Original paper

Evaluation of fatty acid profile of oils/fats by GC-MS through two quantification approaches

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Abstract

For simultaneous separation, detection, identification and quantification of fatty acid methyl esters from oils/fats, a GC-MS method was developed and validated. The method is based on fat extraction and transesterification of fatty acids to fatty acid methyl esters. Samples of sunflower oil, refined non-hydrogenated palm oil, fish oil and lard, demonstrated the applicability of the proposed method. Fatty acid methyl esters determination and quantification was realized by using internal standards, and applying relative response factors, and without using internal standards by applying correction factors. Linearity, sensitivity, precision, accuracy, recovery and robustness were determined. The method is sensitive enough to simultaneously quantify 26 compounds, when using internal standard (absolute concentration), and 40 fatty acids without using internal standard (relative concentration). Accuracy was achieved by using a reference material, peanut butter (SRM®2387). The results have shown that the proposed method could be considered an effective tool for analyzing the fatty acid profile of food.

Keywords

Fat, fatty acids quantification, fatty acid methyl esters, GC-MS, cooking oil.

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Introduction

The main sources of lipids in our diet are oils and fats of vegetable and/or animal origin. In order to analyse the fatty acid (FA) profiles of food, it is needed the extraction of lipids from samples followed by separation, identification and determination through different techniques. For the determination of FAs various methods were developed using different devices such as high-performance liquid chromatography (HPLC) (de la Mata-Espinosa, 2011), silver-ion-HPLC (Ag⁺-HPLC) (Luna, 2008), attenuated total reflection- Fourier transformer infrared spectroscopy (ATR-FTIR) (Vongsvivut, 2012; da Costa Filho, 2014; Lucarini, 2018; Karunathilaka, 2019), capillary zone electrophoresis (De Castro Barra, 2012), nuclear magnetic resonance (NMR) (Castejón, 2013; Mihai, 2018) but the the most used method is gas chromatography (GC) (Chowdhury, 2007; Dikbas, 2011; Manzano, 2012; Chen, 2014; FirL, 2014; Pintilie, 2014; Demirel, 2016; Adjepong, 2017; Mazurek, 2017). GC coupled with mass spectrometry (GC-MS) helps to be a better separation and identification of FA isomers and less overlapping compared to GC-FID (Zhang H., 2015). In GC analysis, fatty acids identification is based on conversion of FAs into corresponding fatty acid methyl esters (FAME) with higher volatility.

An aspect that should be taken into consideration when validating the method for fatty acids determination, is the procedure for derivatisation of fatty acids after extraction of lipids from food samples. The methods for fatty acids derivatisation involve acid or base catalysis. The most used base reagents for fast transformation of FAs into FAMEs are NaOH or KOH in methanol (Simionato, 2010; Chen, 2014; Salimon, 2014; Zhang M., 2015).

Another important aspect of GC method development and validation is the choice of GC column. From the polarity of the column depends the peak resolutions, a highly polar capillary column being a good choice for the separation of geometric isomers of unsaturated FAMES (Delmonte, 2016).

For fatty acids quantification, it can be used the internal or the external standard procedure. In the case of external calibration procedure, the analyte peak area is compared with the peak area of the reference standards of fatty acids with known concentration (SoBrado, 2016). In the method which uses internal standards of triglycerides or fatty acids, which has been used mostly, the results are being expressed in weight (absolute concentration, g/100 g). For expressing the absolute concentration, it is required to use the response factors, which are dependent on the detector response. The response factors for quantification of individual fatty acids are different, being proportional with the number of active carbons in the fatty acid chain (Simionato, 2010; FirL, 2014). Results can also be expressed in weight percentage (relative concentration, weight %). When the results are expressed like this, there are used correction factors which can be theoretical or experimentally determined (Simionato, 2010).

The aim of this study was to validate a GC-MS method for simultaneous determination and quantification of fatty acids from oils/fats of vegetable and/or animal origin, by expressing the results in relative and absolute concentration. In our study it were used both the internal and the external standard procedure in order to quantify the FA content and the results were compared. To demonstrate the suitability of the proposed method, a certified reference material (SRM®2387- peanut butter) was used to study the accuracy and the results were compared.

Materials and Methods

Reference standards, reagents

Two reference standards were used in the validation procedure: F.A.M.E. Mix, C4-C24 (mixture of 37 FAME, Bellefonte, PA, USA) and SRM®2377 (mixture of 26 FAME, NIST certified, USA). Internal standards of triglycerides (TAG-IS, C11:0, C15:0) and fatty acid methyl ester (FAME-IS C23:0) purchased from Sigma-Aldrich (St. Louis, MO, USA) and Laradan AB (Solna, Sweden) were used during the validation procedure.

A standard reference material (SRM®2387- peanut butter, Gaithersburg, MD 20899), NIST certified for fat (extractable), 12 fatty acids, for the sum of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids was used to assess the accuracy of the method and the fat content.

All solvents and reagents were of analytical grade, specially for chromatography and were used for the preparation and analysis of FAME: petroleum ether, 40-60 °C, solvent used for fat extraction (VWR Chemicals, France), 5.4 M methanolic solution of sodium hydroxide (Acros, New Jersey), 14% methanolic solution of boron trifluoride (Sigma Aldrich, Switzerland), sodium chloride (Sigma Aldrich, Denmark), methanol picograde and 2,2,4-trimethylpentane picograde (LGC Standards GmbH, Germany).

Food matrices

Samples from the category of oils/fats of vegetable and/or animal origin were purchased from several local supermarkets and were as follows: vegetable origin oil of sunflower – produced in Romania; refined non-hydrogenated palm oil – produced in Malaysia, animal origin oil of pure fish with omega-3 and lemon flavour – produced in Iceland, vegetable origin fat of peanut butter (SRM®2387) and also animal origin fat of lard, obtained from rural households in the county of Brăila, Romania.

Calibration solution preparation

Calibration solutions from the F.A.M.E. Mix, C4-C24 (N1-N6) and SRM®2377 (S15-S60) were realised in order to
determine correction factors (CFs). From the SRM®2377, calibration solutions (S1-6a: N1-6b) with FAME-IS C23:0 were prepared. The calibration curves were performed by plotting the linear graphical representation of the peak area ratio (ΔFAME/ΔFAME-IS) versus FAME concentration (C_FAME) or graphical representation of the peak area ratio (ΔFAME/ΔFAME-IS) versus concentration ratio (C_FAME/C_IS) of analyte/internal standard (average response).

Calibration curves were performed for 26 FAME components of the reference standard SRM®2377, in relation to FAME-IS C23:0. The concentration of FAME-IS in each calibration level was 50 µg/mL, and 2,2,4-trimethylpentane picograde was used as a solvent to prepare the stock and working solutions of the reference standards.

**Determination of correction factors (CFs) and relative response factors (RRFs)**

**Determination of correction factors (CFs)**

The 6 calibration level solutions (S1-6a: N1-6b) were injected into GC-MS, each in 3 replicates. Correction factors were calculated using the formula from the equation (1).

\[
CF_i = \frac{p}{100} \times \frac{\sum A_i}{A_i}
\]

where: \( p \) - FAME, mass percent from the mix of reference standards based on the certificate of analysis (%); \( \sum A_i \) - sum of FAME areas for all FAME from the chromatogram of reference standards; \( A_i \) - area of FAMEi from the chromatogram of reference standards.

**Determination of relative response factors (RRFs)**

For the determination of experimental RRFi, the calibration levels S1a-S6a were prepared by using the certified reference standard (SRM®2377) and the FAME-IS C23:0. RRFs were determined for the 26 FAMEs of the reference standard SRM®2377, in relation with FAME-IS C23:0.

S1a-S6a calibration solutions were injected into GC-MS in 3 replicates for each level, and it were calculated the RRFi means determined for the 6 calibration levels. These RRFi values were calculated using the formula from the equation (2).

\[
RRF_i = \frac{\text{Area ratio}}{\text{Amount ratio}} = \frac{\Delta \text{FAME}_i / \Delta \text{FAME-IS}_i}{C_{\text{FAME}} / C_{\text{FAME-IS}}} = \frac{\Delta \text{FAME} \times C_{\text{FAME-IS}}}{\Delta \text{FAME-IS} \times C_{\text{FAME}}}
\]

where: Area ratio - the ratio between \( \Delta \text{FAME}_i \) and \( \Delta \text{FAME-IS}_i \) from the chromatogram of the calibration solution; Amount ratio - the ratio between \( C_{\text{FAME}_i} \) and \( C_{\text{FAME-IS}_i} \) from the calibration solution level; \( \Delta \text{FAME}_i \) - area of FAMEi from the chromatogram of calibration solution; \( \Delta \text{FAME-IS}_i \) - area of FAME-IS from the chromatogram of calibration solution; \( C_{\text{FAME}_i} \) - quantity of FAME, from the calibration solution (µg); \( C_{\text{FAME-IS}_i} \) - quantity of FAME-IS from the calibration solution (µg).

**Preparation of FAMEs from oils/fats of vegetable and/or animal origin**

Samples of sunflower oil, non-hydrogenated refined palm oil, fish oil and lard were prepared in accordance with ISO 661 (2003) and the fat of peanut butter sample was extracted based on ISO 17189 (2003). FAMEs were prepared by transesterification of fat extracted from the studied matrices according to ISO 12966-2 (2017) by using the transmethylation procedure with boron trifluoride (BF3) catalyst.

**Recovery determination**

Recovery (Rec.) was determined by using an internal standard of triglyceride (TAG-IS) which is added to the fat sample extracted from the food matrix, before converting it into FAME.

The selection of IS is critical when using GC-MS, TAG-IS and FAME-IS must accomplish the following conditions: not to be included in the sample to be analyzed, to be completely separated from the rest of the components, and not to differ too much from the concentration of the component to be determined.

To determine recovery, a 4 mg/mL TAG-IS (C11:0/C15:0) solution was used. Over the amount of fat weighted into the 50 mL flask was added 250 µL of TAG-IS solution, and the preparation of FAME was performed as described in the procedure.

Recovery, expressed in %, is calculated based on equation (3).

\[
\text{Rec. (%) } = \frac{V \times D \times A_{\text{TAG-IS}} \times C_{\text{FAME-IS}} \times P_{\text{TAG}} \times A_{\text{FAME-IS}}}{\Delta \text{FAME-IS} \times RRF_i \times C_{\text{TAG-IS}}} \times 100
\]

where: \( V \) - volume of the final extract of FAMEi (3 mL); \( D \) - dilution of final extract; \( A_{\text{TAG-IS}} \) - peak area of TAG-IS from the chromatogram of final extract; \( C_{\text{FAME-IS}} \) - quantity of internal standard added to the diluted final extract (µg); \( P_{\text{TAG}} \) - stoichiometric conversion factor of TAG-IS into FAME-IS; \( A_{\text{FAME-IS}} \) - area of FAME-IS from the chromatogram of final extract; \( RRF_i \) - relative response factor of TAG-IS in relation to FAME-IS; \( C_{\text{TAG-IS}} \) - quantity of TAG-IS added to the mass of fat taken into analysis (g).
Quantification of FAMEs/FAi composition of food samples analyzed

Quantification of FAMEi/FAi composition of food samples based on CFs

CFs determined according to equation (1) were used to quantify FAMEs/FAi (relative concentration expressed as weight % of total identified FAMEs/FAIs) in the food matrices studied. The obtained values correspond to the weight percentage of FAME/FAi individually determined, expressed as triacylglycerol per 100 g fat and calculated using the equation (4).

\[ p_i% = \frac{C_{Fi} \times A_i}{\sum(C_{Fi} \times A_i)} \times 100 \]  
(4)

where: \( p \) - weight percent of individually FAMEs, calculated as triacylglycerol per 100 g fat (%); \( C_{Fi} \) - correction factors corresponding to each FAMEi from the reference standard; \( A_i \) - peak area of FAMEi from the chromatogram of fat extract; \( \sum(C_{Fi} \times A_i) \) - sum of the results of CFi \( x A_i \) for the FAME peaks from the extracted fat.

Quantification of FAME/FAi composition of food samples based on RRFs

The RRFs determined according to equation (2) were used for the quantification of FAMEs/FAi (absolute concentration expressed as g/100 g) in the food matrices studied. The values obtained are expressed as weight fraction in g FAME/FAi per 100 g sample and given by equations (5) and (6):

\[ g_{\text{FAMEi}/100g} = \frac{V \times D \times \text{Area ratio} \times C_{\text{FAME-IS}} \times L}{w \times \text{RRF} \times P_{\text{FR}}} \]  
(5)

\[ g_{\text{FAi}} = g_{\text{FAMEi}} \times F_{\text{FAi}} \]  
(6)

where: \( V \) - volume of the final extract of FAMEi (3 mL); \( D \) - dilution of final extract; \( \text{Area ratio} \) - ratio between \( A_{\text{FAME}} \) and \( A_{\text{FAME-IS}} \) from the final extract chromatogram; \( C_{\text{FAME-IS}} \) - quantity of internal standard FAME-IS added to the diluted final extract (µg); \( L \) - lipid content of the food matrix (%); \( w \) - weight of sample taken into analysis (g); \( \text{RRF} \) - relative response factor of FAMEi in relation to FAME-IS determined from the reference standard; \( F_{\text{FAi}} \) - transformation factor from µg in g (10^6); \( F_{\text{FAi}} \) - stoichiometric conversion factor of FAMEi into FAi.

GC-MS analysis

Chromatographic analysis was performed on a Trace GC Ultra/TSQ Quantum XLS system (Thermo Fisher Scientific, USA), a gas chromatograph coupled with a mass spectrometer (MS) TSQ Quantum XLS, autosampler, TriPlus AS. FAMEs/FAIs separation was realized on a high polarity capillary column, TR-FAME (60 m x 0.25 mm x 0.25 µm film thickness) of 70% cyanopropyl and 30% polysilphenyl-siloxane. Analysis of calibration solutions and the derivatized extract samples was performed in the positive electron impact ionization (EI+) mode, selected ion monitoring (SIM) mode, using 24 segments. The ion source temperature was 250°C, the oven temperature was programmed at 100 °C for 0.2 min, increased to 240°C with 2 °C/min and hold for 15 min. The mobile phase was He of purity 99.99995% (5.0), at a constant flow rate of 1 mL/min. A volume of 0.5 µL of extract was injected at 240 °C in split mode with a 1:50 split ratio and a 50 mL/min splitting rate. Instrument control, data acquisition and processing were performed using the Xcalibur Program. The total run time of a GC-MS chromatogram was 85.20 min.

Validation procedure

The evaluation of the performance parameters of the GC-MS method to determine FAMEs/FAIs in oils/fats of vegetable and/or animal origin was made based on the guidelines and recommendations for the validation of chromatographic methods (ISO 5725-6 (1994); Commission Decision 2002/657/EC; Taverniers, 2004; ICH, 2005).

The internal validation procedure of the method, implied the evaluation and optimisation of the performance parameters, by repeated measurements and the interpretation of the results obtained according to the established acceptability criteria. The following performance parameters of the method were evaluated: method linearity, working range, sensitivity, precision, accuracy, recovery, and robustness.

Statistical analysis

The significance of difference was performed by using one-way ANOVA (analysis of variance) followed by Tukey’s test and was considered significant when p value was less than 0.05. Data are expressed as mean ± standard deviation. Analyses were performed by using the SPSS software program (IBM SPSS Statistics 24).

Results and Discussions

The aim of this study was to validate a precise and robust method for FAME/FAi determination from oils/fats of vegetal and/or animal origin. For the identification of FAME, the retention times (RT) of FAME were confirmed by comparing their RT and the mass/charge (m/z) ratio characteristic of each compound with those obtained for each individual FAME from the reference materials.

The elution order of the FAME of the SRM® 2377 is given in the chromatogram from Fig. 1.
The correction factors (CFs) and relative response factors (RRFs) were determined in order to quantify the concentration of each FAME/FAi present in the sample.

CFs and RRFs experimentally determined
The detector's response is different based on the carbon chains of methyl esters and will respond differently, which determines that experimentally determined CFi or RRFi should be used when quantifying FAME/FAi.

CFs were calculated in each calibration level as well as the CF mean for the 6 concentration levels was obtained. By evaluating the CFi values obtained by using the two reference standards (F.A.M.E. Mix, C4-C24 and SRM®2377), it is noted that for the FAMEs common to both standards (23 FAME) there are no major differences between them, and therefore can be determined up to 40 FAME from the food samples (23 FAMEs are common to the reference standards F.A.M.E. Mix, C4-C24 and SRM®2377, 3 are SRM®2377 specific, and 14 are F.A.M.E. Mix, C4-C24 specific). Based on CFs experimentally determined, FAMEs in the food matrices studied can be quantified as relative concentration, expressed as weight %.

RRFs that were obtained experimentally, were calculated based on the equation (2), for each FAME/FAi of the SRM®2377 standard, in relation to FAME-IS C23:0. Based on the experimentally determined RRF, 26 FAMEs can be quantified (absolute concentration, expressed in g/100 g) from the food matrices studied.

Evaluation of performance characteristics
The following performance characteristics of the method were evaluated: linearity, working range, sensitivity (LOD and LOQ), precision, accuracy, recovery, and robustness.

Linearity
Linearity was determined by performing the calibration curves (linear) from different serial dilutions of the FAME stock solutions. Good linearity of the MS detector response was found for all 26 FAME with linear regression coefficients (R^2) higher than 0.99. The detailed results are summarized in Table 1.

Working range
The working range for quantification of FAME/FAi is the lowest working limit equal to the LOQ and the upper working limit given by the maximum concentration of S6a solution in the standard reference material SRM®2377.

Sensitivity
The sensitivity of the method was evaluated by limit of detection (LOD) and limit of quantification (LOQ), calculated based on calibration curves and estimated using the ICH approach (2005). LOD and LOQ were calculated based on the residual standard deviation of the calibration curve (SD) and the slope of the calibration curve (b) for each FAME/FAi of the reference standard SRM®2377, in relation with FAME-IS C23:0, where LOD = 3.3xSD/b and LOQ = 10xSD/b (Table 1).

Precision
To estimate precision, repeatability (equipment precision and method precision) and intra-laboratory reproducibility were determined. The equipment precision was achieved by consecutive injections (n = 9) of the same sample on RT. The CV(r) values obtained experimentally were between 0.01 and 0.04% for all the FAME/FAi and correspond to laboratory requirements (CV(r) ≤ 0.05%), for all of the tested samples.

The intra-day precision was determined on 5-6 replicates, from each matrix, in repeatability conditions, by evaluating the statistical parameters: mean of absolute concentration (g/100 g), mean of relative concentration (%); standard deviation under repeatability conditions, SD(r); coefficient of variation under repeatability conditions, CV(r); the repeatability limit (r) for both individual FAME/FAi and sum of SFA, MUFA and PUFA.

The mean values of the concentration and repeatability limits (r) for the food matrices taken into study are presented in Table 2 and correspond to the required conditions, according to ISO 12966-4 (2017). The repeatability limit (r) for each FAME/FAi, sum of SFA, MUFA and PUFA was below 1.32% for sunflower oil, palm oil and lard quantified based on CFs and was below 8.15 g/100 g when it were calculated based on RRFs. Fish oil was quantified just
based on CFs and the repeatability limit was below 2.68%. This sample was not quantified based on RRFi, as the internal standard, FAME-IS C23:0, was found in this matrix.

The same statistical parameters were also evaluated for intra-laboratory reproducibility by repeated measurements (n=5-9), by different analysts, on identical samples from each food matrix. The results obtained (Table 3) correspond to the requirements for reproducibility according to ISO 12966-4 (2017). The reproducibility limit (R) for each FAME/FA, sum of SFA, MUFA and PUFA was below 2.55% for sunflower oil, palm oil and lard quantified based on CFs and below 8.91 g/100 g when it was calculated based on RRFs. Fish oil was quantified just based on CFs and the reproducibility limit was below 2.10%.

As it can be seen in Table 2, oleic acid (C18:1n9) and linoleic acid (C18:2n6) were the major FAs of sunflower oil, followed by palmitic acid (C16:0). Similar results were obtained by Chowdhury (2007) and Kostik (2013) who studied the composition of sunflower oil and showed a high content of oleic and linoleic acids, and a lower content of palmitic acid. Sunflower oil has a low content of SFA, making it a suitable oil for consumption.

Regarding the fatty acid composition of palm oil, our results are in accordance with the one obtained by Chowdhury (2007), Kamatou (2017), and Montoya (2014) who showed that the major FAs are palmitic and oleic acids, followed by linoleic and stearic acids (C18:0).

| No. | Compound name               | Identification | Regression equations<sup>a</sup> (y=ax+b) | Sensitivity | LOD (µg/mL) | LOQ (µg/mL) |
|-----|-----------------------------|----------------|------------------------------------------|-------------|-------------|-------------|
| 1   | C8:0 caprylic acid          | 7.93           | y = 0.0402x – 0.1054/37.50 – 284.74      | 0.9966      | 12.30       | 37.20       |
| 2   | C10:0 capric acid           | 12.33          | y = 0.0414x – 0.1262/35.89 – 293.07      | 0.9989      | 12.00       | 36.37       |
| 3   | C12:0 lauric acid           | 18.70          | y = 0.0392x – 0.0305/40.81 – 309.91      | 0.9999      | 4.36        | 13.30       |
| 4   | C14:0 myristic acid         | 26.02          | y = 0.0359x + 0.0056/36.59 – 277.84      | 0.9999      | 4.03        | 12.22       |
| 5   | C14:1n5 myristoleic acid    | 27.91          | y = 0.0105x – 0.0006/9.75 – 74.02        | 0.9998      | 1.42        | 4.29        |
| 6   | C16:0 palmitic acid         | 33.36          | y = 0.0336x – 0.063/37.981 – 288.42      | 0.9995      | 7.44        | 22.54       |
| 7   | C16:1n7 palmitoleic acid    | 34.77          | y = 0.0088x – 0.01/25.89 – 196.62        | 0.9991      | 7.23        | 21.92       |
| 8   | C18:0 stearic acid          | 40.32          | y = 0.0311x – 0.1238/39.53 – 300.14      | 0.9993      | 9.87        | 29.90       |
| 9   | C18:1n9 elaidic acid        | 41.00          | y = 0.0073x – 0.0104/10.39 – 78.94       | 0.9988      | 3.28        | 9.94        |
| 10  | C18:1n7 trans- vaccenic acid| 41.19          | y = 0.0088x – 0.0122/12.19 – 92.54       | 0.9990      | 3.59        | 10.86       |
| 11  | C18:1n9 oleic acid          | 41.43          | y = 0.0087x – 0.0233/36.08 – 273.96      | 0.9995      | 9.36        | 11.89       |
| 12  | C18:1n7 vaccenic acid       | 41.74          | y = 0.0089x + 0.005/11.89 – 90.28        | 0.9992      | 2.72        | 8.25        |
| 13  | C18:2n6f linolelaic acid    | 42.43          | y = 0.0117x – 0.0237/10.35 – 79.96       | 0.9990      | 10.86       | 32.92       |
| 14  | C18:2n6 linoleic acid (LA)  | 43.49          | y = 0.0119x – 0.0049/37.22 – 286.46      | 0.9990      | 10.86       | 32.92       |
| 15  | C18:3n6 γ-linolenic acid (GLA)| 44.81   | y = 0.0105x + 0.0112/9.24 – 70.19         | 0.9992      | 2.43        | 7.36        |
| 16  | C18:3n3 α-linolenic acid (ALA)| 45.97  | y = 0.0152x – 0.0387/21.92 – 166.49      | 0.9991      | 6.15        | 18.64       |
| 17  | C20:0 arachidic acid        | 46.78          | y = 0.0272x – 0.069/18.90 – 143.04       | 0.9987      | 6.24        | 18.90       |
| 18  | C20:1n9 gondolic acid       | 47.90          | y = 0.0080x – 0.0115/9.87 – 75.00         | 0.9985      | 3.29        | 9.87        |
| 19  | C20:4n6 arachidonic acid (ARA)| 51.94  | y = 0.0007x – 0.0014/7.78 – 59.05         | 0.9990      | 2.26        | 6.84        |
| 20  | C22:0 behenic acid          | 52.76          | y = 0.0231x - 0.0703/22.03 – 167.27      | 0.9978      | 7.34        | 22.03       |
| 21  | C22:1n9 erucic acid         | 53.88          | y = 0.0072x – 0.0112/11.34 – 86.14       | 0.9979      | 3.78        | 11.34       |
| 22  | C20:5n3 eicosapentanoic acid| 54.30          | y = 0.0102x – 0.0153/7.85 – 59.64         | 0.9988      | 2.25        | 7.73        |
| 23  | IS C23:0 tricosanoic acid   | 55.54          | y = 0.0183x – 0.0337/9.30 – 70.62         | 0.9973      | 3.10        | 9.30        |
| 24  | IS C24:0 lignoceric acid    | 58.30          | y = 0.0065x – 0.0109/9.86 – 68           | 0.9976      | 2.99        | 8.96        |
| 25  | IS C24:1n9 nervonic acid    | 59.43          | y = 0.0092x – 0.0237/10.19 – 105.77      | 0.9987      | 2.45        | 7.34        |
| 26  | IS C22:6n3 docosahexanoic acid(DHA)| 60.29 | y = 0.0090x – 0.0168/3.84 – 63.35        | 0.9987      | 2.78        | 8.34        |

* y: FAMEi peak area/FAME-IS peak area; x: [FAMEi]/[FAME-IS]; IS- internal standard (C23:0); R² – regression coefficient

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Similar results were also obtained by Al-Khusaibi (2012) who studied the fatty acid composition of palm olein. The fish oil taken into study had a high content of omega-3 fatty acids, eicosapentaenoic acid (C20:5n3 – EPA) and docosahexaenoic acid (C22:6n3 – DHA) being the major FAs found. In the case of lard, it can be noticed (Table 2) that it has a high content of omega-6 fatty acids, followed by palmitic, linoleic and stearic acids. Piasentier (2009) studied the fatty

Table 2. Mean concentration and repeatability limit (r) for FAi/SFA/MUFA/PUFA from the composition of food samples taken into study

| Compound name             | Sunflower oil, n = 5 | Palm oil, n=6 | Fish oil, n = 6 | Lard, n=5 |
|---------------------------|----------------------|---------------|----------------|-----------|
|                           | Concentration (g FAi/100g) | r (r) | Concentration (g FAi/100g) | r (r) | Concentration (g FAi/100g) | r (r) | Concentration (g FAi/100g) | r (r) |
| C16:0 palmitic acid       | (5.90) | (0.15) | (42.67) | (1.26) | (11.87) | (0.62) | (25.33) | (24.23) | (1.32) |                |
| C16:1n7 palmitoleic acid  | (0.12) | (0.01) | - | - | (7.49) | (0.51) | (2.72) | (2.67) | (0.13) |                |
| C17:0 margaric acid       | - | - | - | - | (1.08) | (0.68) | - | - | - |                |
| C18:0 stearic acid        | (2.80) | (0.15) | (3.83) | (0.18) | (2.19) | (0.23) | (10.71) | (10.39) | (0.60) |                |
| C18:1n9 oleic acid        | (46.92) | (0.51) | (41.73) | (0.71) | (5.71) | (0.49) | (43.40) | (43.21) | (0.74) |                |
| C18:1n7 vaccenic acid     | - | - | (0.69) | (0.08) | (2.48) | (0.25) | (3.22) | (3.2) | (0.06) |                |
| C18:2n6 linoleic acid (LA)| (43.60) | (0.33) | (9.72) | (0.45) | (0.98) | (0.10) | (11.08) | (10.44) | (0.74) |                |
| C18:3n6 γ-linolenic acid  | - | - | - | - | (0.45) | (0.27) | - | - | - |                |
| C18:3n3 α-linolenic acid  | (0.05) | (0.02) | (0.16) | (0.02) | (1.42) | (0.87) | (0.37) | (0.35) | (0.06) |                |
| C20:0 arachidic acid      | (0.18) | (0.07) | (0.27) | (0.04) | (0.29) | (0.18) | (0.15) | (0.15) | (0.01) |                |
| C20:1n9 gondolic acid     | (0.16) | (0.01) | - | - | (1.63) | (0.96) | (0.71) | (0.72) | (0.10) | (0.15) |                |
| C21:0 hexacosanoic acid   | - | - | - | - | (0.06) | (0.04) | - | - | - |                |
| C20:2n6 eicosadienonic acid | (0.31) | (0.19) | - | - | (0.31) | (0.19) | - | - | - |                |
| C20:3n6 dihomo-γ-linolenic acid (DGLA) | (0.56) | (0.34) | - | - | (0.56) | (0.34) | - | - | - |                |
| C20:4n6 arachidionic acid | - | - | - | - | (2.55) | (1.53) | (0.12) | (0.12) | (0.01) | (0.01) |                |
| C20:3n6 eicosatrienonic acid (ETE) | (0.15) | (0.09) | - | - | (0.15) | (0.09) | - | - | - |                |
| C22:6n3 docosa hexaenoic acid | (0.58) | (0.09) | (0.10) | (0.10) | (0.16) | (0.10) | - | - | - |                |
| C22:1n9 erucic acid       | - | - | - | - | (0.26) | (0.16) | - | - | - |                |
| C20:5n3 eicosapentaenoic acid (EPA) | - | - | - | - | (27.72) | (0.08) | - | - | - |                |
| C23:0 tetracosenonic acid | - | - | - | - | (0.04) | (0.02) | - | - | - |                |
| C22:2n6 docosadienonic acid | - | - | - | - | (0.03) | (0.02) | - | - | - |                |
| C24:0 lignoceric acid     | (0.23) | (0.03) | (0.05) | (0.01) | (0.07) | (0.04) | - | - | - |                |
| C24:1n9 nervonic acid     | - | - | - | - | (0.99) | (0.60) | - | - | - |                |
| C22:5n3 docosapentaenoic acid (DPA) | - | - | - | - | (1.88) | (0.25) | - | - | - |                |
| C22:6n3 docosa hexaenoic acid (DHA) | - | - | - | - | (22.62) | (2.68) | - | - | - |                |
| SFA                        | (9.1) | (0.25) | (47.7) | (1.11) | (22.5) | (1.30) | (38.4) | (36.75) | (1.12) |                |
| MUFA                       | (9.00) | (0.66) | (56.62) | (4.83) | (18.8) | (0.95) | (50.0) | (49.80) | (0.68) |                |
| PUFA                       | (47.2) | (4.30) | (52.34) | (5.91) | (85.7) | (2.24) | (11.6) | (10.91) | (0.69) | (2.21) |
acid composition of heavy pig back fat and the results are in agreement with the one obtained in this study.

By evaluating the two FAMEs quantification approaches, with and without IS in the matrices studied, it was noticed that the values are close. RRFs quantification is more accurate, but the cost of the method is higher due to IS used. Sample preparation and processing by using IS is much more expensive than quantification based on CFs (without IS).

Table 3. Mean concentration and reproducibility limit (R) for FA/SFA/MUFA/PUFA from the composition of food samples taken into study

| Compound name                  | Sunflower oil, n = 5 | Palm oil, n = 6 | Fish oil, n = 6 | Lard, n=5 |
|--------------------------------|----------------------|----------------|----------------|-----------|
|                                | Concentration (°), [**] | R (°), [**] | Concentration (°), [**] | R (°) | Concentration (°), [**] | R (°), [**] | Concentration (°), [**] | R (°), [**] |
| C4:0 butyric acid              | -                    | -              | -              | (0.01) | -                    | -              | -                    | -              |
| C6:0 caprylic acid             | -                    | -              | -              | -      | -                    | -              | -                    | -              |
| C10:0 capric acid              | -                    | -              | -              | -      | -                    | -              | -                    | -              |
| C12:0 lauric acid              | -                    | -              | -              | -      | -                    | -              | -                    | -              |
| C13:0 tridecanoic acid         | -                    | -              | -              | -      | -                    | -              | -                    | -              |
| C14:0 myristic acid            | (0.07), [0.06]       | (0.01), [0.01] | (0.94), [1.07] | (0.15) | (5.62), (0.10)       | (1.92), [1.98] | (0.17), [0.33] |
| C14:1n5 myristoleic acid       | -                    | -              | -              | -      | (0.23), (0.12)       | -              | -                    | -              |
| C15:0 pentadecanoic acid       | -                    | -              | -              | (0.74) | (0.39)               | -              | -                    | -              |
| C15:1n7 pentadecenoic acid     | -                    | -              | -              | (0.05) | (0.03)               | -              | -                    | -              |
| C16:0 palmitic acid            | (5.84), [5.57]       | (0.21), [0.29] | (42.79), [51.06] | (0.25) | (11.88), (0.22)     | (25.17), [26.76] | (1.61), [4.57] |
| C16:1n7 palmitoleic acid       | (0.13), [0.12]       | (0.01), [0.02] | -              | -      | (7.40), (0.21)       | (2.73), [2.98]  | (0.11), [0.50] |
| C17:0 margaric acid            | -                    | -              | -              | (0.92) | (0.48)               | -              | -                    | -              |
| C18:0 stearic acid             | (2.53), [2.17]       | (0.03), [0.16] | (3.84), [4.72] | (0.36) | (2.22), (0.13)       | (10.83), [11.65] | (0.62), [2.04] |
| C18:1n9 oleic acid             | (47.07), [45.06]     | (0.19), [0.04] | (41.57), [52.13] | (1.47) | (5.82), (0.34)       | (43.36), [47.94] | (0.37), [7.70] |
| C18:1n7 vaccenic acid          | -                    | -              | -              | (0.05) | (0.05)               | -              | (0.12), [0.57] |
| C18:2n6 linoleic acid (LA)     | (43.39), [39.22]     | (0.07), [0.01] | (9.72), [11.59] | (0.89) | (1.01), (0.06)       | (11.25), [11.75] | (0.82), [2.15] |
| C18:3n6 γ-linolenic acid (GLA) | -                    | -              | -              | (0.39) | (0.21)               | -              | -                    | -              |
| C18:3n3 α-linolenic acid (ALA) | (0.05), [0.05]       | (0.02), [0.01] | (0.17), [0.20] | (0.03) | (1.22), (0.63)       | (0.36), [0.39]  | (0.05), [0.07] |
| C20:0 arachidic acid           | (0.18), [0.17]       | (0.01), [0.16] | (0.27), [0.34] | (0.03) | (0.25), (0.13)       | (0.15), [0.16]  | (0.01), [0.03] |
| C20:1n9 gondoic acid           | (0.16), [0.15]       | (0.02), [0.02] | -              | -      | (1.40), (0.70)       | (0.71), [0.80]  | (0.07), [0.18] |
| C20:1n9 heicocosanonic acid    | -                    | -              | -              | (0.05) | (0.03)               | -              | -                    | -              |
| C20:2n6 eicosadienonic acid    | -                    | -              | -              | (0.27) | (0.14)               | -              | -                    | -              |
| C20:3n9 dhomo-γ-linolenic acid | (0.48), [0.25]       | -              | -              | -      | -                    | -              | -                    | -              |
| C20:4n6 arachidonic acid (ARA) | -                    | -              | -              | (2.18) | (1.12), (0.12)       | (0.14), [0.14]  | (0.01), [0.02] |
| C20:3n3 eicosatrienoic acid (ETE) | -                    | -              | -              | (0.13) | (0.07)               | -              | -                    | -              |
| C22:0 behenic acid             | (0.56), [0.52]       | (0.03), [0.06] | -              | (0.14) | (0.07)               | -              | -                    | -              |
| C22:1n9 erucic acid            | -                    | -              | -              | (0.22) | (0.11)               | -              | -                    | -              |
| C20:5n3 eicosapentaenoic acid (EPA) | -                    | -              | -              | (28.38) | (1.92)               | -              | -                    | -              |
| C23:0 tricosanoic acid         | -                    | -              | -              | (0.03) | (0.02)               | -              | -                    | -              |
| C22:2n6 docosadienonic acid    | -                    | -              | -              | (0.03) | (0.01)               | -              | -                    | -              |
| C24:0 lignoceric acid          | (0.23), [0.22]       | (0.02), [0.03] | -              | (0.06) | (0.03)               | -              | -                    | -              |
| C24:1n9 nervonic acid          | -                    | -              | -              | (0.86) | (0.43)               | -              | -                    | -              |
| C22:5n3 docosapentaenoic acid (DPA) | -                    | -              | -              | (1.93) | (0.18)               | -              | -                    | -              |
| C22:6n3 docosahexaenoic acid (DHA) | -                    | -              | -              | (23.32) | (2.10)               | -              | -                    | -              |
| SFA                            | (9.2), [8.51]        | (0.18), [0.47] | (47.8), [57.21] | (2.31) | (22.1), (1.05)       | (38.3), [40.74] | (1.19), [6.77] |
| MUFA                           | (47.4), [45.33]      | (0.20), [2.78] | (42.3), [53.00] | (1.46) | (16.5), (1.75)       | (50.0), [55.28] | (0.43), [8.91] |
| PUFA                           | (43.4), [39.27]      | (0.07), [2.46] | (9.9), [11.79] | (0.87) | (59.4), (1.79)       | (11.7), [12.28] | (0.80), [2.20] |

(*) – relative concentration (% FAi from total FAs); [**] – absolute concentration (g FAi/100 g); R - limit of reproducibility.

\[ R = 2.8 \times SD(R) \]
Accuracy

The accuracy of the method was achieved by using a NIST certified reference material, peanut butter (SRM®2387). The fatty acids composition was evaluated by applying the developed method and comparing the results with the certified values of SRM®2387. The determined fat content of SRM®2387 was compared to the certified value. This consists in comparing the absolute difference between the certified value and the measured value ($\Delta m$, with the expanded uncertainty ($U_A$) (LINSINGER, 2010).

As shown in Table 4, the experimentally measured values did not show significant differences ($p > 0.05$) from the NIST-certified values except for the C16:1n7 acid, where the value found was below LOQ (21.92 µg/mL = 0.13 g/100 g) and higher than LOD (7.23 µg/mL = 0.04 g/100 g). Results are presented as mean of repeated measurements (n = 6).

Recovery

Recovery was calculated for sunflower oil, peanut butter and lard. The obtained mean recoveries for the FAME/FAi determined in the food matrices studied is shown in Table 5. The addition of an internal standard has been largely used in the analysis of fatty acids, as it allows the expression of results as weight (g/100 g). This method is less susceptible to errors, because the internal standard and the sample are injected together. Recovery determined using the two internal standards, TAG-IS and FAME-IS, complies with the required conditions (100% ± 20%). When recovery is not between these limits, the origin of the problem could be due to shorter reaction time, lower reaction temperature, incomplete transesterification of internal standard, partial degradation or loss due to evaporations of the internal standard. If the recovery values are higher or lower than the required values, the transesterification performance is not considered optimal and the entire procedure should be repeated in order to obtain satisfactory results (CRUZ-HERNANDEZ, 2013). All recovery values were above 80%, indicating that the method used is effective and can be successfully applied in the quantification of FAME/FAi, from vegetable and/or animal oil/fat samples.

Table 4. Accuracy test results of SRM®2387 (peanut butter)

| Fat and FAi profile | Concentration (g FAi/100 g) |
|--------------------|-----------------------------|
|                    | Certified values | Measured values (n= 6) |
| Fat                | 51.60 ± 1.4       | 51.72 ± 0.49          |
| Saturated fatty acids (SFA) | 10.4 ± 0.2     | 10.6 ± 0.56          |
| C14:0 myristic acid | 0.024 ± 0.002    | 0.022 ± 0.00          |
| C16:0 palmitic acid | 4.94 ± 0.15      | 5.09 ± 0.24          |
| C18:0 stearic acid | 2.13 ± 0.008     | 2.18 ± 0.12          |
| C20:0 arachidic acid | 0.710 ± 0.029  | 0.685 ± 0.05         |
| C22:0 behenic acid | 1.81 ± 0.08      | 1.89 ± 0.18          |
| C24:0 lignoceric acid | 0.781 ± 0.044    | 0.765 ± 0.08         |
| Monounsaturated fatty acids (MUFA) | 24.4 ± 0.9   | 26.5 ± 1.3          |
| C16:1n7 palmitoleic acid | 0.044 ± 0.010  | < LOQ                 |
| C18:1n9 oleic acid | 23.38 ± 0.90     | 25.44 ± 1.26         |
| C18:1n7 vaccenic acid | 0.255 ± 0.016 | 0.277 ± 0.01         |
| C20:1n9 gadolinic acid | 0.643 ± 0.031  | 0.622 ± 0.044        |
| Polyunsaturated fatty acids (PUFA) | 13.2 ± 0.4  | 14.0 ± 0.7          |
| C18:2n6 linoleic acid (LA) | 13.15 ± 0.41 | 13.93 ± 0.69        |
| C18:3n3 α-linolenic acid (ALA) | 0.030 ± 0.001 | 0.033 ± 0.004      |

Table 5. Mean recoveries for the food samples

| Food sample | Internal standards used | Number of samples, n | Recovery ± SD, % |
|-------------|-------------------------|----------------------|------------------|
|             | TAG-IS                  | FAME-IS              |                  |
| Sunflower oil | C15:0                  | C23:0 | 15 | 89.86 ± 6.21 |
| Peanut butter  | C15:0                  | C23:0 | 14 | 94.30 ± 13.66 |
| Lard        | C11:0                  | C23:0 | 14 | 96.16 ± 14.37 |
Similar results with the ones presented in table 6 were obtained by Petrovic (2010) who varied the sample weight of pumpkin oil and showed that there is no significant change when varying this parameter.

The FA composition of sunflower oil was analyzed by both CF and RRF quantification approaches. The results obtained (Table 6) by the temperature variation of the detector (245°C, 255°C) did not show significant

### Table 6. Robustness test results for weight and detector temperature variation for sunflower oil

| Compound name                | Concentration (*) | Sample weight (± 0.005 g) | Detector temperature (± 5°C) |
|------------------------------|-------------------|---------------------------|------------------------------|
| C14:0 myristic acid          | (0.05), [0.05]    | (0.06), [0.06]            | (0.05), [0.06]               |
| C16:0 palmitic acid          | (5.60), [5.92]    | (5.65), [5.74]            | (5.58), [6.00]               |
| C16:1n7 palmitoleic acid     | (0.11), [0.12]    | (0.12), [0.13]            | (0.12), [0.13], (0.11), [0.12], (0.12), [0.13] |
| C18:0 stearic acid           | (2.24), [2.40]    | (2.25), [2.32]            | (2.30), [2.45]               |
| C18:1n9 oleic acid           | (47.63), [47.44]  | (47.42), [47.42]          | (47.49), [47.62]             |
| C18:2n6 linoleic acid (LA)   | (43.35), [43.42]  | (43.42), [43.42]          | (43.45), [43.37]             |
| C18:3n3 α-linolenic acid (ALA)| (0.05), [0.05]  | (0.05), [0.05]            | (0.05), [0.05], (0.05), [0.05] |
| C20:0 arachidic acid         | (0.16), [0.18]    | (0.17), [0.18]            | (0.18), [0.18], (0.16), [0.18] |
| C20:1n9 gondolic acid        | (0.15), [0.16]    | (0.14), [0.16]            | (0.16), [0.17], (0.15), [0.16] |
| C22:0 behenic acid           | (0.49), [0.53]    | (0.52), [0.53]            | (0.55), [0.56], (0.47), [0.52] |
| C24:0 lignoceric acid        | (0.16), [0.18]    | (0.19), [0.20]            | (0.19), [0.20], (0.16), [0.18], (0.15), [0.16] |
| SFA                          | (8.7), [9.25]     | (8.8), [9.03]             | (8.8), [9.44], (8.8), [9.29], (8.7), [9.21] |
| MUFA                         | (47.9), [52.64]   | (47.7), [50.59]           | (47.7), [52.65], (47.7), [52.49], [52.23] |
| PUFA                         | (43.4), [43.44]   | (43.5), [43.45]           | (43.5), [43.5], (43.4), [44.99] |

(*) – relative concentration (% FAi from total FAs); [**] – absolute concentration (g FAi/100 g)

### Table 7. Robustness test results for the fat extraction approaches from SRM®2387 (peanut butter)

| Compound name                | Concentration [**] (n= 4) | Certified values | Measured values | ISO 17189 (2003) | Soxhlet extractor | Soxhlet hot extractor | Certified values | Measured values | ISO 17189 (2003) | Soxhlet extractor | Soxhlet hot extractor |
|------------------------------|---------------------------|-------------------|-----------------|------------------|------------------|-------------------|-------------------|-----------------|------------------|------------------|---------------------|
| C14:0 myristic acid          | 0.024 ± 0.002             | 0.022 ± 0.002     | 0.024 ± 0.002   | 0.022 ± 0.01     |
| C16:0 palmitic acid          | 4.94 ± 0.15               | 4.94 ± 0.07       | 5.12 ± 0.05     | 5.04 ± 0.20      |
| C16:1n7 palmitoleic acid     | 0.044 ± 0.010             | < LOQ             | < LOQ           | < LOQ            |
| C18:0 stearic acid           | 2.13 ± 0.08               | 2.10 ± 0.09       | 2.15 ± 0.11     | 2.15 ± 0.19      |
| C18:1n9 oleic acid           | 23.38 ± 0.9               | 24.65 ± 0.66      | 25.23 ± 0.55    | 24.92 ± 1.23     |
| C18:1n7 vaccenic acid        | 0.255 ± 0.016             | 0.267 ± 0.011     | 0.257 ± 0.009   | 0.273 ± 0.023    |
| C18:2n6 linoleic acid (LA)   | 13.15 ± 0.41              | 13.52 ± 0.51      | 14.07 ± 0.41    | 14.09 ± 0.90     |
| C18:3n3 α-linolenic acid (ALA)| 0.030 ± 0.001            | 0.031 ± 0.003     | 0.028 ± 0.001   | 0.029 ± 0.001    |
| C20:0 arachidic acid         | 0.710 ± 0.029             | 0.663 ± 0.054     | 0.678 ± 0.050   | 0.696 ± 0.091    |
| C20:1n9 gondolic acid        | 0.643 ± 0.031             | 0.602 ± 0.047     | 0.604 ± 0.054   | 0.628 ± 0.071    |
| C22:0 behenic acid           | 1.81 ± 0.08               | 1.84 ± 0.18       | 1.90 ± 0.16     | 1.97 ± 0.30      |
| C24:0 lignoceric acid        | 0.781 ± 0.044             | 0.741 ± 0.082     | 0.789 ± 0.068   | 0.85 ± 0.164     |
| SFA (g/100 g)                | 10.4 ± 0.2                | 10.3 ± 0.5        | 10.7 ± 0.4      | 10.7 ± 0.9       |
| MUFA (g/100 g)               | 24.4 ± 0.9                | 25.5 ± 0.7        | 26.1 ± 0.6      | 25.8 ± 1.3       |
| PUFA (g/100 g)               | 13.2 ± 0.4                | 13.5 ± 0.5        | 14.1 ± 0.4      | 14.1 ± 0.9       |

[**] – absolute concentration (g FAi/100 g)
differences ($p > 0.05$) in FAE composition in the test sample compared to the working procedure variant ($250^\circ$C).

The composition in FA$_i$ of peanut butter in the three fat extraction variants was compared with the certified values of the certified reference material (LINSINGER, 2010). The obtained results (Table 7) showed that there are no significant differences ($p > 0.05$) between the measurement results and the certified value ($\Delta m \leq U_{\alpha}$) and demonstrated that the method is robust.

**Conclusions**

A precise and robust method for the simultaneous determination of FAME$_i$/FA$_i$ from oils/fats of vegetable and/or animal origin has been developed and validated. The proposed method allows FAME$_i$/FA$_i$ analysis by two quantification approaches. The first is based on correction factors, by simultaneous determination of max. 40 FAME (relative concentration expressed as weight % of total identified FAME) and/or as the sum of SFA, MUFA, PUFA from oils/fats of vegetable and/or animal origin. The second one is based on the relative response factors, by simultaneous determination of max. 26 FAME (absolute concentration expressed in g/100 g), as individual FAME$_i$/FA$_i$ and/or as the sum of SFA, MUFA, PUFA from oils/fats of vegetable and/or animal origin, using GC-MS. The quality of the calibration curves ($R^2$), detection limit, quantification limit, precision, accuracy, recovery tests, robustness of the developed method have provided sufficient evidence suggesting that the proposed method is an appropriate alternative for determination of FAME$_i$/FA$_i$ composition of oils/fats of vegetable and/or animal origin, with a view to nutritional characterization of foods.

**Abbreviations**

CF, correction factors; FA, fatty acids; FAME, fatty acid methyl esters; IS, internal standard; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Rec., recovery; RRF, relative response factors; RT, retention time; SFA, saturated fatty acids.

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