C16i	0.2	0.01	0.2	0.01	0.2	0.01	0.2	0.03	0.2	0.01	0.2	0.02
C16	18	0.04	19	1	18	1	19	0.20	19	1	19	0.20
C17ai	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.02	0.1	0.02	0.1	0.01
C16:1 c7	0.3^{BC}	0.01	0.4^{AB}	0.01	0.4^{A}	0.03	0.3^{C}	0.03	0.4^{AC}	0.01	0.4^{AB}	0.01
C16:1 c9	1^{BC}	0.03	2^{A}	0.04	2^{A}	0.10	1^{C}	0.02	2^{B}	0.04	2^{AB}	0.01
C16 PhyAc	3^{B}	0.03	2^{C}	0.02	2^{C}	0.01	3^{A}	0.20	2^{C}	0.04	1.3^{D}	0.10
C17ai	0.3	0.01	0.4	0.01	0.4	0.01	0.4	0.20	0.3	0.01	0.3	0.01
C18 DMA	0.1^{C}	0.01	0.1^{C}	0.01	0.1^{C}	0.01	0.1^{BC}	0.02	0.1^{AB}	0.01	0.2^{A}	0.01
C17:1 unk	0.2^{BC}	0.01	0.2^{A}	0.01	0.2^{A}	0.01	0.1^{C}	0.01	0.2^{AB}	0.01	0.2^{AB}	0.01
C17:1 c8	0.1^{B}	0.01	0.1^{B}	0.01	0.2^{A}	0.01	0.1^{B}	0.02	0.1^{B}	0.01	0.1^{A}	0.01
C17:1 c9	0.1	0.02	0.1	0.02	0.2	0.01	0.1	0.02	0.1	0.01	0.2	0.04
C18	14^{A}	0.01	13^{B}	0.10	13^{B}	0.20	14^{A}	0.02	13^{AB}	0.40	13^{B}	0.01
C18:1 t11	0.2	0.01	0.2	0.01	0.3	0.01	0.2	0.03	0.2	0.01	0.2	0.01
C18:1 c9	22	0.04	21	0.01	21	0.04	22	0.20	22	0.40	21	0.10
C18:1 c11	0.8	0.02	1	0.03	0.9	0.01	0.8	0.10	0.8	0.01	0.8	0.01
C18:1 c12	0.1^{A}	0.01	0.1^{A}	0.01	0.2^{A}	0.03	$n.d^{B}$	n.a	0.1^{A}	0.01	0.1^{A}	0.01
C18:2 t, t	0.1	0.01	0.1	0.01	0.2	0.03	0.1	0.02	0.2	0.01	0.1	0.01
C18:2 c9, c12	25	0.10	26	0.30	26	0.40	24	0.30	25	0.30	25	0.20
C18:3 c6, c9, c12	0.3^{C}	0.03	0.4^{AB}	0.01	0.4^{A}	0.01	0.2^{D}	0.01	0.3^{C}	0.01	0.3^{BC}	0.02
C18:3 c9, c12, c15	5^{CD}	0.01	6^{B}	0.10	6^{A}	0.04	4^{E}	0.10	5^{D}	0.03	5^{C}	0.01
C18:4 n3	0.1	0.01	0.2	0.02	0.2	0.02	0.1	0.10	0.1	0.01	0.1	0.01
C20:2 n6	0.1^{A}	0.01	0.05^{A}	0.01	0.05^{A}	0.01	$n.d^{B}$	n.a	0.05^{A}	0.01	0.1^{A}	0.02
C20:3 n6	0.9	0.01	0.8	0.04	0.9	0.04	0.8	0.04	0.8	0.06	0.8	0.01
C20:4 AA	1^{A}	0.01	1.3^{A}	0.10	1^{A}	0.04	1^{B}	0.01	1^{B}	0.03	1^{B}	0.05
C20:4 n3	0.9^{A}	0.10	0.7^{B}	0.04	0.7^{B}	0.02	0.8^{A}	0.10	0.6^{B}	0.05	0.6^{B}	0.01
C20:5 EPA n3	0.9^{A}	0.02	0.8^{AB}	0.05	0.9^{A}	0.01	0.6^{C}	0.03	0.7^{C}	0.01	0.7^{BC}	0.04
C22:5 DPA n3	0.4	0.02	0.4	0.05	0.4	0.02	0.5	0.01	0.5	0.10	0.4	0.10
C22:6 DHA	0.2	0.02	0.2	0.01	0.2	0.01	0.3	0.04	0.3	0.10	0.4	0.10
μg FA/ mL serum	658	60	683	90	707	60	666	77	684	40	705	40

DMA: dimethylactetal; ***ai***: anteiso; **i**: iso; ***PhyAc***: Phytanic acid; ***c***: cis double bond; ***t***: trans double bond; ***n3***: omega 3 fatty acid; ***n6***: omega 6 fatty acid; ***AA***: Arachidonic acid; ***EPA***: eicosapentanoic acid; ***DPA***: docosapentanoic acid; ***DHA***: docosahexanoic. Superscript letter for significant differences in the fatty acid composition among samples ($p < 0.0.5$). $n=3$ in all tests.

added. MBF has been assayed in the analysis of the FA composition in human milk [27] and human plasma with emphasis on CLA contents [28]. According to the present research work that methodology can yield a slight production of *trans* isomers and erroneous oleic acid concentration.

Among the assayed methods, the proposed MHS showed the lowest capacity of alteration of the CLA profile in the assayed methods. Such modifications were corrected with the utilization of DMF.

3.4. Experiment 4

The feasibility of the obtained FA composition was tested by means of calculations of response factors (RF) of a skimmed milk added with a reference butterfat from the analysis using the DT methods BF3, ACL, MBF and MHS. KOH was used as the reference method.

According to the obtained results (data not shown), MHS presented the best RF values, close to 1 for most of the FA (except for C4 and C6, 2.72 and 1.80, respectively) including CLA isomers (0.97). Nevertheless when MBF and BF3 methods were applied the RF values for short-chain FA (C4–C10) ranged from 5.99 (C6 with BF3) to 1.62 (C10 with MBF) while C4 was not detected. The results are the consequence of loses of these compounds. The opposite occurred for ACL (RF < 0.5), pointing out the presence of artifacts or co-elution in short FA moieties.

3.5. Experiment 5

According to the results obtained in the previous experiments of the current research work, MHS and MBF methods showed the best results in the derivatization of lipids into FA. Thus, they were tested in the analysis of an animal-based control serum at three different sample volumes (Table 4).

Finally the MHS and MBF methods were selected according to completeness of reaction and lack of alteration of the fatty acid profile to be tested with animal plasma samples at three different volumes (100, 250 and 500 μL).

The derivatization performance (Dp) was calculated as EM was added to plasma prior to derivatization. Dp values were not significantly different in any of the assayed sample amounts with MBF (mean value 74% ± 5). However for MHS, Dp was 77% ± 0.8 for 100 μL, 87% ± 2 in 500 μL ($p > 0.05$) while 93% ± 2 using 250 μL ($p < 0.05$). Except using 100 μL, Dp values were significantly better

with MHS than with MBF. These results confer a superior reliability to the composition obtained using MHS: without application of Dp, total concentration was lower in MBF ($p < 0.05$). After correction, a slight increase was found with the sample volume but differences among methods and amounts were not significant. At the lowest sample level C14:1 *c*9, C18:1 *c*12 and C20:2 n6 were not detected using MBF. Using higher volumes did not result in any difference in their concentrations when comparing methods.

On the other hand C16 PhyAc and C18 showed higher concentrations when using 100 μL than with 250 μL and 500 μL in both methods ($p < 0.05$). It could be attributed to low Pt in these samples but the same effect was not found in MBF with 250 μL and 500 μL, which also registered Dp < 75%. Some minor compounds (C14 DMA, C15i DMA, C15ai DMA, C16 DMA, C18 DMA) showed lower amounts in MHS while some PUFA (C18:3 *c*6, *c*9, *c*12, C18:3 *c*9, *c*12, *c*15, C20:4 AA and C20:5 EPA n3) had higher contents ($p < 0.05$). Such differences could be attributable to a difficulty of MBF in the derivatization of lipids containing PUFA.

Results showed that in general there are no major differences when using 250 μL or 500 μL as sample size in both compared methods. However, utilization of the lowest sample amount possible is a desirable matter in order to accomplish further or complementary determinations.

4. Conclusion

The results of the present research work showed that among the assayed DT methods, the proposed MHS was the only one suitable for the analysis of total FA in foodstuff and biological samples as it showed a high derivatization performance, absence of alteration of the FAME profile mainly CLA isomers, good RF values for short, medium and long chain FA and allowed the measurement of total cholesterol.

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