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# Fast derivatization of fatty acids in different meat samples for gas chromatography analysis

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ABSTRACT

In order to analyze the composition of fatty acids employing gas chromatography as the separation method, a derivatization of lipids using esterification and transesterification reactions is needed. The methodologies currently available are time consuming and use large amounts of sample and reagents. Thus, this work proposes a new procedure to carry out the derivatization of fatty acids without the need for prior extraction of lipids. The use of small amounts of sample (100 mg) allows the analysis to be performed in specific parts of animals, in most cases without having them slaughtered. Another benefit is the use of small amounts of reagents (only 2 mL of NaOH/Methanol and $H_2SO_4$/Methanol). The use of an experimental design procedure (Design Expert software) allows the optimization of the alkaline and acid reaction times. The procedure was validated for five minutes in both steps. The method was validated for bovine fat, beef, chicken, pork, fish and shrimp meats. The results for the merit figures of accuracy (from 101.07% to 109.18%), precision ($RSD_{intra\text{-}day}$ (from 0.65 to 3.93%), $RSD_{inter\text{-}day}$ (from 1.57 to 5.22%)), linearity ($R^2 = 0.9864$) and robustness confirmed that the new method is satisfactory within the linear range of 2–30% of lipids in the sample. Besides the benefits of minimizing the amount of samples and reagents, the procedure enables gas chromatography sample preparation in a very short time compared with traditional procedures.



## 1. Introduction

The analysis of fatty acids in food, both qualitative and quantitative, using gas chromatography (GC) as a separation technique requires the use of sample preparation procedures. The use of lipid extraction followed by derivatization to methyl esters by esterification and/or transesterification reactions, is necessary due to the high boiling points of the substances involved (fatty acids, triacylglycerols, phospholipids, and others) [1]. After conversion of these compounds to methyl esters, the reduction in boiling point promotes their volatilization, improving the chromatographic resolution [2].

The methodology proposed by Bligh and Dyer [3] is widely used for the extraction of lipids from food (more than 34,000 citations according to the Web of Knowledge), and it is carried out at room temperature in order to maintain the integrity of the lipid material, avoiding oxidation reactions. However, this methodology, established for specific samples (fish with 1% lipid and 80% moisture), is currently used for any type of food sample. Other drawbacks of Bligh and Dyer extraction are the use of large quantities of sample (100 g per replicate) and the use of large quantities of solvents, such as methanol and chloroform (200 mL of each per replicate). Moreover, extensive sample manipulation (agitation, phase separation, filtration and evaporation of solvent) increases the likelihood of error in the results.

After extraction, the lipids must be derivatized to methyl esters and to this end many methods can be adopted [4–9]. The choice of procedure depends on characteristics such as percentage of acidity and moisture. For lipids with lower acidity and moisture levels of 1%, alkaline catalysis methodologies are recommended as they are faster, more cost-effective and do not use heating [10]. Where these conditions are not applicable, acid catalysis methodologies are recommended, being the most often used for food analysis. The esterification/transesterification proposed by Hartman and Lago [4], modified by Maia and Amaya later [5], is widely used, and proposes the use of fatty acid saponification reactions (alkaline catalysis) followed by esterification/transesterification (acid catalysis). However, this procedure has the limitation that samples rich

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in polyunsaturated fatty acids can easily undergo lipid degradation, mainly due to the use of high temperatures [9,11].

Therefore, this work proposes a new procedure for fatty acid derivatization and separation in gas chromatography, without the need for prior extraction of lipids and using low amounts of samples and reagents.

## 2. Experimental

### 2.1. Reagents and materials

A fatty acid methyl esters standard mixture (FAMEs 189-19) and methyl tricosanoate (23:0me) were purchased from Millipore-Sigma (St. Louis, MO, USA). The other reagents such as chloroform, methanol, *n*-heptane, sulfuric acid, and hydrochloric acid were also purchased from MilliporeSigma (Darmstadt, Germany) and used without further purification.

Bovine fat located in the longissimus dorsi muscle, boneless top loin steak, chicken breast meat, pork steak, Piracanjuba fish (*Brycon orbignyanus*) and Seven-beards shrimp (*Xiphopenaeus kroyeri*), were obtained from a local food store (Maringa, Parana, Brazil). Samples were packed under vacuum and kept in a freezer at −18 °C. Before carrying out the analysis, samples were triturated in a knife mill until they became homogeneous. During food analysis it is common to use larger sample sizes in order to reduce the effect of non-homogeneous material. When using small amounts of samples for the analysis, it is necessary to have a homogeneous test material before sampling.

### 2.2. Traditional procedure of lipid extraction and esterification/transesterification

Lipid extraction was conducted according to Bligh and Byer [3]. Primarily, about 100 g of triturated sample was weighed, then 100 mL of chloroform and 200 mL of methanol were added and homogenized in a Waring Blendor for 2 min. After that, 100 mL of chloroform was added and then blended for 30 s. Next, 100 mL of water was added and blended for 30 s. The mixture was filtered through Whatman paper no 1 with vacuum, and the filtrate was transferred to a separatory funnel. After separation, the chloroform phase was collected and the solvent evaporated by using a rotary evaporator. It is important to remember that the Bligh and Dyer method was established for fish with 80% water and 1% fat, so whenever possible, these factors need to be adjusted.

The esterification/transesterification reactions of the fatty acids were carried out according to the methodology of Maia and Amaya [5]. Primarily, about 100 mg of lipid extract was weighed in a test tube, after that 4 mL of NaOH/MeOH 0.5 mol $L^{-1}$ was added. Then, the tube was heated in a water bath for 5 min, with subsequent cooling under running water. About 5 mL of esterifying reagent ($NH_4Cl/H_2SO_4$/MeOH) was added to the same tube, and the system was once again heated in a water bath for 5 min and cooled under running water. The mixture was transferred to a separatory funnel, then it was added 4 mL of saturated NaCl solution and vigorously shaken for 30 s. After that, 5 mL of hexane was added and vigorously shaken for 30 s. Finally, the internal standard (23:0me) was added, and after phase separation the supernatant was collected and injected on GC.

### 2.3. New procedure of lipid extraction and esterification/transesterification

Primarily, about 100 mg of triturated sample was weighed in a 10 cm test tube. Then, it was added 2.0 mL of NaOH (1.5 mol $L^{-1}$ in methanol). After that, the sample was crushed with a glass stirring rod to form a thin film in order to increase the contact surface. Then, test tubes were placed in an ultrasonic bath (using different reaction times according to an experimental design, Section 2.4). After the alkaline reaction time was over, 2.0 mL of $H_2SO_4$ or HCl (1.5 mol $L^{-1}$ in methanol for both cases) was added, and the test tube was once again placed in the ultrasonic bath during the specific times which were determined by the same experimental design mentioned above. Then, 1 mL of *n*-heptane was added, and the tubes were vortexed for 30 s and later centrifuged at 2000 rpm for 1 min. After that, 500 μL of internal standard (23:0me) of concentration 1 mg/mL was added, the upper phase was collected and injected on GC.

The procedure was performed in an Eco-Sonics Q 5.9/25 ultrasonic bath model (Unique, Sao Paulo, Brazil) with 165 W potency and 25 kHz frequency.

### 2.4. Experimental design

A central composite rotary design generated by Design Expert 7 software was used to evaluate the influence of reaction time with alkali (NaOH in methanol) and with acid, as well as the type of acid ($H_2SO_4$ and HCl in methanol; categorical variable), both in ultrasound. The alkali time levels −1 and +1 were 6 and 11 min, and acid time levels −1 and +1 were 8 and 15 min, respectively. The axial points (±α) given for the rotational system ($k < 5$) were ±1.4142 and they were used in order to extend the response surface. Five replicates were performed at the center point (0), totaling 26 experiments.

### 2.5. Gas chromatography with flame ionization detector analysis (GC-FID)

Chromatographic analysis was carried out on a Thermo Scientific GC equipped with a flame ionization detector (FID), a split/splitless injector and a fused silica capillary column CP-7420 (Select FAME, 100 m size, 0.25 mm internal diameter and 0.25 μm film thickness of the cyanopropyl stationary phase). The operation parameters were: column temperature of 165 °C for 18 min and then ramped to 235 °C (4 °C $min^{-1}$) for 20 min. The injector and detector temperatures were kept at 230 and 250 °C, respectively. The gas flow rates used were 1.2 mL $min^{-1}$ for the carrier gas ($H_2$), 30 mL $min^{-1}$ for the make-up gas ($N_2$) and 30 and 300 mL $min^{-1}$ for the FID gas $H_2$ and synthetic air, respectively. The sample was injected in split mode with a 40:1 split ratio. The injection volume was 1 μL. FAMEs were identified by comparison of the retention times of the sample constituents with Sigma FAMEs. Theoretical FID correction factor values were used in the calculations to obtain fatty acid concentration values according to Visentainer [12] and the fatty acid contents were calculated as mg $g^{-1}$ of sample. The FID correction is necessary because the magnitude of the signal generated by detector is proportional to the number of active carbon atoms which are bonded to hydrogen atoms. However, there are intrinsic factors of a FAME molecule that alter FID response, like the presence of oxygen, which diminishes such response [13].

### 2.6. Reaction efficiency as assessed by Atmospheric Solids Analysis Probe/Mass Spectrometry (ASAP/MS)

An ASAP (Atmospheric Solids Analysis Probe) coupled to a Xevo ACQUITY® TQD mass spectrometer (Waters®, Milford, MA) was used to analyze the efficiency of the new procedure compared to the traditional methodologies of lipid extraction and esterification/transesterification. A glass capillary (100 mm length and 1.7 mm outer diameter) was first dipped into the sample, and any excess sample was wiped off with a lint-free paper towel. The capillary was then attached to the ASAP probe and loaded directly into the source enclosure of the Xevo TQD/MS. For the 150–500 Da mass