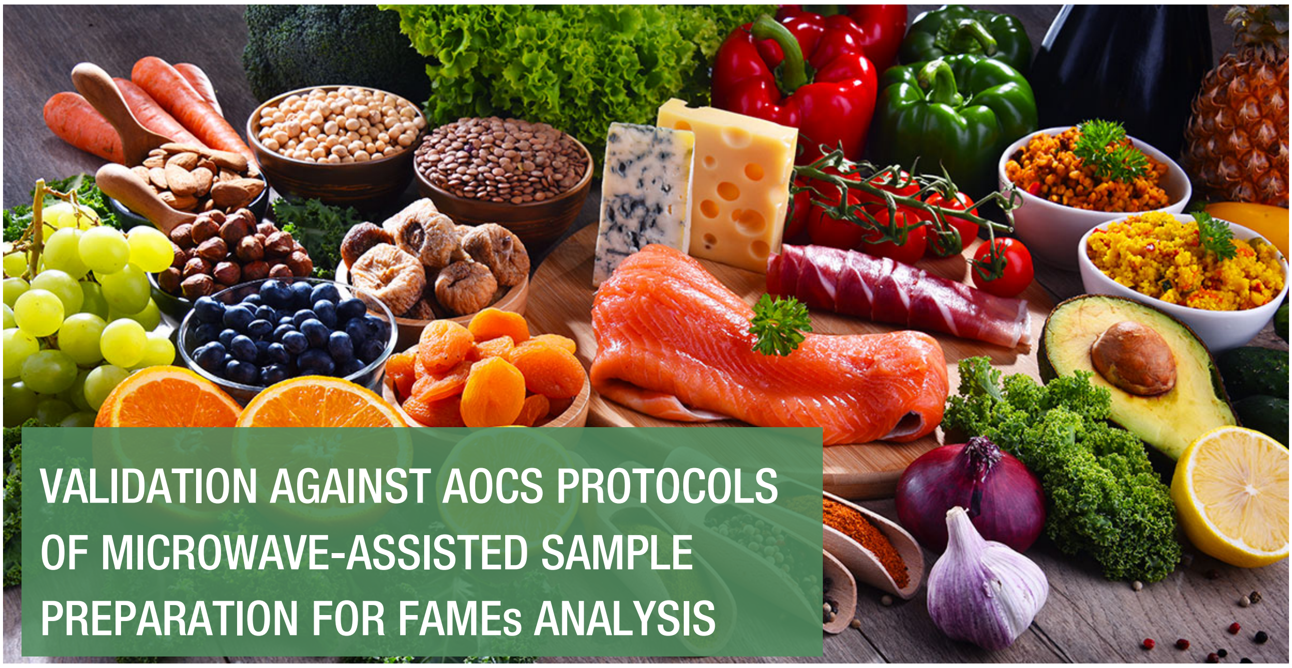


# VALIDATION AGAINST AOCS PROTOCOLS OF MICROWAVE-ASSISTED SAMPLE PREPARATION FOR FAMEs ANALYSIS

Fatty Acid Methyl Ester (FAME) analysis is commonly used to calculate saturated, monounsaturated, and polyunsaturated fat contents in both food and feed industries and related third-party quality control laboratories. A great number of these laboratories follow various AOCS methods, which rely on the use of standard and outdated techniques, usually employing hazardous reagents. FAME protocols based on the Milestone's ETHOS X Microwave-assisted technology are a solid alternative to the conventional technique. This work aims to provide a demonstration of the reliability of this microwave-assisted method against the conventional AOCS procedures.

## INTRODUCTION

In the U.S. and EU, fat determination in food is subjected to regulatory standards that ensure accurate labeling and quality control. In the U.S., FDA and USDA are the organizations in charge of the fat analysis legislation [1], specifying methods based on food type, such as total fat extraction. Similarly, EU follows standardized protocols under EFSA and has adopted the subject under Regulation (EU) No 1169/2011 [2].

One of the most widely used techniques for fat characterization is the conversion of fat to fatty acid methyl esters (FAMEs), which allows detailed profiling of the fatty acid composition through gas chromatography (GC). This process, subject of AOCS methods (AOCS Ce 2b-11 and 2c-11), involves lipid extraction followed by transesterification to form FAMEs, enabling precise measurement of individual fatty acids. Despite its accuracy, traditional FAME analysis methods involve lengthy and solvent-intensive steps, which are not efficient and not the best choice for the environment.

Recent advancements have drawn the attention to microwave-assisted extraction (MAE) as a potential improvement over the conventional method. In fact, MAE reduces both solvent usage and extraction time by applying microwave energy to accelerate lipid extraction and transesterification. This technique has been shown to produce comparable or superior accuracy to standard methods and has the added benefit of lowering operational costs.

The aim of the work presented in this report is the comparison between different ways to get FAMEs profile in different food matrices.

Specifically, we compared two AOCS methods for FAMEs preparation (alkaline and acid conditions) with MAE derivatization conducted on fat extracted with or without hydrolysis, and the innovative all-in-one-stage MAED technique.

Overall, seven different methods on six different food matrices were evaluated, followed by a common analysis by GC (GC x GC)- FID.

## | EXPERIMENTAL

### EQUIPMENT

- Milestone's ETHOS X[3]
- SR-15 easyTEMP extraction rotor
- Aluminum caps
- SFS-24 filtration system
- Vacuum system with condensation module
- Analytical balance (with direct interface to the terminal)
- Conventional methods were performed with Soxhlet extraction apparatus with associated glassware

Figure 1 – Milestone's ETHOS X with SR-15 extraction rotor (left) and RAR-15 evaporation rotor (right).

### SOLVENT AND REAGENTS

Solvents and reagents were purchased from Merck KGaA (Germany). 25% solution of sulphuric acid, 14 % boron trifluoride (BF3) solution in methanol, acidic methanol (1.25 M HCl) and ACS reagent grade cyclohexane, pentane, hexane, petroleum ether, methanol, sodium sulphate anhydrous and sodium hydroxide were used.

### SAMPLES

For this study, certified reference materials and labeled foodstuff (purchased at the grocery store) were used.
The reference materials used were cordon-bleu (RCIL n° 2022-2023-0544) and factory-made pastry (RCIL n° 2022-2023-0446) provided by BIPEA, as they have a certified value of the FAMEs' reference profile.
The commercial foodstuff includes spreadable cream, infant formulas, cheese (gouda) and oat flakes.
The samples of commercial foodstuff should be homogenized before the weighing step, in order to get a representative aliquot of the sample. The sample has been used untreated, avoiding any drying step.

### PROCEDURES FOR EXTRACTION

#### MAHE - Microwave-assisted hydrolysis extraction

Approximately 3 g of sample was directly weighed into the SR-15 eT extraction vessels; 10 mL of sulphuric acid (25%) and 25 mL of cyclohexane were subsequently added, recording its final mass. Magnetic stirring bars were added to each vessel. The SR-15eT was properly assembled. The microwave program is reported in Table 1.

| STEP | TIME | T | POWER | Stirrer |
|---|---|---|---|---|
| 1 | 00:03:00 | 90°C | 1400w | 80% |
| 2 | 00:04:00 | 135°C | 1400W | 80% |
| 3 | 00:40:00 | 135°C | 1400W | 80% |
| Cooling | | | | |

Table 1 – ETHOS X MAEH program

At the end of the program, the SR-15 eT vessels were opened, and the aliquots of the organic phase were transferred into aluminum caps and then weighed. After a fast solvent evaporation using the RAR-15 evaporation rotor, the aluminum caps were newly weighed for total fat determination.
The preparation of FAME was sequentially performed on an aliquot of 100 mg of the extracted residue using both the official derivatization procedure AOCS Ce 2b-11 (MAEH-BF3) and a derivatization using microwave in acid conditions (MAEH-HCl/MeOH) described after.

#### MAE - Microwave-assisted solvent extraction

Approximately 3 g of sample was directly weighed into the SR-15 eT extraction vessels. Then 30 mL of pentane was subsequently added. Magnetic stirring bars were added to each vessel. The SR-15 eT was properly sealed. The microwave program is reported in Table 2.

| STEP | TIME | T | POWER | Stirrer |
|---|---|---|---|---|
| 1 | 00:10:00 | 120°C | 1400W | 80% |
| 2 | 00:40:00 | 120°C | 1400W | 80% |
| Cooling | | | | |

Table 2 –ETHOS X MASE program

At the end of the program, the SR-15 eT vessels were opened. The solution was then filtered into the Milestone SFS-24 on 0.45 μm PTFE filters using sodium sulfate as filtering agent.
The filtered solutions were transferred into the RAR-15 for a fast evaporation. The final residue inside the aluminum caps was weighed for the determination of free fat.

The preparation of FAME was sequentially performed on an aliquot of 100 mg of the extracted residue using both the official derivation procedure AOCS Ce 2b-11 (MASE-BF3) and a derivatization using microwave in acid conditions (MASE-HCl/MeOH) described after.

### PROCEDURES FOR DERIVATIZATION

#### Official procedures for derivatization

Two official methods were tested in this study, described by the AOCS Ce 2b-11 and 2c-11.

**Official method Ce 2b-11** is a direct methylation from foodstuff using alkaline condition.
The sample was weighed according to the method table. 5 mL of NaOH/MeOH (0.5 M) was added and kept in reflux for 15 minutes. Thereafter, 5 ml of 14% $BF_3$ solution in MeOH was added, and maintained under reflux for an additional 2 minutes.
At the end 5 ml of hexane was added and kept under stirring without heating. After cooling, the aliquot of supernatant with FAMEs was collected and sent to GC analysis, in duplicate.

**Official method Ce 2c-11** is a direct methylation from foodstuff using alkali acidic condition.
The sample was weighed according to the method table. 5 mL of acidic methanolic solution (1.25 M) was added and kept in reflux for 15 minutes. Following, 5 ml of NaOH/MeOH (2.3 M) and kept in reflux for a further 15 minutes.
10 ml of 14% $BF_3$ solution in MeOH was added and maintained under reflux for two additional minutes.
At the end 5 ml of hexane was added and kept under stirring without heating. After cooling, the aliquot of supernatant with FAMEs was collected and sent to GC analysis, in duplicate.

#### MAED – Microwave assisted extraction and derivatization

This innovative procedure combines extraction and derivatization, starting directly from sample taking advantage of the microwave heating. 500 mg of sample was directly weighed into the SR-15 eT extraction vessels. Then 10 mL HCl methanolic solution (1.25 ml) and 25 ml of cyclohexane were added. Magnetic stirring bars were added to each vessel. The SR-15 eT was properly sealed. The microwave program is reported in Table 3.

| STEP | TIME | T | POWER | Stirrer |
|---|---|---|---|---|
| 1 | 00:02 00 | 120°C | 1400W | 80% |
| 2 | 00:15:00 | 120°C | 1400W | 80% |
| Cooling | | | | |

Table 3 –ETHOS X MAED program

At the end of the program, the SR-15 eT vessels were opened and, after cooling, the aliquot of supernatant with FAMEs was collected and sent to GC analysis, in duplicate.

### ANALITICAL CONDITIONS

A GC (GC x GC) equipped with a split/splitless injector, autosampler and FID detector were used. The sample injection volume was 1 µl. The two columns used were SepSolve 1D-FAME 20 m x 0.18 mm x 0.1 um for first dimension and SepSolve 2D-FAME 5 m x 0.25 mm x 0.1 um for second dimension.
The detector was maintained at 250°C. A two-step ramp oven program was used.

| RATE (°C/min) | T (°C) | PLATEAUS (min) |
|---|---|---|
| 10 | 40 | 2 |
| 11 | 250 | 2 |

Table 4 – GC oven program

Helium was used as carrier gas.

## I RESULTS AND DISCUSSION

In this study, we aimed to clarify the contribution of microwave technology in all the steps of sample preparation of FAMEs, that are extraction of fat and derivatization.
Extraction of fat is not a mandatory step, but in control laboratories it is an essential parameter to be determined. Therefore, for such laboratories it is certainly beneficial to adopt a sample preparation device able to perform both fat determination and fatty acid profiling through derivatization and GC analysis. Fat extraction can be either conducted with hydrolysis (MAEH) to give an indication of Total Fat parameter, or with only solvent extraction (MASE) to give an indication of the Free or Crude Fat parameter.
Consequently, all the six chosen matrices were tested both with one-step direct derivatization (AOCS methods and MAED) and two-steps procedure including the preliminary extraction.
The FAMEs were then assessed using 2D dimensions GC, which helps to discriminate the different FAMEs with a FID detector. All the GC plots were integrated, and the FAMEs profile were calculated, considering sums of saturated (SFA) mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fats.

## One-step methods comparison

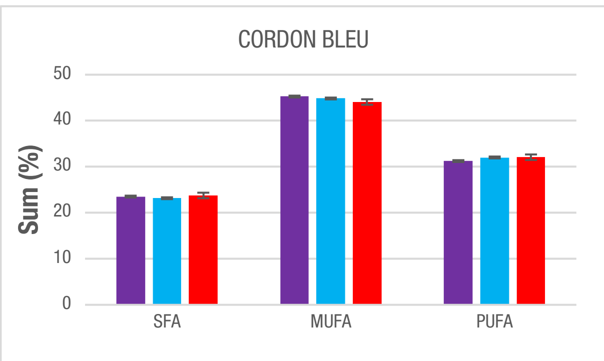
The following tables and graphs compare the single-step procedures to obtain FAMES: the two AOCS methods and MAED microwave method.

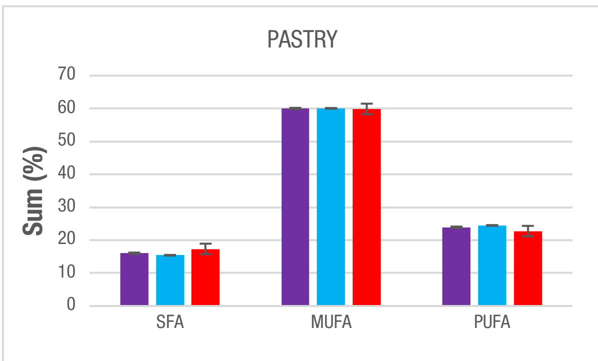
| SFA | REFERENCE VALUE (%) | AOCS Ce 2c-11 (%) | AOCS Ce 2b-11 (%) | MAED (%) |
|---|---|---|---|---|
| CORDON BLEU | 23.6 ± 0.2 | 23.5 ± 0.2 | 23.2 ± 0.1 | 23.8 ± 0.1 |
| PASTRY | 15.1 ± 0.2 | 16.0 ± 0.02 | 15.4 ± 0.1 | 17.4 ± 0.2 |
| CHEESE | - | 66.5 ± 0.01 | 66.9 ± 0.01 | 66.3 ± 0.01 |
| INFANT FORMULA | - | 18.7 ± 0.2 | 19.0 ± 0.2 | 19.4 ± 0.03 |
| SPREAD CREAM | - | 19.7 ± 0.03 | 22.3 ± 2.8 | 19.6 ± 0.03 |
| OAT | - | 19.3 ± 0.3 | 17.0 ± 1.3 | 22.8 ± 0.1 |

| MUFA | REFERENCE VALUE (%) | AOCS Ce 2c-11 (%) | AOCS Ce 2b-11 (%) | MAED (%) |
|---|---|---|---|---|
| CORDON BLEU | 44.1 ± 0.2 | 45.3 ± 0.02 | 44.8 ± 0.2 | 44.1 ± 0.6 |
| PASTRY | 60.4 ± 0.4 | 60.0 ± 0.2 | 60.1 ± 0.1 | 59.9 ± 1.6 |
| CHEESE | - | 30.5 ± 0.1 | 30.2 ± 0.04 | 30.7 ± 0.1 |
| INFANT FORMULA | - | 63.9 ± 0.2 | 63.4 ± 0.2 | 62.9 ± 0.1 |
| SPREAD CREAM | - | 63.3 ± 0.1 | 66.7 ± 3.8 | 62.5 ± 0.02 |
| OAT | - | 39.2 ± 0.4 | 35.8 ± 2.9 | 34.9 ± 0.2 |

| PUFA | REFERENCE VALUE (%) | AOCS Ce 2c-11 (%) | AOCS Ce 2b-11 (%) | MAED (%) |
|---|---|---|---|---|
| CORDON BLEU | 32.0 ± 0.1 | 31.2 ± 0.2 | 32.0 ± 0.2 | 32.1 ± 0.6 |
| PASTRY | 24.4 ± 0.4 | 23.9 ± 0.2 | 24.5 ± 0.002 | 22.8 ± 1.5 |
| CHEESE | - | 2.9 ± 0.1 | 2.9 ± 0.05 | 3.0 ± 0.002 |
| INFANT FORMULA | - | 17.4 ± 0.02 | 17.7 ± 0.01 | 17.8 ± 0.1 |
| SPREAD CREAM | - | 17.1 ± 0.03 | 18.0 ± 7.8 | 17.9 ± 0.003 |
| OAT | - | 41.5 ± 0.1 | 47.2 ± 4.2 | 42.4 ± 0.1 |

Tables 5-7 – Results of saturated (SFA) mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fats in the six matrices, expressed as sum, for the single-step methods.



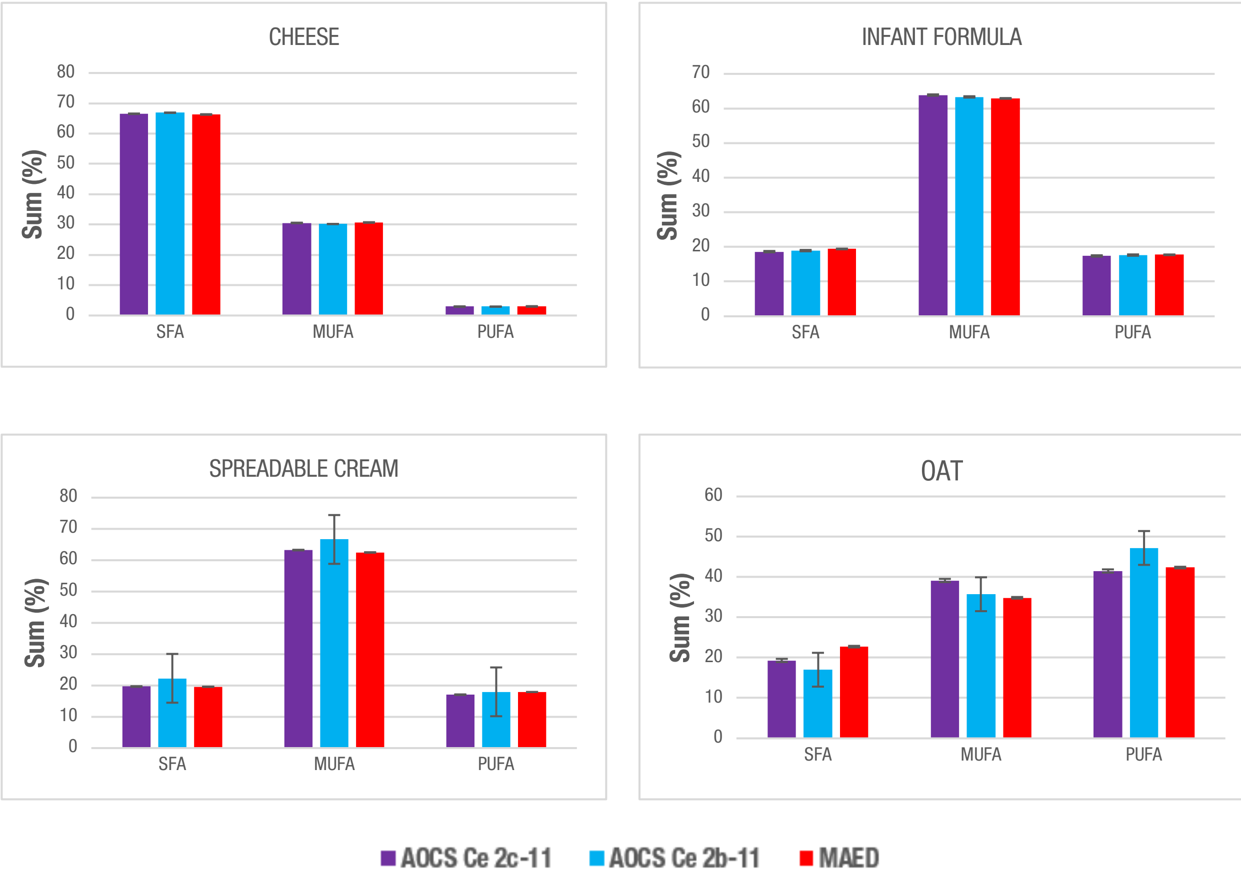



Figure 2-7 – Histograms of comparison for sum of fatty acids for the three evaluated one-step methods

*Two-step methods comparison*

The two common ways to perform fat determination are a simple solvent extraction and a solvent extraction preceded by hydrolysis (commonly done with sulfuric acid). Both these methods can be performed efficiently using a microwave. In addition, two different ways to conduct derivatization were explored: the first one in a simple Soxhlet using $BF_3$ as catalyst, while the second one in the microwave using acidic methanol (HCl/MeOH), a less hazardous derivatization agent.

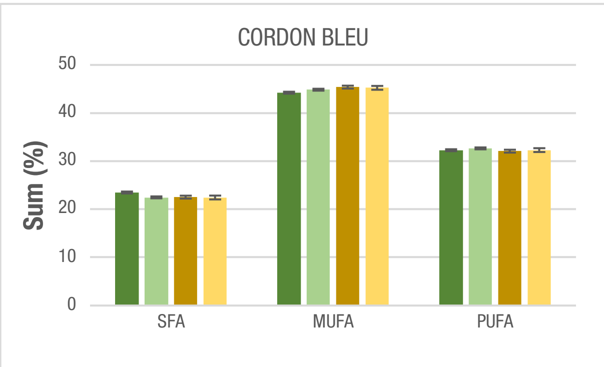
| *SFA* | REFERENCE VALUE (%) | MAEH-HCl/MeOH (%) | MAEH-$BF_3$ (%) | MASE-HCl/MeOH (%) * | MASE-$BF_3$ (%) |
|---|---|---|---|---|---|
| CORDON BLEU | 23.6 ± 0.2 | 23.5 ± 0.2 | 22.5 ± 0.1 | 22.5 ± 0.2 | 22.5 ± 0.3 |
| PASTRY | 15.1 ± 0.2 | 16.1 ± 0.1 | 15.6 ± 0.01 | 14.8 ± 0.1 | 14.1 ± 0.2 |
| CHEESE | - | 66.5 ± 0.5 | 65.6 ± 0.2 | 66.1 ± 0.05 | 67.5 ± 0.5 |
| INFANT FORMULA | - | 19.4 ± 0.2 | 18.7 ± 0.02 | 17.7 ± 0.1 | 17.3 ± 0.1 |
| SPREAD CREAM | - | 19.0 ± 0.2 | 19.1 ± 0.05 | 19.5 ± 0.1 | 19.0 ± 0.2 |
| OAT | - | 21.6 ± 0.02 | 20.6 ± 0.2 | 18.7 ± 0.2 | 18.5 ± 0.2 |

| MUFA | REFERENCE VALUE (%) | MAEH-HCl/MeOH (%) | MAEH-BF$_3$ (%) | MASE-HCl/MeOH (%) * | MASE-BF$_3$ (%) |
|---|---|---|---|---|---|
| CORDON BLEU | 44.1 ± 0.1 | 45.3 ± 0.02 | 44.8 ± 0.1 | 44.2 ± 0.1 | 44.9 ± 0.1 |
| PASTRY | 60.4 ± 0.4 | 59.4 ± 0.03 | 59.8 ± 0.04 | 60.9 ± 0.04 | 61.3 ± 0.5 |
| CHEESE | - | 30.4 ± 0.5 | 31.5 ± 0.2 | 31.0 ± 0.1 | 30.1 ± 0.5 |
| INFANT FORMULA | - | 63.0 ± 0.1 | 63.3 ± 0.03 | 64.4 ± 0.05 | 64.3 ± 0.1 |
| SPREAD CREAM | - | 63.0 ± 0.3 | 62.8 ± 0.1 | 62.6 ± 0.01 | 62.8 ± 0.02 |
| OAT | - | 37.2 ± 0.2 | 37.9 ± 0.1 | 39.9 ± 0.1 | 41.0 ± 0.3 |

| PUFA | REFERENCE VALUE (%) | MAEH-HCl/MeOH (%) | MAEH-BF$_3$ (%) | MASE-HCl/MeOH (%) * | MASE-BF$_3$ (%) |
|---|---|---|---|---|---|
| CORDON BLEU | 23.6 ± 0.2 | 23.5 ± 0.2 | 22.5 ± 0.1 | 22.5 ± 0.2 | 22.5 ± 0.3 |
| PASTRY | 15.1 ± 0.2 | 16.1 ± 0.1 | 15.6 ± 0.01 | 14.8 ± 0.1 | 14.1 ± 0.2 |
| CHEESE | - | 3.1 ± 0.01 | 3.0 ± 0.01 | 2.9 ± 0.02 | 2.8 ± 0.02 |
| INFANT FORMULA | - | 17.7 ± 0.1 | 18.0 ± 0.01 | 18.1 ± 0.1 | 18.4± 0.2 |
| SPREAD CREAM | - | 18.0 ± 0.1 | 18.2 ± 0.1 | 17.9 ± 0.1 | 18.2 ± 0.2 |
| OAT | - | 41.2 ± 0.2 | 41.5 ± 0.05 | 41.4 ± 0.2 | 40.5 ± 0.6 |

Table 8-10 – Results of saturated (SFA) mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fats in the six matrices, expressed as sum, for the two-steps methods

* The maximum temperature of extraction was 100 °C for these determinations



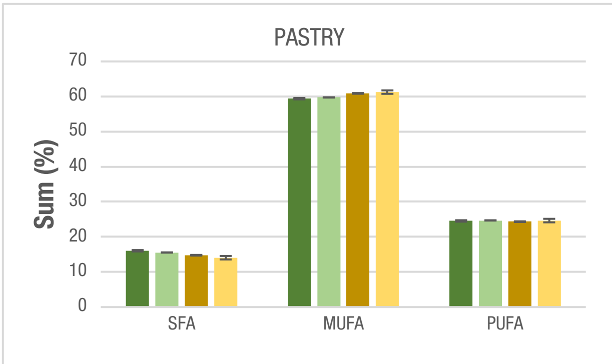




Figure 8-13 – Histograms of comparison for sum of fatty acids for the four evaluated two-steps methods

## CONCLUSIONS

The results extracted for the one-step methods showed that MAED aligned perfectly with the conventional AOCS methods. For the reference material the results for microwave are consistent also with the reference value reported in BIPEA certificates.
The comparison of the two-steps methods MASE and MAEH with the two different derivatization conditions further demonstrates that both microwave approaches give the same outcome, and the derivatization techniques are equivalent.
This means that the MeOH/HCl method for derivatization could be a feasible alternative for the $BF_3$ derivatization, which is hazardous and not safe for the operator and is ultimately more expensive for the laboratory.
Overall, the results obtained employing ETHOS X, both through the single-step MAED or two-steps MASE and MAEH methods, showed that microwaves technology is the best one to obtain reliable and accurate results, matching the results obtained with conventional AOCS methods but using a safer condition and environment for the operator, saving time of analysis and cutting the cost for reagents.
ETHOS X has also the great benefit to be flexible.
In case the laboratory needs to perform total fat or free fat as a common practice, they can then recover a part of the extracted oil to perform a green derivatization with HCl/MeOH in a two-steps approach.
Instead, if the laboratories want to go straight for the fatty acid profile, a one-step MAED approach should be preferred.
The ETHOS X microwave approach for FAME determination has proved to be precise and accurate on a wide working range and on several food matrices.

## ACKNOWLEDGEMENTS
Milestone wants to thank Dr. Donatella Ferrara and prof. Giorgia Purcaro for their precious work carried out to obtain the results reported in this report. [4]

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