MICROWAVE-ASSISTED METHODOLOGY FEASIBILITY FOR ONE-STEP EXTRACTION AND TRANSMETHYLATION OF FATTY ACIDS IN MILK FOR GC-MASS SPECTROMETRY

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Abstract

Fatty acids (FAs) play many essential roles in bio- logical systems, and they are the aim of different research studies due to their benefits on human health. Milk and dairy products contribute significantly to the consumption of FAs in the human diet. In consequence, manipulation of FA compo- sition of cows' milk via nutritional strategies has been an important target for the dairy industry and a challenge from an analytical point of view. Milk FA composition is complex, and their analysis involves multiple steps (extraction proce- dure, methylation, FA methyl ester extraction and gas chro- matography (GC) determination) that turn it into a tedious and time-consuming procedure. In recent years, some efforts have been made to develop an analytical approach with simulta- neous extraction and derivatization of FAs. In this sense, mi- crowave (MW)-assisted digestion and extraction methods have been used for many years and today can be considered standard operating procedures in many laboratories. It is a powerful tool for different analytical methodology develop- ment. This study is focused on one-step extraction/ transmethylation MW-assisted methodology feasibility for FA analysis in milk compared to a reference method.

Keywords Fatty acids . Microwave-assisted digestion, Milk . Acidic methodology . Basic methodology

INTRODUCTION

Fatty acids (FAs) play many essential roles in biological sys- tems, providing energy sources, serving as signalling mole- cules and being the major structural components in complex lipids of cellular membranes. On the other hand, cardiovascu- lar diseases are statistically associated with an excessive in- take of saturated fatty acid (SFA) (mainly palmitic C16:0) as compared with unsaturated (UFA), specifically oleic (C18:1) and essential FAs (C20:5 and C22:6) in food (Kalinin and Krasheninnikov 2014). Thus, they are the aim of multiple research studies due to their impact on human health.

Milk and dairy products represent a significant source of FAs in the human diet, and FAs play a critical role in the sensory attributes of these foods (Demment and Allen 2004; Chen et al. 2004; Chilliard and Ferlay 2004). Taking into account milk and dairy product intake on human diets, manip- ulation of the fat content and FA composition of cows milk via nutritional strategies has been an important target for the dairy industry and, as a consequence, there are many studies aiming to rise up its content in UFA (as CLA and n-3) (Elgersma et al. 2006; Collomb et al. 2006; Glasser et al. 2008; Morales-Almaraz et al. 2011; Hernández-Ortega et al. 2014).

Milk FA composition is complex, with chain lengths rang- ing from C4 to C26, including branch-chain FAs and many positional and geometric isomers of mono-, di- and tri- UFA, many of them present in very low concentrations (Kramer et al. 1997; Kramer et al. 2008). In total, milk fat has been estimated to contain over 400 different FAs (Delmonte et al. 2012). FA analysis involves multiple steps (extraction procedure, methylation, FA methyl ester extraction and gas chromatography (GC) determination) turning it into a tedious and time-consuming procedure. Due to the increasing impor- tance of FAs in human health, many studies have been devel- oped over the past decade focusing on the steps previously described for their analysis (Feng et al. 2004; Kramer et al. 2004; Chen et al. 2007; Moltó-Puigmartí et al. 2007; Araujo et al. 2008; Luna et al. 2008; Delmonte et al. 2012).

Some efforts have been made in recent years to develop an analytical approach with simultaneous extraction and deriva- tization of FAs (Liu et al. 2012). However, the use of micro- wave (MW) heating to enhance the efficiency of GC deriva- tization protocols has so far found limited use and acceptance in the scientific community. The latter were carried out for the first time in the early 1990s, mainly applied to clinical and forensic toxicology

and drug monitoring/doping control, but also involving examples related to food and environmental samples (Söderholm et al. 2010). MW-assisted digestion and extraction methods have been in use for many years, and today, they can be considered standard operating procedures in many laboratories. Moreover, the applications of MW- assisted process to prepare methyl ester FAs (FAMEs) can be found in different types of biological samples (Khoomrung et al. 2012). However, there has been no attempt to establish a proper method for FAME preparation in milk samples.

Taking advantage of efficient microwave dielectric heating mechanisms, reaction times can be reduced from hours to minutes, using sealed vessel MW heating (Söderholm et al. 2010). The inherent ability of MW-assisted process to rapidly heat the sample solvent mixture is the main advantage of this technique. In conventional heating, a finite period of time is needed to heat the vessel before the heat is transferred to the solution, while microwave heats the solution directly. This keeps the temperature gradient to a minimum and accelerates heating speed. The principle of heating using this energy is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation (Eskilsson and Bjorklund 2000). In many applications, these two mechanisms take place simultaneously. The samples subjected to these methodolo- gies can be immersed in a single solvent or mixture of solvents that strongly absorbs MW energy (mechanism I) or in a com- bined solvent containing solvents with both high and low dielectric losses mixed in various proportions (mechanism II), or when the sample has a high dielectric loss, it can be immersed in a microwave transparent solvent (mechanism III).

The specific aim of this work was to establish the feasibility of MW irradiation as an alternative to carry out extraction and derivatization steps and effectively combining these two pro- cesses into a single one (namely microwave-assisted one-step extraction-derivatization (MAED)) and to estimate accuracy degree to reference methodology (ISO15884/IDF182) for de- termination of FAs in milk and milk products.

MATERIALS AND METHODS

Samples, Reagents and Chemicals

Milk samples were provided by dairy cows belonging to SERI DA home herd (Lat 43° 28′ 50″ N, Long 5° 26′ 27″ W). Milk FAs were analysed under basic and acidic conditions applying MW-based methodology and were compared to reference one. Basic

methodology was carried out using hexane (95 %, HPLC grade, J.T. Baker, Mallinckrodt Baker, Inc., London, UK) as extraction solvent, methyl acetate (synthesis grade) and sodium methylate (30 % solution in methanol, synthesis grade) as transesterification agents. Reaction is stopped adding a saturated solution of 1 g of oxalic acid (synthesis grade) in 30 mL diethyl ether (synthesis grade). All reagents were supplied by Merck (Hohenbrunn, Germany) except hexane.

Acidic methodology was carried out using hexane as ex- traction solvent, methanolic chlorhydric acid (10 %) prepared using acetylchloride (98 %, reagent grade, Sigma-Aldrich, Inc., St Louis, MO 63178, USA) in methanol (95 %, HPLC grade, J.T. Baker, Mallinckrodt Baker, Inc., London, UK) and potassium carbonate (6 %, GR for analysis, Merck, Hohenbrunn, Germany). Sodium sulphate anhydrous (GR for analysis, Merck, Hohenbrunn, Germany) was used to re- move water in organic layer after solvent layer separation.

GC mass selective detector (GCMS) peaks were identified by comparison of column retention times and mass spectra obtained between the samples and the standards: compounds FAME mix 10 mg/mL in dichloromethane (99.0–99.9 % pu- rity ref. 47885-U) and 11*t*-C18:1 methyl ester (99.9 % purity; 10,000 ppm in *n*-heptane, ref. 46905-U) from SUPELCO (SUPELCO, Bellefonte, PA, USA) and 9*c*,11*t*-C18:2 methyl ester (98 % purity, ref. MT-001255) from Matreya (Matreya LLC, Pleasant Gap, PA, USA). Methyl nonadecanoate (C19:0) was used as internal standard (minimum 98 % GC, Sigma-Aldrich, Inc., St Louis, MO 63178, USA). Table 1 showed the FA names and abbreviations.

Butter fat matrix sample with reference values for caprylic acid (C8:0), myristic (C14:0), α-linoleic (9*c*12*c*-C18:2) and linolenic (9*c*12*c*15*c*C18:3) was used to control performance and to assess result accuracy. The reference material was dis- tributed by the Spanish Reference Laboratory for milk and milk products whose commitment to quality and efficiency has been demonstrated through accreditation UNE-EN ISO17025 (by EA Search Facility, no. 517/LE1040). See Table 2 for butter composition.

Instrumentation

Sample digestion was performed on MW digestion unit ETHOS One (Milestone, Srl, Sorisole, Italy) equipped with a rotor for ten TFM Teflon (chemically modified PTFE vessels. Temperature parameter is controlled by a temperature sensor (ATC-400). The

thermocouple is placed into the refer- ence vessel, simply sliding-in the sensor through the hole in the HTC (new high-performance plastic) screw of the refer- ence segment. The hole for the thermocouple is aligned with the thermowell, allowing the temperature sensor to be fully introduced in the vessel.

FAs were separated and quantified on a VARIAN 3800 GC equipped with a 4000 mass spectrometer detector (Varian, Inc. Palo Alto, CA, USA). A CP-Sil 88 column (100 m×0.25 mm, 0.20 μm i.d.; Varian, Inc.) was selected for analytical separations.

Milk centrifugation step was carried out on Biofuge Stratos (Heraeus Instruments, Hanau, Germany). Top fat-cake layer centrifugation was performed using an Eppendorf Centrifuge 5415R (Hamburg, Germany).

Lipid Milk Obtention and Fatty Acid Methyl Esthers

Milk fat was obtained following Feng et al.'s (2004) methodology. Briefly, 45 mL of milk was subjected to a first centrifugation step (17,800×*g*, 30 min, 4 °C, Biofuge Stratos, Heraeus Instruments, Hanau, Germany). An aliquot of the top fat-cake layer was removed, placed into an Eppendorf vial and subjected to a second centrifugation step (19,300×*g*, 20 min, room temperature, Eppendorf Centrifuge 5415R, Hamburg, Germany). The lipid top layer was used for FAME quantification.

Taking into account that milk represents a complex matrix for FAs, two strategies were selected: a basic methodology applied on lipid layer milk (Christie 1982) and an acidic meth- odology (adapted from Palmquist and Jenkins 2003) applied on milk and lipid layer milk. No information was available about basic methodology applied on liquid milk without pre- vious fat extraction.

All MW optimization experiments were carried out using maximum power of 700 W.

Acidic Transesterification

An aliquot of 0.5–1g milk or 0.2–0.1 g of top layer lipid milk was accurately weighted in the TFM MW vessels. Four millilitres of methanolic chlorhydric acid and 4 mL of hexane were added. MW vessels were closed, and MW process was carried out under different experimental conditions (see Table 3). After MW step, 6 mL of K_2CO_3 and2 mL of hexane were added. Solution was placed in a Pyrex tube with Teflon- lined screw cap and subjected to centrifugation (1500 rpm, 30 min, room temperature). Top organic solvent was trans- ferred to another tube containing 0.5 g of Na_2SO_4 anhydrous, vortexed and left

for water absorption during 1 h. Then, it was subjected to centrifugation (1500 rpm, 5 min, room tempera- ture). Top organic solvent was transferred to spider issue for evaporation (Heidolph, laborota 4011 digital, Schwabach, Germany). Two millilitres of hexane were added and filtered by 0.2 μm Teflon filters (Teknokroma, Barcelona, Spain). This extract was diluted before GCMS analysis.

Basic Transesterification

An aliquot of 40–160 mg of top layer lipid milk was accurate- ly weighted in the TFM MW vessels. Eight millilitres of hex- ane, 160 μL of methyl acetate and 160 μL of sodium methyl- ate were added. MW vessels were closed and MW process was carried out under different experimental conditions (see Table 3). After MW step, solution was placed in a culture tube with Teflon-lined screw cap and 240 μL of saturated oxalic acid solution were added and vortexed. The samples were then subjected to centrifugation (1500 rpm, 5 min, room tempera- ture). Top organic solvent was transferred to spider issue for evaporation. Two millilitres of hexane were added and filtered by 0.2 μm Teflon filters (Teknokroma, Barcelona, Spain). This extract was diluted before GCMS analysis.

Reference Method

Reference method is a basic transesterification method, based on ISO15884/IDF182 using saturated oxalic acid solution fol- lowing Chouinard et al.'s (1999) modification. Briefly, 40 mg of lipid layer milk or standard butter fat were weighted in a Pyrex tube with Teflon-lined screw cap. After that, FAs were extracted with 2 mL of hexane and esterified with 40 μL of methyl acetate and 40 μL of sodium methylate. After a 10-min reaction time, 60 μL of saturated oxalic acid solution were added and vortexed. The samples were then subjected to cen- trifugation (1500 rpm, 5 min, room temperature). Top organic solvent was filtered using 0.2 μm Teflon filters. This extract was diluted before GCMS analysis.

MW Parameter Optimization

The most commonly studied parameters in MW process are solvents, temperature and time. There are some considerations to take into account related with MW-absorting properties, interaction of the solvent with the matrix, the analyte solubility and solvent extraction compatibility with the analytical meth- od used for the final analysis step. For example, water has been known as the best solvent for MW extraction compared to other solvents;

however, its presence in esterification reac- tions could significantly affect FAME yield. In the present study, solvents used are compatible with MW process and with those used in the acidic and basic methodologies chosen for FAME preparation. Hexane is transparent in MW and is used in the reaction to maintain the solubility of lipids and fatty acids and also to trap FAMEs after transesterification process. However, the combination of solvents implies a mechanism II previously described (a solvent mixture con- taining solvents with both, high and low dielectric losses) or a mechanism III, using solvents with low dielectric constant but matrix with high water content, as milk or lipid layer milk. For temperature optimization, nine samples (eight samples and a blank) of milk or lipid layer under basic or acidic transesterification conditions were subjected to MW irradia- tion during 5 min at different temperatures (see Table 3). Ex- tracts obtained for the temperatures assayed were analysed by GCMS.

Once the best temperature conditions were chosen, reaction time was studied. Nine samples (eight samples and a blank) of milk or lipid layer under basic or acidic transesterification conditions were subjected to MW irradiation at the best tem- perature conditions during 10, 20 or 30 min (see Table 3). Extracts obtained at different times were analysed by GCMS. Finally, sample/solvent ratio optimization was carried out.

Different ratios were studied. Under the best temperature and reaction time conditions, 0.5/1.0 g of milk and 0.1/0.2 g of lipid layer for acidic conditions (using 8 mL of solvent correspond- ing to 4 mL of methanolic chlorhydric acid and4 mL hexane) and 40/160 mg of lipid layer for basic transesterification con- ditions (using 8.32 mL of solvent corresponding to 4 mL hex- ane, 160 µL of methyl acetate and 160 µL of sodium methyl- ate) were subjected to MW irradiation (see Table 3). Extracts obtained for different conditions were analysed by GCMS.

GCMS Analysis

The FAs were separated and analysed using helium as carrier gas at a flow rate of 1 mL/min. The temperature of injector and detector were 250 °C. The column temperature was held at 40 °C for 1.20 min; from 40 to 140 °C at 30 °C min−1 and held at 140 °C for 25 min; from 140 to 190 °C at 1 °C min−1 and held for 15 min; from 190 to 215 °C at 1 °C min−1 and held for 8 min; and finally, from 215 to 240 °C at 30 °C min−1 and held during 1 min. The mass spectrometry detection system was operated at full scan from 50 to 500 m/z. The composition of individual FAs (%) in the sample was calculated by comparing the peak area of each FA with the total peak area of all FAs obtained from GCMS. Peaks were identified by

comparison of column retention times and mass spectra obtained between samples and the standards previously described.

Statistical Analysis

Differences in the FAME content under different conditions for temperature (T), time (t) and sample/solvent ratio (R) were ex- amined using GLM procedure with the LSmeans statement pro- vided by the SAS (1999) statistical analysis according to the model $Y = a + \text{compound}_a + (b + \text{compound}_b) \times \text{covariable} + E$, where Y is the GCMS analysis result for each FA, a is the inter- cept, b is the line slope, compound_a and compound_b are FAME effect of each FAME over a and b, covariable is temperature (T), time (t) and sample/solvent ratio (R) and E_{ij} is the residual error.

RESULTS AND DISCUSSION

The ideal method used for sample preparation should be sim- ple, rapid, precise and accurate. Besides these essential fac- tors, sample preparation rate (number of samples that can be performed per hour or per day) is also important to establish a methodology in routine analysis. MW irradiation can be an alternative to promote extraction and derivatization and effec- tively combining these two processes into a single one (name- ly microwave-assisted one-step extraction-derivatization (MAED)), to establish a reliable method for rapid determina- tion of FA profiles in milk samples by GCMS.

Focusing on MW parameter optimisation, results obtained at different temperatures (30, 40 and 50 °C) with 5 min as fixed reaction time are showed in Table 4. As it can be seen, under acidic digestion (AD) conditions, there are more signif- icative differences than for basic digestion (BD). For AD and liquid milk samples, significative differences have been found with a negative effect for caprylic acid (C8:0, $P<0.05$; 2.22– 1.65 %), and using lipid layer samples, the same negative effect was observed for palmitic (16:0, $P < 0.001$; 38.15– 29.12 %) and oleic (9*c*-18:1; 10.43–8.41 %). Positive influ- ence by higher temperature is detected for other FAs involved in the study, all of them major compounds, with content larger than 7 %. On the other hand, under BD conditions, lipid layer samples were not affected by temperature parameter, with the exception of palmitic acid (C16:0; $P<0.05$; 37.15–36.70 %). Based on results showed in Table 4, 50 °C was selected as reaction temperature for all the evaluated methodologies (AD and BD). This temperature is lower than those showed in

other experimental conditions for testing MW effect on FAMEs (Herzallah et al. 2005; Giua et al. 2013) to avoid undesirable effects on FA distribution. They studied the effect of different heating treatments, pasteurized (85 °C), boiled (96.3 °C), UHT (140 °C) or MW (95 °C), obtaining in the latter an increase in trans isomers formation due to MW heating. This study tests temperature lower than those previously cited, avoiding the results described, due to reference methodology is carried out under room temperature conditions.

After fixing 50 °C as reaction temperature, the second parameter to be optimized for the success of the MAED was reaction time (see Table 5). A complex matrix sample may require longe r r e action time t o complete transesterification. Analysis of the results showed that for milk and lipid layer AD, all those FAs affected by temper- ature are influenced by reaction time, with the exception of caprylic acid (C8:0). Liquid milk samples were positively affected by higher reaction times on myristic (14:0), palmitic (16:0) and stearic (18:0) acids. In lipid layer sam- ples, capric (10:0), lauric (12:0), myristic (14:0) and stearic (18:0) acids were also positively affected whereas palmitic (16:0) and oleic (9*c*-18:1) acids were negatively affected, showing negative slope. BD showed no significativ differences for different reaction time assayed, even palmitic acid (16:0).

Previous researches (Herzallah et al. 2005; Giua et al. 2013) observed that heating for a prolonged period of time, 30 min and 63 °C of temperature under aerobic conditions, seems to contribute to lipid oxidation more than heating at higher temperature for 5 min. This unexpected result may be explained as follows: heating at 62 °C is not effective in ex- pelling the dissolved oxygen in the liquid milk, whereas above 80 °C caused a rapid escape of dissolved oxygen. However, this effect was not observed in these experimental conditions, due to the mild temperature (up to 50 °C in the highest case) applied. According to these experimental results, 30 min was chosen as reaction time for AD and 10 min for BD.

After establishing 50 °C and 30 min for AD and 50 °C and 10 min for BD, as optimal conditions, the amount of solvent needed for a single sample was evaluated (see Table 6). Sample/solvent ratio is also an important parameter for effi- cient reaction. The solvent volume must be sufficient to ensure that the entire sample is immersed, especially when the matrix will swell during the process. Investigations led to the conclu- sion that the proportion of sample in the mixture should not exceed 30–34 % (*w*/*v*) (Sparr and Björklund 2000). In con- ventional techniques, a higher volume of solvent will increase the recovery, but in

MAED, a higher solvent volume may give lower recoveries, thus, low sensitivity and precision in analy- sis. This effect can probably be due to inadequate stirring of the solvent by the MWs. In this research work, results of sample/solvent ratios studied ranged from 0.5 to 12 % and are shown in Table 6. In MW AD, the amounts of samples assayed were 0.5 and 1 g of liquid milk and 0.1 and 0.2 g of lipid layer. For milk samples, myristic (C14:0; $P<0.001$), palmitic (C16:0; $P<0.001$) and stearic (C18:0; $P<0.05$) acids showed significative differences with higher values using 0.5 than 1 g. This positive effect is observed for major compounds with content higher than 9 %. Only caprylic (C8:0; $P<0.01$) and capric acid (C10:0; $P<0.001$) showed higher values for 1 g (2.27 and 7.95 %) than for 0.5 g (1.36 and 6.53 %). In lipid layer samples plus AD, only two FAs showed significant dif- ferences. Miristic acid (14:0; $P<0.01$) content was higher using 0.1 g of sample than 0.2 g (13.54±0.36 vs. 14.06±0.40) and oleic acid (9c-C18:1; $P<005$) showed a content of 10.91 ± 0.24 % for 0.2 g and 10.42 ± 0.24 % using 0.1 g of sample. In lipid layer MW BD case, 40 and 160 mg of samples were tested. Caprylic (C8:0) and capric (C10:0) acids showed higher values for 160 mg than for 40 mg (1.25 and 5.38 % vs. 1.12 and 5.25 %, respectively). As it happens for milk AD, major compounds (concentration higher than 9 %) affected by sample/solvent ratio showed lower values for higher amount of sample, such as palmitic (C16:0) and stearic (C18:0) acids (41.29 and 11.25 % for 40 mg and 40.61 and 11.12 % for 160 mg, respectively). Taking into account these results, dif- ferent effects can be observed for major compounds compared with other FAs analysed. An excess of sample amount could produce a saturation effect decreasing the extraction and transesterification of major FA compounds. From the basis of these results, 0.5 g of milk and 0.1 g of lipid layer were selected as sample amount for AD and 40 mg was chosen as sample amount for BD conditions.

In summary, the experimental conditions selected for the MW-optimized procedures were 50 °C, 30 min and 0.5 g of liquid milk and 0.1 g of lipid layer for AD and 50 °C, 10 min and 40 mg of lipid layer for BD.

MW-Assisted Reaction vs. Reference Methodology

Conditions selected for MW methodologies and suitable for a butter fat sample (this means 0.1 g under AD and 40 mg under BD) were used to analyse the reference material previously described in the BMaterials and Methods^ section. Results were compared with those obtained with the reference method.

The values for repeatability and reproducibility limits cover the preparation of fatty acid methyl esters and their analysis by GCMS in accordance with ISO15885/IDF184. Capric acid (C6:0) was discarded in this study due to this FA elutes close to solvent front in the GCMS chromatographic conditions.

Accuracy was evaluated comparing results obtained with reference method vs. MW BD and AD conditions (see Table 7). MW BD method showed accuracy in the range between 80 and 120 % for values lower than 0.5 %, between 90 and 110 % for values in the ranges 0.5–5% and 95–105 % accuracy range for values higher than 5 %. As can be observed in Table 7, MW AD shows worse accurate values for C8:0, C10:0, C12:0, C16:0, C17:0, C18:0, 9c-C18:1, 9c12c-C18:2 and 9c11t-C18:2 than MW BD.

Thus, to check the final status and to estimate the goodness of MW, these methodologies were compared to reference standard values (see Table 2). BD procedure, optimized and proposed for one-step extraction and methylation methodolo- gy, provided the best recoveries, defined as the ratio of the observed mean test result to the true value. The results obtain- ed were 91, 112, 91 and 89 % for C8:0, C14:0, 9c12c-C18:2 and 9c12c15c-C18:3, respectively, under MW BD and 109, 114, 71, and 71 %, respectively, for MW AD.

Precision was evaluated for AD and BD analysing coeffi- cient of variation (CV), repeatability values and maximum differences. Repeatability was calculated as 2 × squared root 2 × standard deviation of repeatability. For fatty acid compo- nents present in excess of 5 %, maximum of 5 % (with an absolute maximum of 1 g per 100 g) and for fatty acid com- ponents present in amounts of 1–5 %, maximum of 12 % with an absolute maximum of 0.5 g per 100 g, is allowed (ISO15885/IDF184). As shown in Table 8, MW BD presents values in compliance with repeatability criteria; however, MW AD is not in compliance with these criteria for many com- pounds, not only saturated (C14:0, C15:0, C16:0 and C18:0) but also unsaturated (9c-C16:1, 9c-C18:1, 9c12c-C18:2, 9c12c15c-C18:3, 11t-C18:1) fatty acids. These results are similar to those obtained for lipid layer MW BD and MW AD, also shown in Table 8.

On the first glance, MW AD methodology must be discarded for further implementation at routine laboratory analysis. By contrast, precision and accuracy results obtained for BD are similar to those of reference methodology and according with the requirements of ISO15885/IDF184.

Once MW BD was selected as the best one for MAED, reproducibility was also evaluated with nine experiments. Three TFM Teflon vessels were used for 40 mg BD of refer- ence

material in each one, and three MW programmes were run in different days.

Results showed that MAED analysis of the FAs generated reproducible data in compliance with reproducibility require- ments of ISO15885/IDF184: 15 % reproducibility with maximum difference of 4 g per 100 g for fatty acids present in excess of 5 g per 100 g and 20 % reproducibility with maxi- mum difference of 1 g per 100 g for fatty acids present in amounts of 1 g to 5 g per 100 g.

CONCLUSIONS

In the present study, it has been observed that, considering MW methodology, the major FAs present in milk are the most affected FAs by temperature, time and sample/solvent ratio parameters.

Comparing MW-assisted methodology applying BD to ref- erence methodology, using a reference material with reference values provided by external accredited laboratory, we can conclude that the novelty proposed methodology meets accep- tance criteria for all the evaluated compounds (ISO15885/ IDF184). AD methodology does not meet these criteria for linoleic (9*c*12*c*-C18:2) and α-linolenic (9*c*12*c*15*c*-C18:3) acids, being necessary to discard this procedure for further applications.

This methodology improves the sample pretreatment for milk FA analysis, minimizing sample manipulation and, as a result, time analysis. This procedure greatly facilitated the analysis process in a simple, rapid and high-throughput way.

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Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects

Conflict of Interest

The authors have no financial relationship with the organization that sponsored the research. All authors declare that they have no conflict of interest.

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TABLES

Table 1. Fatty Acid abbreviations and names

FAs	FA names
8:0	Caprylic acid, methyl ester
10:0	Capric acid, methyl ester
12:0	Lauric acid, methyl ester
14:0	Myristic acid, methyl esther
9*c*-14:1	Myristoleic methyl esther
15:0	Pentadecanoic acid, methyl ester
16:0	Palmitic acid, methyl ester
9*c*-16:1	Palmitoleic acid, methyl ester
17:0	Heptadecanoic acid, methyl ester
18:0	Stearic acid, methyl ester
9*c*-18:1	Oleic acid, methyl ester
9*c*12*c*-18:2	Linoleic acid, methyl ester
9*c12c15c*-18:3	α-Linolenic acid, methyl esther
11*t*-18:1	Trans vaccenic acid, methyl ester
9*c*11*t*-18:2	Rumenic acid, methyl ester

Table 2. Butter fat FA content used as internal standard

	Ref value[a] (%)	Min	Max
C8:0	1.40	1.12	1.68
C14:0	10.94	9.30	12.58
9*c*12*c*-18:2	3.02	2.42	3.62
9*c*12*c*15*c*-18:3	0.38	0.30	0.46

[a] Reference values provided by Spanish Reference Laboratory for milk and milk products (EA Search Facility, no. 517/LE1040)

Table 3. MW parameters optimization under acidic/basic conditions

I		II		III
Temperature (700 W)		Time (700 W)		Sample/Solvent (700W)
T(C)	Hold time (min)	T selected (C)	Hold time (min)	50 °C-30 min-700 W-acidic
30	5	50	10	0.5/1.0 g milk
40	5	50	20	0.1/0.2 g top layer lipid milk
50	5	50	30	50 °C-10 min-700 W-basic
				40/160 mg top layer lipid milk

I-II MW programmes for temperature and time optimization; *III* sample amount optimization for acidic and basic conditions selected

Table 4. Statistical analysis for temperature evaluation on fatty acid profiles in milk samples

	M-AD (%)			F-AD (%)			F-BD (%)					
FAs	T1	T2	T3	T1	T2	T3	T1	T2	T3	M-AD	F-AD	F-BD
										T	*T*	*T*
8:0	2.22±0.29	1.75±0.41	1.65±0.11	1.40±0.05	1.57±0.14	1.84±0.19	0.90±0.04	0.92±0.03	0.89±0.14	*	NS	NS
10:0	7.67±0.24	7.21±0.38	7.51±0.62	5.64±0.22	6.01±0.36	7.15±0.50	4.57±0.10	4.55±0.14	4.60±0.19	NS	*	NS
12:0	7.16±0.14	6.95±0.26	7.50±0.41	5.85±0.16	6.30±0.40	7.52±0.44	4.75±0.26	4.85±0.08	4.93±0.09	NS	**	NS
14:0	15.57±0.49	16.25±0.50	17.51±0.27	15.42±0.62	16.27±0.44	18.83±0.70	15.68±0.35	15.81±0.31	15.99±0.09	***	***	NS
9c-14:1	0.92±0.08	0.93±0.08	1.09±0.02	0.98±0.05	1.10±0.12	1.42±0.16	1.03±0.06	1.03±0.08	1.07±0.09	NS	NS	NS
15:0	1.05±0.11	1.17±0.04	1.31±0.07	1.16±0.11	1.35±0.15	1.69±0.19	1.38±0.03	1.33±0.02	1.36±0.06	NS	NS	NS
16:0	36.40±0.95	38.36±0.81	37.40±0.67	38.15±1.69	35.83±3.48	29.12±4.33	37.15±1.48	37.41±0.71	36.70±1.26	**	***	*
9*c*-16:1	0.48±0.12	0.48±0.07	0.54±0.15	0.54±0.12	0.61±0.12	0.72±0.13	0.67±0.03	0.64±0.03	0.67±0.04	NS	NS	NS
17:0	0.30±0.04	0.29±0.04	0.34±0.09	0.40±0.07	0.47±0.23	0.67±0.14	0.70±0.03	0.66±0.03	0.72±0.06	NS	NS	NS
18:0	8.30±0.47	8.98±0.73	9.54±0.45	10.91±1.40	11.82±0.68	13.57±0.56	14.31±0.35	14.17±0.11	14.35±0.50	***	***	NS
9*c*-18:1	9.97±0.16	9.85±0.54	9.72±0.14	10.43±0.53	10.32±1.60	8.41±0.64	11.83±0.39	11.63±0.14	11.73±0.24	NS	**	NS
9*c*12*c*-18:2	0.89±0.36	0.84±0.23	1.01±0.35	0.93±0.42	1.07±0.28	1.04±0.38	1.26±0.10	1.21±0.05	1.29±0.16	NS	NS	NS
9*c12c15c*-18:3	0.47±0.06	0.40±0.18	0.29±0.08	0.34±0.07	0.37±0.14	0.47±0.23	0.50±0.06	0.43±0.02	0.50±0.14	NS	NS	NS
11*t*-18:1	0.72±0.08	0.80±0.03	0.97±0.15	1.04±0.23	1.31±0.32	1.88±0.22	1.34±0.66	1.54±0.03	1.61±0.09	NS	NS	NS
9*c*11*t*-18:2	0.38±0.10	0.43±0.11	0.48±0.14	0.47±0.15	0.68±0.23	0.97±0.28	0.80±0.05	0.71±0.03	0.82±0.17	NS	NS	NS

Values are presented as mean±standard error of mean (*n*=6; three samples and two replicates)

M milk, *F* lipid layer, *AD* acidic digestion, *BD* basic digestion, *T1* 30 °C, *T2* 40 °C, *T3* 50 °C, *NS* nonsignificant

*P<0.05; **P<0.01;***P<0.001

Table 5. Statistical analysis for reaction time evaluation on fatty acid profiles in milk samples

	M-AD (%)			F-AD (%)			F-BD (%)			M-AD	F-AD	F-BD
	t1	t2	t3	t1	t2	t3	t1	t2	t3			
FAs				.						*t*	*t*	*t*
8:0	1.45±0.36	1.16±0.38	1.06±0.62	1.39±0.03	1.45±0.06	1.47±0.05	1.04±0.06	1.10±0.09	1.03±0.17	NS	NS	NS
10:0	7.57±0.16	6.94±0.26	7.07±0.36	5.26±0.03	5.47±0.11	5.52±0.09	4.83±0.22	4.97±0.18	4.83±0.27	NS	*	NS
12:0	7.69±0.44	7.41±0.28	7.88±0.09	5.53±0.04	5.77±0.11	5.84±0.12	4.68±0.33	4.98±0.20	4.69±0.24	NS	*	NS
14:0	16.52±1.17	16.95±0.61	17.87±0.33	14.59±0.22	15.12±0.37	15.26±0.18	16.19±1.02	16.58±0.82	16.35±0.80	***	***	NS
9c-14:1	1.18±0.10	1.15±0.08	1.30±0.02	1.05±0.02	1.12±0.06	1.15±0.04	1.12±0.07	1.12±0.10	1.13±0.11	NS	NS	NS
15:0	1.24±0.08	1.38±0.04	1.44±0.03	1.24±0.05	1.36±0.03	1.41±0.03	1.47±0.08	1.46±0.11	1.42±0.10	NS	NS	NS
16:0	32.75±1.43	33.33±0.87	31.93±1.39	35.61±0.49	34.87±0.75	34.57±0.70	35.12±3.53	33.93±2.83	34.90±2.89	**	***	NS
9*c*-16:1	0.76±0.03	0.77±0.02	0.80±0.01	0.68±0.03	0.71±0.02	0.73±0.01	0.74±0.04	0.76±0.07	0.75±0.07	NS	NS	NS
17:0	0.40±0.05	0.47±0.04	0.51±0.03	0.55±0.04	0.63±0.04	0.67±0.03	0.75±0.05	0.71±0.09	0.67±0.13	NS	NS	NS
18:0	9.06±0.36	9.83±0.37	10.01±0.24	12.13±0.14	12.36±0.26	12.50±0.16	14.62±0.80	14.53±0.89	14.34±0.99	**	**	NS
9*c*-18:1	11.14±0.15	11.32±0.27	11.57±0.37	11.79±0.13	11.53±0.20	11.36±0.21	12.10±0.67	12.46±0.60	12.50±0.58	NS	**	NS
9*c*12*c*-18:2	1.82±0.14	1.79±0.05	1.98±0.05	1.47±0.13	1.48±0.07	1.48±0.04	1.22±0.10	1.16±0.10	1.11±0.20	NS	NS	NS
9*c12c15c*-18:3	0.72±0.20	0.77±0.02	0.90±0.05	0.65±0.10	0.71±0.06	0.75±0.07	0.60±0.08	0.53±0.08	0.51±0.16	NS	NS	NS
11*t*-18:1	1.02±0.08	1.11±0.11	1.17±0.02	1.39±0.08	1.51±0.06	1.55±0.03	1.41±0.10	1.52±0.20	1.33±0.17	NS	NS	NS
9*c*11*t*-18:2	0.62±0.08	0.62±0.01	0.74±0.03	0.84±0.10	0.92±0.07	0.97±0.07	0.85±0.09	0.78±0.09	0.72±0.21	NS	NS	NS

Values are presented as mean±standard error of mean (*n*=6; three samples and two replicates)

M milk, *F* lipid layer, *AD* acidic digestion, *BD* basic digestion, *t1* 10 min, *t2* 20 min, *t3* 30 min, *NS* nonsignifican

*P<0.05; **P<0.01; ***P<0.001

Table 6. Statistical analysis for sample/solvent ratio evaluation on fatty acid profiles in milk samples

	M-AD (%)		F-AD (%)		F-BD (%)				
	R1 (0.5 g)	R2 (1 g)	R1 (0.1 g)	R2 (0.2 g)	R1 (40 mg)	R2 (160 mg)	M-AD	F-AD	F-BD
FAs							*R*	*R*	*R*
8:0	1.36±0.06	2.27±0.05	1.54±0.04	1.48±0.05	1.12±0.03	1.25±0.04	**	NS	*
10:0	6.53±0.04	7.95±0.11	5.16±0.14	4.91±0.11	5.25±0.05	5.38±0.10	***	NS	*
12:0	6.18±0.48	6.79±0.11	4.79±0.21	4.51±0.33	5.42±0.08	5.49±0.07	NS	NS	NS
14:0	14.91±0.57	12.82±0.24	14.06±0.40	13.54±0.36	15.84±0.03	15.86±0.25	***	**	NS
9c-14:1	0.97±0.04	1.01±0.08	0.85±0.10	0.69±0.08	0.94±0.05	0.97±0.01	NS	NS	NS
15:0	1.15±0.08	1.03±0.03	1.27±0.14	1.18±0.02	1.36±0.01	1.34±0.04	NS	NS	NS
16:0	34.89±2.90	31.69±0.35	38.09±1.01	38.20±0.78	41.29±0.21	40.61±0.29	***	NS	***
9*c*-16:1	0.73±0.01	0.75±0.05	0.63±0.05	0.62±0.02	0.67±0.01	0.67±0.02	NS	NS	NS
17:0	0.38±0.03	0.37±0.03	0.49±0.13	0.44±0.07	0.61±0.01	0.59±0.04	NS	NS	NS
18:0	9.46±0.54	8.56±0.16	12.53±0.52	12.54±0.20	11.25±0.14	11.12±0.14	*	NS	*
9*c*-18:1	10.91±0.71	10.42±0.07	10.42±0.24	10.91±0.24	10.61±0.04	10.54±0.08	NS	*	NS
9*c*12*c*-18:2	2.22±0.07	2.69±0.12	1.10±0.09	1.27±0.05	0.87±0.02	0.84±0.03	NS	NS	NS
9*c12c15c*-18:3	0.81±0.09	1.07±0.13	0.37±0.13	0.36±0.10	0.36±0.01	0.38±0.06	NS	NS	NS
11*t*-18:1	1.07±0.06	1.14±0.19	0.98±0.13	0.87±0.07	1.26±0.04	1.26±0.04	NS	NS	NS
9*c*11*t*-18:2	0.73±0.05	0.87±0.06	0.37±0.08	0.34±0.04	0.30±0.02	0.35±0.09	NS	NS	NS

Values are presented as mean±standard error of mean (*n*=6; three samples and two replicates)

M milk, *F* lipid layer, *AD* acidic digestion, *BD* basic digestion, *R* sample weight, *NS* nonsignificant

*P<0.05; **P<0.01; ***P<0.001

Table 7. MW-assisted reaction: accuracy

FAs	MW-AD (%) x±s.e.	MW-BD (%) x±s.e.	Ref method (%) x±s.e.	Accuracy (%) MW-AD	Accuracy (%) MW-BD
8:0[a]	1.53±0.10	1.28±0.06	1.30±0.02	118	90
10:0	5.27±0.08	4.52±0.15	4.35±0.24	121	104
12:0	6.39±0.06	6.05±0.10	5.83±0.07	110	104
14:0[a]	12.42±0.52	12.31±0.31	12.18±0.91	102	101
9c-14:1	0.61±0.30	0.75±0.03	0.72±0.03	85	104
15:0	1.36±0.20	1.58±0.04	1.49±0.02	91	106
16:0	44.38±0.99	41.57±0.55	41.50±0.63	107	100
9*c*-16:1	0.75±0.10	0.90±0.02	0.83±0.02	90	108
17:0	0.43±0.21	0.70±0.03	0.62±0.02	69	113
18:0	12.00±1.32	14.39±0.19	13.89±0.15	86	104
9*c*-18:1	9.84±0.42	10.56±0.13	10.46±0.12	94	101
9*c*12*c*-18:2[a]	2.13±0.43	2.76±0.11	2.55±0.07	84	108
9*c12c15c*-18:3[a]	0.27±0.08	0.34±0.03	0.33±0.03	96	113
11*t*-18:1	0.51±0.02	0.63±0.03	0.58±0.02	88	109
9*c*11*t*-18:2	0.24±0.01	0.35±0.03	0.32±0.02	75	109

AD-MW acidic MW digestion, *BD-MW* basic MW digestion, *CV* coeffi- cient of variation, *x* mean value, *s.e.* standard error of mean (*n*=18; nine samples and two replicates)

[a] FAs with reference values

Table 8. MW-assisted reaction: precisión

	Reference Material						Milk lipid layer					
	BD-MW			AD-MW			BD-MW			AD-MW		
FAs	CV	Repeatability	MAX-Dif	CV	Repeatability	MAX-Dif	CV	Repeatability	MAX-Dif	CV	Repeatability	MAX-Dif
8:0	2.26[a]	0.06[a]	0.06[a]	6.78[a]	0.29[a]	0.28[a]	2.60[a]	0.08[a]	0.08[a]	2.45[a]	0.11[a]	0.12[a]
10:0	*1.39[b]*	*0.18[b]*	*0.18[b]*	*1.57[b]*	*0.23[b]*	*0.20[b]*	*1.04[b]*	*0.15[b]*	*0.14[b]*	*2.67[b]*	*0.39[b]*	*0.32[b]*
12:0	1.49[a]	0.23[a]	0.23[a]	0.99[a]	0.18[a]	0.15[a]	*1.55[b]*	*0.24[b]*	*0.17[b]*	*4.39[b]*	*0.59[b]*	*0.61[b]*
14:0	*0.84[b]*	*0.36[b]*	*0.36[b]*	*4.15[b]*	*1.46[b]*	*1.23[b]*	*0.22[b]*	*0.10[b]*	*0.10[b]*	*2.88[b]*	*1.14[b]*	*1.19[b]*
9c-14:1	2.87[a]	0.07[a]	0.08[a]	4.18[a]	0.09[a]	0.08[a]	5.21[a]	0.14[a]	0.10[a]	12.06[a]	0.29[a]	0.25[a]
15:0	3.00[a]	0.13[a]	0.11[a]	14.97[a]	0.58[a]	0.55[a]	0.39[a]	0.01[a]	0.01[a]	10.85[a]	0.39[a]	0.35[a]
16:0	*0.89[b]*	*1.01[b]*	*0.78[b]*	*7.91[b]*	*9.93[b]*	*9.08[b]*	*0.50[b]*	*0.59[b]*	*0.61[b]*	*2.65[b]*	*2.85[b]*	*2.92[b]*
9*c*-16:1	1.99[a]	0.05[a]	0.05[a]	13.83[a]	0.29[a]	0.27[a]	0.97[a]	0.02[a]	0.02[a]	7.14[a]	0.13[a]	0.10[a]
17:0	5.66[a]	0.10[a]	0.10[a]	7.03[a]	0.10[a]	0.09[a]	1.82[a]	0.03[a]	0.03[a]	25.89[a]	0.36[a]	0.30[a]
18:0	*1.42[b]*	*0.54[b]*	*0.52[b]*	*11.03[b]*	*3.74[b]*	*3.41[b]*	*1.23[b]*	*0.39[b]*	*0.30[b]*	*4.16[b]*	*1.47[b]*	*1.37[b]*
9*c*-18:1	*0.86[b]*	*0.27[b]*	*0.26[b]*	*4.23[b]*	*1.18[b]*	*1.08[b]*	*0.39[b]*	*0.12[b]*	*0.11[b]*	*2.27[b]*	*0.67[b]*	*0.58[b]*
9*c*12*c*-18:2	4.06[a]	0.26[a]	0.24[a]	20.34[a]	1.23[a]	1.14[a]	1.89[a]	0.05[a]	0.05[a]	8.40[a]	0.26[a]	0.24[a]
9*c12c15c*-18:3	10.50[a]	0.09[a]	0.08[a]	30.38[a]	0.23[a]	0.24[a]	3.08[a]	0.03[a]	0.02[a]	34.56[a]	0.36[a]	0.33[a]
11*t*-18:1	5.46[a]	0.08[a]	0.08[a]	49.14[a]	0.60[a]	0.53[a]	3.53[a]	0.13[a]	0.09[a]	13.44[a]	0.37[a]	0.31[a]
9*c*11*t*-18:2	7.02[a]	0.05[a]	0.04[a]	4.64[a]	0.03[a]	0.03[a]	6.54[a]	0.06[a]	0.04[a]	21.42[a]	0.23[a]	0.21[a]

BD-MW basicMWdigestion, AD-MWacidicMWdigestion, CV coefficient of variation, MAX-Dif maximum difference between the highest and lowest value for that FA

[a] FA 1–5 %

[b] FA >5 %