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Comprehensive comparison of fatty acid methyl ester profile in different food matrices using microwave-assisted extraction and derivatization methods and comprehensive two-dimensional gas chromatography coupled with flame ionization detection

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ABSTRACT

Analyzing fatty acids provides key insights into fat composition for industrial applications and their implications for nutrition and health. Typically, fatty acid analysis involves extracting lipids from the matrix and converting them into fatty acid methyl esters (FAME) through a derivatization process before gas chromatography (GC) analysis. Either one-step or two-step procedures can be found in the literature and as official methods. In this work, different methods exploiting microwave-assisted processes were compared with two official methods from the American Oil Chemical Society (AOCS). Especially, two types of microwave-assisted extractions were employed: solvent extraction and extraction with hydrolysis. The extracts were derivatized using either BF_3 or a microwave-assisted methanolic hydrogen chloride solution. These combinations of extraction and derivatization methods were compared also with one-step microwave-assisted extraction and derivatization, and two AOCS reference methods, resulting in seven different methods applied to six different food matrices. The performance of the different procedures was compared based on the FAME profile obtained from the comprehensive two-dimensional GC (GC × GC)-FID analysis.

Microwave-assisted processes were shown to be effective, yielding results comparable to the official methods in both the one-step and two-step methods. Moreover, it was shown that the BF_3 derivatization could be safely replaced with microwave-assisted derivatization with methanolic hydrogen chloride, providing equivalent performances while enhancing operator safety and environmental friendliness. Some discrepancies in the FAMEs profile were highlighted for the sample of oats, the only explicitly requiring acidic hydrolysis for lipid extraction. Further studies are required to understand the reasons behind these differences and develop a suitable modified method. In conclusion, all the methods were evaluated for greenness and blueness with two specific tools: AGREEprep and BAGI.

1. Introduction

Fatty acids (FAs) are the most abundant components of the lipid fraction, mainly present in the esterified form (e.g., triglycerides, phospholipids, sterol esters, waxes, etc.). Although FAs determination is usually considered an easy procedure to implement in the laboratory and routinely performed in a simple gas chromatograph (GC) coupled with a flame ionization detector (FID), both sample preparation and chromatographic separation can become critical steps. Due to their acid functional groups and low volatility (especially for the medium-long

Abbreviations: FAME, Fatty Acid Methyl Esters; AOCS, American Oil Chemical Society; AOAC, Association of Official Analytical Chemists; FA, Fatty Acids; PUFA, Polyunsaturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; SFA, Saturated Fatty Acids; MAED, Microwave-assisted extraction and derivatization; MASE, Microwave-assisted solvent extraction; MAEH, Microwave-assisted Hydrolysis Extraction; RFF, reversed fill/flush flow modulator; BAGI, Blue applicability grade index.

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chain FAs in their native form), FAs need to be derivatized before analysis in GC-FID. Among the common derivatization reactions (silanization, acylation, and alkylation), the latter is the most commonly applied to transform FAs in their methyl ester form (FAMEs) [1]. FAMEs are generally formed either in acidic or alkaline conditions on extracted lipid fraction or in a single step combining extraction and derivatization [2–5]. Alkaline-catalyzed derivatization (e.g., using KOH, NaOH, or CH_3NaO) provides the benefit of being faster than acidic conditions, but it requires strictly anhydrous conditions and fails to esterify free FAs, which are not reactive to nucleophilic attack by alcohol or bases. On the other hand, acidic-catalyzed derivatization (e.g., BF_3, HCl, or H_2SO_4 in anhydrous methanol) is suitable for both free and bound FAs. The reaction mechanism involves the protonation of the free FAs to form an oxonium ion, which can undergo an exchange reaction with the alcohol, typically MeOH, and the loss of the proton to give the corresponding ester. In the transesterification mechanism, the initial protonation is followed directly by the addition of the exchanging alcohol [6]. Therefore, a large excess of methanol is required to favor the formation of the FAMEs and the presence of water should be avoided. In the case of acidic-catalyzed derivatization, the choice of the acid used as a catalyst is very important, particularly in the presence of sensitive functional groups, such as cyclic structures or epoxy groups. Particularly aggressive results in the use of H_2SO_4, which also leads to the degradation of polyunsaturated fatty acids (PUFA)[6]. The use of BF_3 as a catalyst is instead probably the most widespread procedure thanks to its efficiency in the derivatization yield, nevertheless, it is unstable (fresh reagent should be prepared before use) and it has been of concern for the formation of artifacts [5–7]. Christie clearly defined it as overrated in his milestone book on lipid analysis [6].

Finally, methanolic hydrogen chloride consists of a solution of 5 % anhydrous hydrogen chloride obtained by bubbling dry gaseous hydrogen chloride into dry methanol. The reagent has a rather good stability if stored refrigerated. A main advantage is that the fatty acids are esterified approximately at the same rate, thus avoiding preferential losses of specific fatty acids during the derivatization step. Nevertheless, it has been reported in the literature that its derivatization yield is low, thus requiring longer reaction times [2,3,6].

Besides the possible selectivity and biases related to the kind of derivatization, the extraction method from solid samples also plays an important role in determining the FAs profile [8]. Nevertheless, most of the studies focused on the comparison of total fat extraction yield rather than the impact on the FAMEs profile [2,9] or only on derivatization procedures [2,3]. A few works investigate the combination of extraction and derivatization, mainly in biomedical studies (from blood or tissue) [3,10–13], and in the food domain method comparisons are reported separately for specific food matrices [14,15].

In the context of routine food analysis, the goal is to have a rapid, robust, green, and possibly matrix-independent method for analyzing FAMEs. To the best of the authors' knowledge, only a few papers report a comprehensive study on different food and related-food matrices proposing a more performing analytical method involving a one-step microwave-assisted extraction and derivatization (MAED) as a reliable alternative to AOCS (American Oil Chemical Society) official method [16,17].

It should be highlighted that when dealing with methods that aim to replace existing official methods, such as the AOAC (Association of Official Analytical Chemists) or AOCS methods for FAMEs analysis, the paradigm to evaluate the results should consider the optimal conditions as the one that provides the most similar results compared to the reference method. Therefore, the evaluation of the ability of the proposed method to replace the official ones should also be done considering other parameters, such as productivity, greenness, sustainability, and easiness. In this regard, microwave-assisted techniques are considered among green strategies to increase the productivity of laboratories [18].

The goal of this work is to compare different extraction and derivatization workflows on different classes of food matrices. Two official AOCS methods, namely AOCS Ce 2b-11 and Ce 2c-11 [19,20], were used as references. Workflows involving the use of MAE involving a dual-step procedure, i.e., extraction (with hexane or after hydrolysis) followed by derivatization (using BF_3 or methanolic hydrogen chloride) after total fat determination and a single-step MAED procedure recently validated [16] were compared, with the references and among them.

The percentage characterization of FAMEs was done using a comprehensive two-dimensional GC (GC × GC) equipped with a reversed fill/flush (RFF) flow modulator and coupled with an FID detector as previously optimized [16,17].

2. Material and methods

2.1. Samples and sample treatment

The samples targeted for the study were cordon bleu, factory-made pastry (later called simply pastry), spreadable cream, oats, infant formula, and cheese (gouda). All products were bought from a local supermarket, except the cordon bleu (RCIL n° 2022-2023-0544) and pastry (RCIL n°2022-2023-0446) that were samples from BIPEA (Paris, France), provider of proficiency testing programs and external reference materials, having certified value for the FAME profile (**Table S1–2**).

2.2. Chemicals and reagents

All chemicals, including solvents (cyclohexane > 99 %, hexane and methanol in HPLC grade), reference standard (Supelco C37 FAME Mix), and derivatization agents, i.e., 14 % boron trifluoride (BF_3) / methanol solution and HCl/MeOH solution 1.25 M, were acquired from Merck KGaA (Darmstadt, Germany).

2.3. Direct extraction and derivatization methods

2.3.1. One-step microwave-assisted extraction and derivatization (MAED)

MAED procedure was previously described [16]. Briefly, 500 mg of the sample was weighed in the SR-12 vessels of an ETHOS X system from Milestone srl (Sorisole, Bergamo, Italy). Subsequently, 10 mL of methanolic hydrogen chloride solution (HCl/MeOH, Merk KGaA) and 25 mL of cyclohexane were added. The vessels were sealed and placed inside the microwave oven, where the samples were heated under continuous stirring with the following temperature program: 120 °C in 2 min, 15 min holding. After cooling, an aliquot of the supernatant containing FAMEs was collected for the following chromatographic analysis. All samples were analyzed in duplicate.

2.3.2. Official method CE 2b-11 by AOCS

For the direct methylation of lipids in food by Alkali Hydrolysis, the AOCS Official Method 2b-11 was applied [19]. Briefly, the samples were weighted following the guidance of the table included in the reference method. Then, 5 mL of NaOH/MeOH (0.5 M) was added and the solution was heated under reflux for 15 min. Afterward, 5 mL of 14 % BF_3 in MeOH was added, maintaining the solution under reflux for an additional 2 min. Finally, 5 mL of hexane was added and then everything was removed from the heating source. After cooling, an aliquot of the supernatant containing FAMEs was collected for the following analysis. All samples were analyzed in duplicate.

2.3.3. Official method CE 2c-11 by AOCS

For the direct methylation of lipids in food by Acid–Alkali Hydrolysis, the AOCS Official Method 2c-11 was applied [20]. The samples were weighted following the guidance of the table included in the reference method. Then, 5 mL of acidic-methanolic solution (1.25 M) was added and heated under reflux for 15 min. Subsequently, 5 mL of NaOH/MeOH (2.3 M) was added and the solution was heated under reflux for an additional 15 min. Then, 10 mL of 14 % BF_3 in MeOH was

added, maintaining the solution under reflux for another 2 min. Finally, 5 mL of hexane was added and then everything was removed from the heating source. After cooling, an aliquot of the supernatant containing FAMEs was collected for the following analysis. All samples were analyzed in duplicate.

2.4. Extraction and derivatization methods with intermediate total fat determination

2.4.1. Microwave-assisted hydrolysis extraction (MAEH)

Approximately 3 g of sample was weighed in the SR-12 extraction vessel of the ETHOS X microwave system; 10 mL of sulfuric acid 25 % and 25 mL of cyclohexane were added. The vessels were closed and heated under continuous stirring with the following temperature program: reaching 90 °C in 3 min, reaching 135 °C in 4 min, and holding 135 °C for 40 min. At the end of the program, the vessels were cooled and the organic phase was evaporated using the RAR 15 evaporation rotor from Milestone connected to a pump and heated for 20 min at 110 °C [21]. The total fat was determined before weighing 100 mg for the following derivatization step. The derivatization step was performed either following the procedure described in **2.3.2** using BF_3 **(MAEH-BF_3)** or the procedure described in **2.3.1** using HCl/MeOH **(MAEH-HCl/MeOH).**

2.4.2. Microwave-assisted solvent extraction (MASE)

Approximately 3 g of the sample was weighed in the SR-12 extraction vessel of the ETHOS X microwave system; 30 mL of hexane was added. The vessels were closed and heated under continuous stirring with the following temperature program: reaching 100 °C in 10 min and holding that temperature for 40 min. At the end of the program, the vessels were cooled and the organic phase was evaporated using the RAR 15 evaporation rotor from Milestone connected to a pump and heated for 20 min at 110 °C. The crude fat was determined before the derivatization step that was performed either following the procedure described in **2.3.2** using BF_3 **(MASE-BF_3)** or the procedure described in 2.3.1 using HCl/MeOH **(MASE-HCl/MeOH).**

2.5. GC × GC-FID instrumentation

All the samples were analyzed by GC × GC-FID. The system (Nexis GC-2030, Shimadzu) was equipped with an AOC-30i autoinjector (Shimadzu). The first dimension (^{1}D) column was a SepSolve 1D-FAMEs 20 m × 0.18 mm × 0.1 μm polar fused silica capillary column; the second dimension column (^{2}D) was SepSolve 2D-FAMEs 5 m × 0.25 mm × 0.1 μm non-polar fused silica capillary column. The two columns were connected through an INSIGHT reversed fill/flush flow (RFF) modulator (SepSolve Analytical Ltd, UK). The oven temperature program was: 40 °C (2 min), to 250 °C (2 min) at 11 °C/min. The temperature program was optimized using the standard mixture Supelco C37 FAMEs. The flow rates were as follows: the first dimension flow was set at 0.5 mL/min, and the second dimension flow at 20 mL/min through an auxiliary flow controller (APC). Helium was used as carrier gas. The modulation period was set at 4 s, including 100 ms of reinjection time. The injection was performed in split mode (1:50 ratio), injecting 1.0 μL at 250 °C. Detection was performed using an FID set at 250 °C (airflow: 300 mL/min, H_2: 30 mL/min; make-up gas: 10 mL/min). Data acquisition frequency was set to 100 Hz. Data was acquired by LabSolution Verison 5.111 and processed by ChromSpace Version 1.5.1 by Markes International Limited (Bridgend, UK).

3. Results and discussion

The one-step MAED method was previously validated in comparison with the AOCS Ce 2b-11 method for a variety of food commodities (n = 11) [16] and edible marine organisms (n = 7) [17]. Nevertheless, from discussions with many routine laboratories, it emerged that, an intermediate step is often needed to determine the total/crude fat of the sample for which the FAME profile is requested. Indeed, fat content is often a crucial parameter in food analysis. Determining total fat, along with SFA, MUFA, and trans-fat content, is essential for dosing ingredients accurately, complying with nutritional standards, producing high-quality, low-fat foods, and optimizing processing conditions to set product quality.

Therefore, we decided to evaluate microwaves'role in the different sample preparation steps, based on the most common procedures used, both for a one-step procedure and a two-step one (i.e., extraction + derivatization). The final results in terms of FAMEs profile were compared to evaluate possible bias due to the different procedures applied against the reference methods. It was decided to also extend the previous comparison of MAED against the AOCS Ce 2b-11 with other matrices and compare it against the AOCS Ce 2c-11. The latter method is specific for some food categories, such as oat-based products, which require acid hydrolysis for the extraction of the total fat.

Therefore, six food matrices complementary to the ones previously used [16,17] were selected, namely: pastry, cordon bleu, oat, cheese (gouda), spreadable cream, and infant formula. All of them were analyzed using both one-step (i.e., the two AOCS and the MAED), and the two-step procedures. In the latter cases, the extraction was energy-assisted by microwave. Once the lipid fraction was extracted, the total/crude fat underwent two acid-based derivatization methods: I) Conventional BF_3 derivatization [19]; II) HCl/MeOH procedure, with the support of the microwave [16]. The complete scheme of the study is reported in Fig. 1.

FAMEs were tentatively identified by comparing their retention time with the Supelco C37 FAME Mix standards, examining their elution on a polar column, and, most importantly, using the position of the FAMEs in a 2D chromatogram [16,22,23]. Indeed, the main advantage of GC × GC is its ability to produce 2D plots that reveal clear chemical group patterns, helping with the identification without the need to use a mass spectrometer as a detector. In this study, the use of GC × GC enabled a detailed and sensitive characterization of FAMEs profile in less than 30 min, and the use of an RFF flow modulator provided good re-injection, resulting in satisfied 2D peak width and symmetry [16,17], and comparable separation to cryogenic modulators. All these advantages are also accompanied by a significant cost reduction since the flow modulator is consumable-free and does not require the use of cryogenic fluids like a cryogenic modulator. Surely, the consumption of carrier gas is higher than 1D analysis, but the cost can be further reduced by moving to hydrogen.

All the GC × GC chromatograms were integrated, and the FAMEs profile was calculated to compare the different procedures. A quantitative comparison was performed on the FAMEs, which were ≥0.1 %. The 2D-GC chromatogram of the infant formula with the identified FAME is reported in **Fig. S1** in supplementary material as an example of the separation obtained.

3.1. One-step methods: comparison of MAED and official methods AOCS CE 2b-11 and CE 2c-11

A first comparison was performed considering only the one-step methods to further confirm the value of the MAED method previously optimized. Fig. 2A shows the summary of the saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) present in the different samples (error bars correspond to the standard deviation (n = 2)) and Fig. 2B shows the radar plot comparison of the different FAMEs. All the values for the FAMEs for all the matrices are reported in **Table S1–6** in the Supplementary material.

The results obtained performing the MAED procedure aligned perfectly with the two official methods, both in terms of total SFA, MUFA, and PUFA (Fig. 2A); as well as looking at the single FAMEs (Fig. 2B) for all the matrices, except the oat. For the particular samples of pastry and cordon bleu, the results are also consistent with those

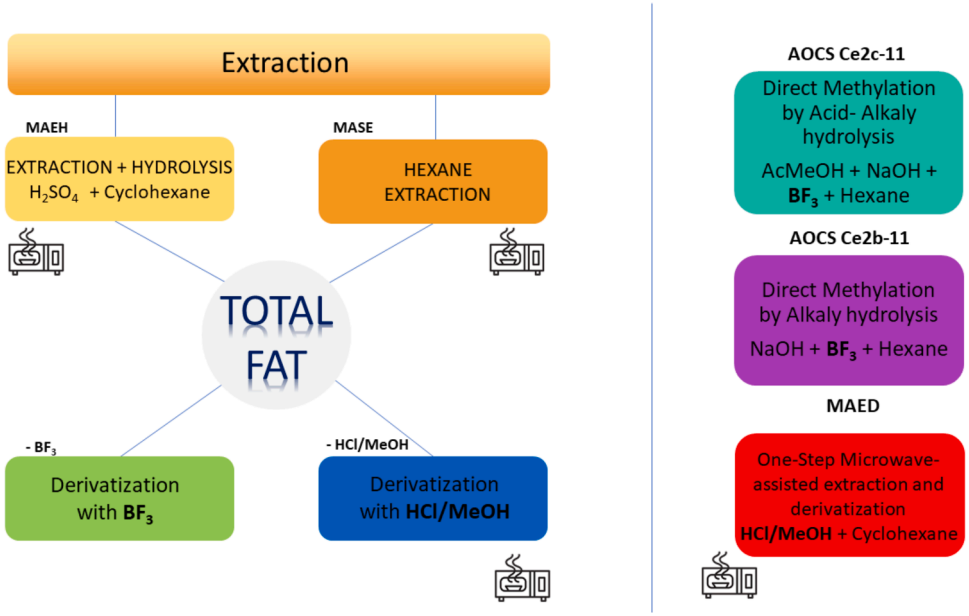

Fig. 1. General scheme of the compared methods.

reported in the BIPEA reports, whose comparison is shown in **Table S1–2** in Supplementary material. These outcomes confirmed the results obtained by Fina et al. [16] on the broad and efficient applicability of the MAED method. At the same time, it is confirmed that the AOCS Ce 2b-11 method fails for oat-based samples, where acidic hydrolysis is needed. The special case of the oat is later discussed (Section 3.3).

3.2. Two-step procedure: comparison of BF_3 and HCL/MEOH derivatizations and MASE and MAEH extractions

From a survey among different laboratories, two main extraction procedures were indicated as the most common, namely, Soxhlet extraction using hexane and the extraction with cyclohexane after previous hydrolysis with H_2SO_4. Both extraction procedures can easily be performed with the support of microwave technology, thus simplifying the procedure, improving the throughput, and reducing the overall energy consumption. Therefore, two equivalent extraction methods, named microwave-assisted solvent extraction (MASE) to replace the Soxhlet and microwave-assisted extraction with hydrolysis (MAEH) were applied. The two extracts obtained were derivatized in two different ways:

1. Using the most used derivatization agents BF_3 (MASE-BF_3 and MAEH-BF_3), without the microwave-assisted derivatization.
2. Using less hazardous derivatization agents, HCl/MeOH (MASE-HCl/MeOH and MAEH-HCl/MeOH), and performing microwave-assisted derivatization.

3.2.1. Determination of total and crude fat

Various methods exist for analyzing fat in food samples [21]. The efficiency of lipid extraction depends on the polarity of both the lipids and the solvent. Polar lipids, such as glycolipids and phospholipids, are more soluble in polar organic solvents (e.g., alcohols). On the other hand, nonpolar lipids (e.g., triacylglycerols, and sterol esters) are more soluble in nonpolar solvents (e.g., hexane and heptane). The Soxhlet method considered the "gold standard" method for fat and oil extractions, is a traditional technique for extracting lipids from foods. It is widely used due to its simplicity and the ability to operate unattended [2,24]. However, it has several disadvantages, including the use of hazardous and flammable organic solvents, the need for more expensive and higher purity solvents, and being time- and solvent-consuming.

Modifications of the methods developed in the 1950s, such as Folch, Bligh and Dyer, have become increasingly common in lipid extraction in food samples due to the use of solvents with different polaries during extraction (a combination of chloroform, MeOH, and water). In both methods, a monophasic solvent system of chloroform and methanol is used to extract and dissolve fats. Subsequently, water is added to create a biphasic system, separating polar and nonpolar compounds into an upper and lower phase, respectively [25,26]. However, even if modifications of Folch and Bligh and Dyer methods are frequently used, they can involve several steps in the sample preparation and high consumption of solvents, especially at the routine level. The use of microwave energy for lipid extraction has been demonstrated to be faster with less solvent involved, giving equivalent results to Soxhlet [21], it has been widely used in sample preparation also elsewhere [21,27,28] and is used also in this work for total fat determination.

Fat was determined in all food matrices using two different methods, MAEH and MASE described in 2.4.1 and 2.4.2, respectively. The first method utilized a combination of sulfuric acid and cyclohexane. This approach allows for a more thorough breakdown of complex food matrices, facilitating the extraction of fat by disrupting both protein and carbohydrate structures. Sulfuric acid acts as a strong acid hydrolyzing agent, while cyclohexane serves as the nonpolar solvent to dissolve the liberated fats. With MAEH what is called the "Total fat" is obtained. The second method is simple solvent extraction, so without the aid of an acid catalyst, the result is a measure of what is called "crude fat" or free fats, which is not bound to complex molecules such as proteins and carbohydrates.

Table 1 reports the value obtained with the two different methods and the value reported on the product labels.

The results of total fat obtained from MAEH closely matched the value reported on the nutritional labels, except for a little overestimation of the total fat in the infant formula and cheese, while the information about the total fat of BIPEA samples (i.e., pastry and cordon bleu) were not disclosed. The variance observed for infant formula and cheese (gouda) can be attributed to the different methods used for the determination of the total fat or to the fact that food products can vary between production batches; in fact, differences in raw materials or production processes can cause variations in fat content. Additionally, changes in fat content may occur also during product storage, affecting

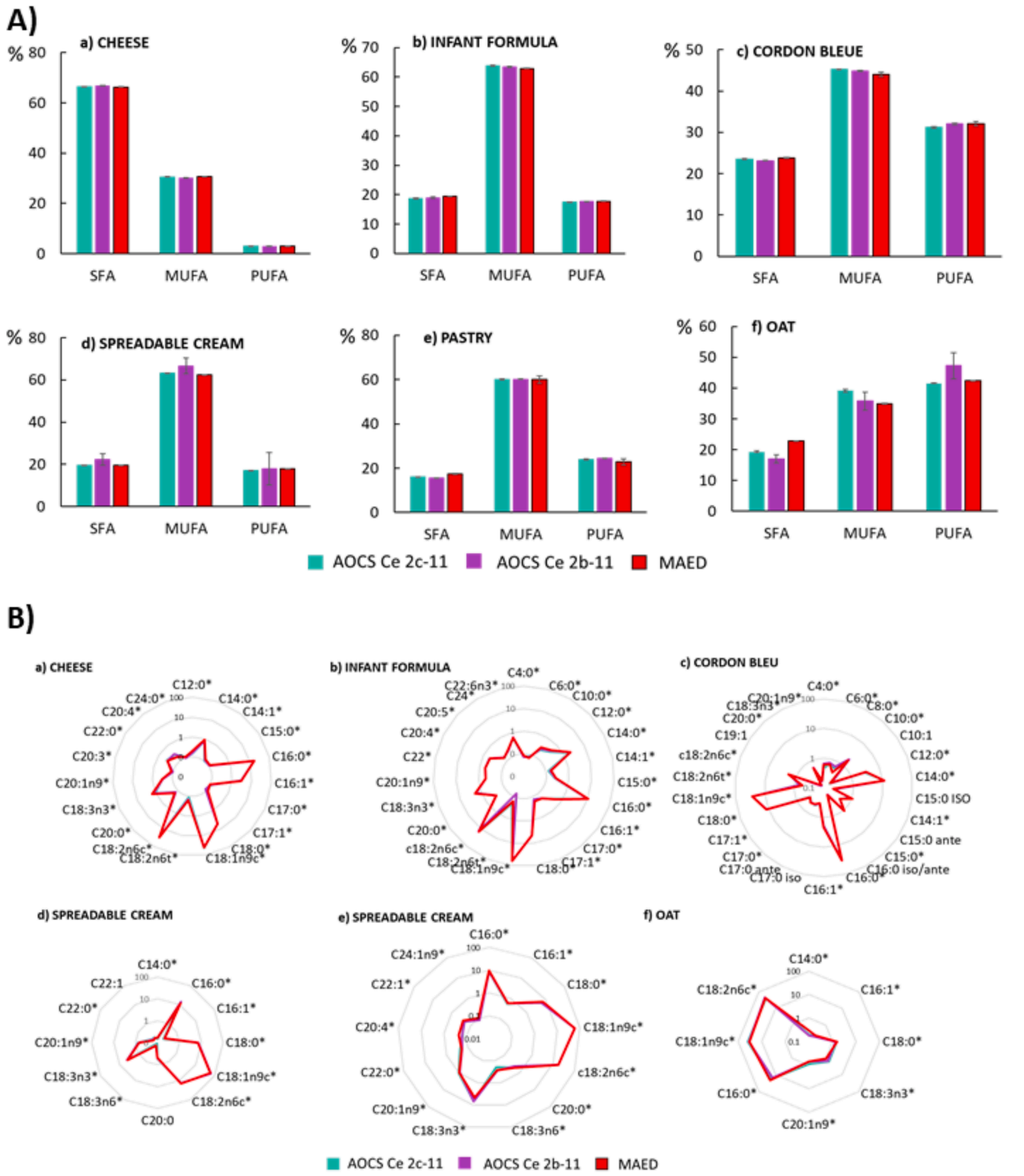

Fig. 2. A)) Percentage profile of Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated fatty acids (PUFA) in a) cheese, b) infant formula, c) cordon bleu, d) spreadable cream, e) pastry, and f) oat performed with Official methods AOCS Ce 2b-11 (purple), Ce 2c-11 (green), and MAED (red). B) Percentage profile of all detected fatty acids in a) cheese, b) infant formula, c) cordon bleu, d) spreadable cream, e) pastry, and f) oat performed with Official methods AOCS Ce 2b-11 and 2c-11 and MAED. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of the total fat, the crude fat, and the label values (g/100 g).

	Cheese	Pastry	Cordon bleu	Oat	Spreadable cream	Infant formula
TOTAL FAT	36.7 ± 1.4	25.3 ± 0.9	18.8 ± 2.8	7.6 ± 0.9	34.8 ± 1.6	29.3 ± 0.6
CRUDE FAT	19.0 ± 5.4	19.6 ± 0.2	13.3 ± 0.4	4.6 ± 0.4	21.6 ± 0.7	10.1 ± 0.2
LABEL	28	-	-	7	32	24

-: no information available

the results compared to the values on the label [28].

3.2.2. *FAME profile*

The percentage profile of FAMEs of the two derivatizations on the two different extracts is compared in Fig. 3 in terms of SFA, MUFA, and PUFA and in Fig. 4 in terms of the percentage profile of each FAME detected in each sample.

Comparing the same extraction method (i.e., MAEH or MASE, represented by yellow and orange, respectively, on the top of the bars) with the two different derivatization procedures (different color in the bottom of the bars, blu for HCl/MeOH and green for BF_3), no difference is observed for any matrix and any FAME (Fig. 4). The results showed that the HCl/MeOH solution can efficiently convert FAs into their methyl esters derivatives, providing comparable results to the overrated BF_3 while being safer for the operator, more stable, and cost-effective. In fact, the HCl/MeOH solution is much less hazardous to handle and use compared to BF_3, which is highly toxic and corrosive. Moreover, it is known that BF_3 can be unstable and difficult to store, whereas HCl/MeOH solutions are generally more stable and easy to manage over time [6]. In the end, considering the throughput of an analytical laboratory, using an HCl/MeOH solution can be a more economical choice for routine use. Indeed, the HCl/MeOH derivatization can also be performed in the microwave, speeding up the overall derivatization procedure.

Considering that the derivatization procedure did not impact the results at all, to simplify the visualization the two extraction modes (i.e. MAEH and MASE) were compared (considering only one derivatization method, i.e., HCl/MeOH for consistency) with the one-step procedure (Fig. 5). Similarly, for the one-step procedure the MAED is retained, considering that all the matrices gave perfectly comparable results (except for oat discussed later) with the two official AOCS methods.

The results showed a very good comparability, except for oat, for which a more thoughtful discussion is presented in the next section. For all the other matrices, a slight difference can be observed for the percentage of C20:4 in pastry, where the MASE-HCl/MeOH method showed lower extraction. This is clearly the effect of the extraction method, harsher on one side, while only capable of extracting the crude fat (alias "free lipids") in the case of the MASE.

Additionally, the C18:2n6t in the cordon bleu showed a discrepancy among the methods. A lower amount was found with the two-step method. Unfortunately, this FA is presented at a very low concentration, ~ 0.1 % and it is not reported in the BIPEA certificate. The value found using the MAED is comparable with both official AOCS methods, suggesting the reliability of the result compared to the two-step methods. It is not clear what could have caused this discrepancy, but the occurrence of this FA was also very low, impacting the reliability of the results.

Generally, the methods resulted in equivalent results, pointing toward flexibility in choosing the method based on specific laboratory requirements and available resources.

3.3. *The special case of oat*

Interestingly, in the case of the oat-based food, the MAED method did not align with the two AOCS methods (Fig. 2A). According to the AOCS

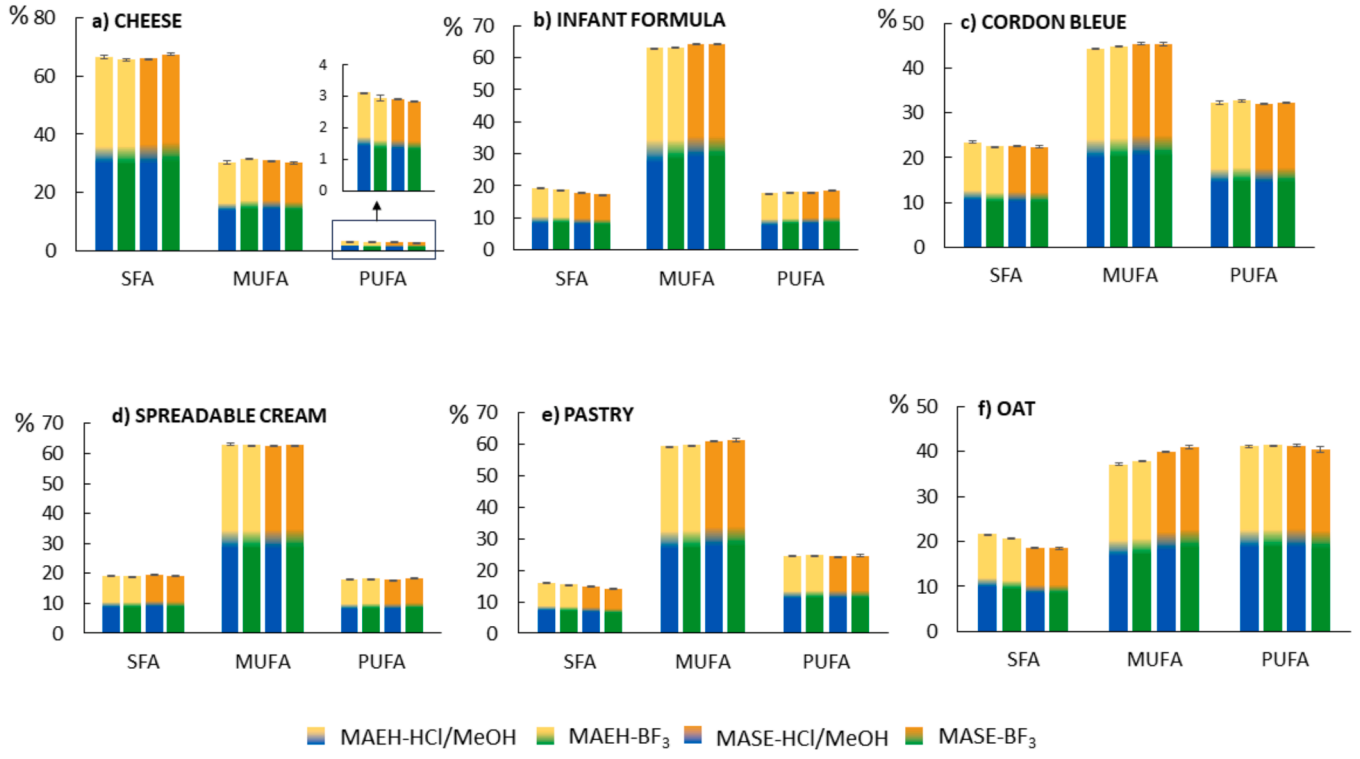

Fig. 3. Percentage profile of Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated fatty acids (PUFA) in all matrices obtained with two different extractions (MAEH, yellowish top bar; MASE, orangish top bar) and derivatization (BF_3, greenish bottom bar; HCl/MeOH, bluish bottom bar) methods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

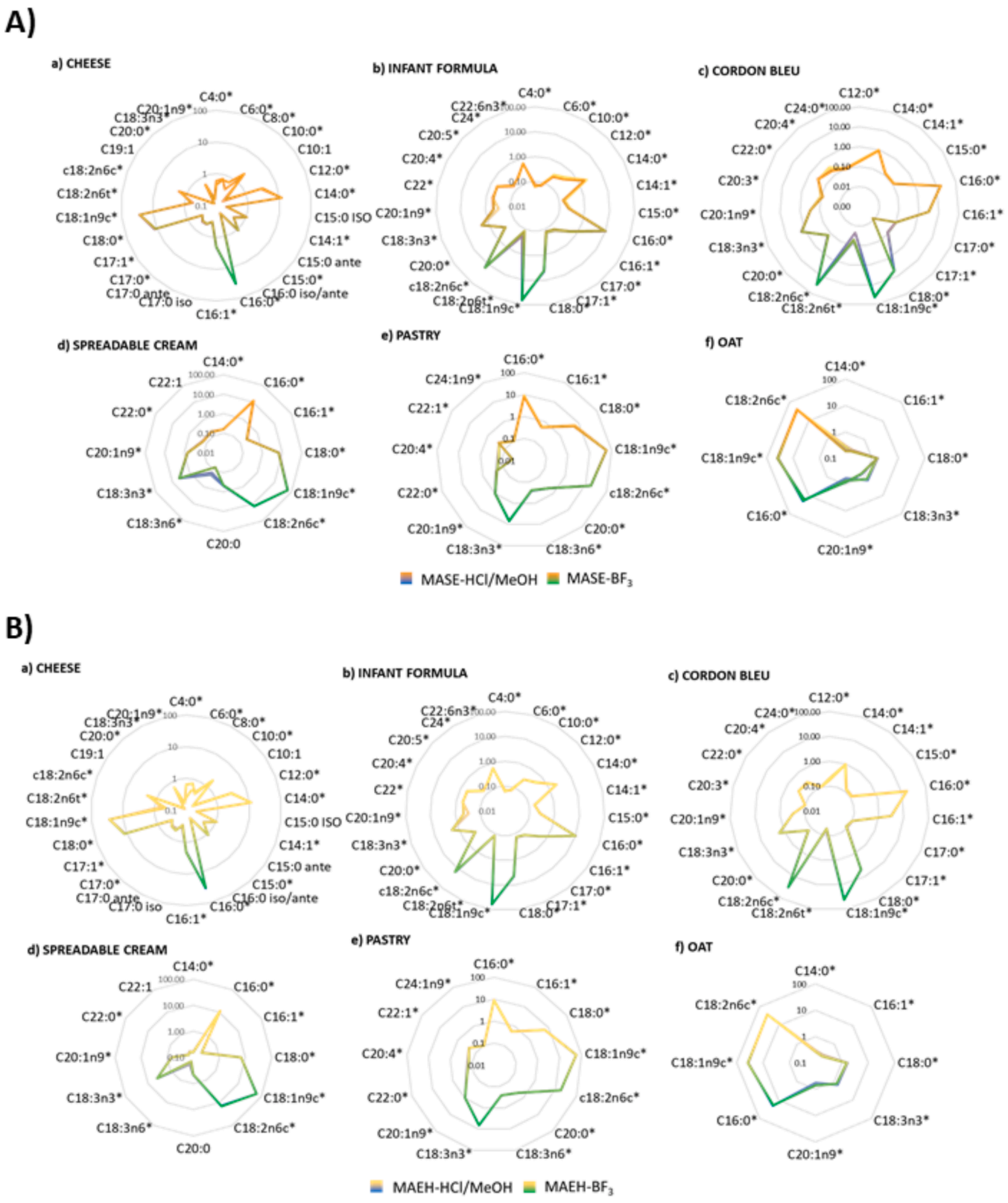

Fig. 4. Percentage profile of FAMEs in all matrices obtained with A) the MASE extraction followed by the BF_3 or HCl/MeOH derivatization; B) MAEH extraction followed by the BF_3 or HCl/MeOH derivatization.

protocols, the AOCS Ce 2c-11 should be used in this case. The PUFA obtained with MAED were similar to the results obtained using AOCS Ce 2b-11, but both were lower than those using the AOCS Ce 2c-11. For the MUFA, the MAED was very similar to AOCS Ce 2c-11, while AOCS Ce 2b-11 was higher, while MAED gave higher amount of SFA compared to the other two reference methods, with the lowest value obtained using the AOCS Ce 2b-11. The same trend is observed in the three major FAMEs, namely C16:0, C18:1n9c, and C18:2n6c (Fig. 2B and Table S6). A clear

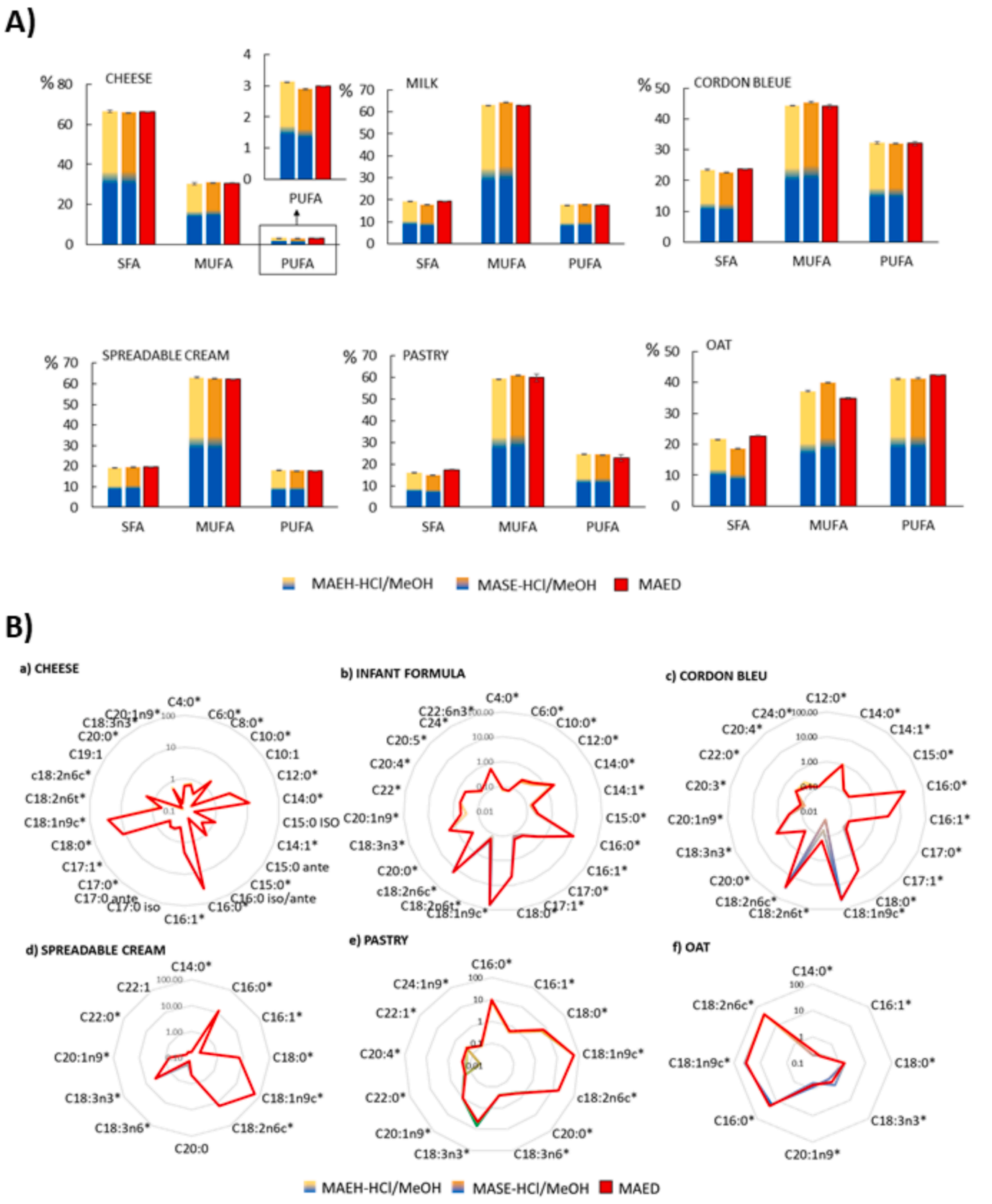

Fig. 5. A) Percentage profile of Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated fatty acids (PUFA) in all matrices obtained with two different extractions and HCl/MeOH derivatization methods. B) percentage profile of FAMEs in all matrices obtained with two different extractions (MAEH, yellowish line; MASE, orangish line) and the same derivatization (HCl/MeOH, blu), and the MAED one-step procedure (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

difference is also shown in the two-step methods, but this was expected as MASE is not efficient in breaking the complex interaction of the lipids with the matrix in this kind of sample. Indeed, in the case of oats, the simple solvent extraction is not sufficiently effective in breaking the complex structures of oat cells. In fact, in oats, lipids are bonded to other macromolecules, such as proteins and carbohydrates, thus requiring harsher conditions to be released [29]. The use of MAEH allowed breaking down these structures and releasing lipids for a more comprehensive analysis. Fig. 6 compares the AOCS Ce 2c-11 Official method suggested for the oat, which implicates acidic pre-treatment, MAEH-HCl/MeOH, and MAED. As aforementioned, the MAED method was not capable of liberating the lipids comparably to acid hydrolysis with H_2SO_4. However, the MAEH-HCl/MeOH also showed some differences in the FAMEs profile. Differently than MAED, it gave similar results than the AOCS Ce2c-11 method for C18:2n6 and C18:3n3, while it gave higher results for C16:0 and lower for C18:1n9.

In Sahasrabudhe's work [30], the lipid class composition of oats is analyzed with particular attention to the various lipid classes present and their fatty acid distribution. According to this work, oats contain a significant amount of lipids compared to other cereals, generally ranging from 4 % to 9 % of the dry weight, with triglycerides representing the majority of the lipid content, followed by phospholipids and glycolipids while free FAs and sterols are present in smaller amounts. Referring to the fatty acids distribution, the work shows that C14:0 and C18:3 are more present in glycolipids, C16:0 is more present in phospholipids, C18:0 is equally distributed in glycolipids and phospholipids, and C18:2 in triglycerides, glycolipids and phospholipids. It appears evident that the different methods are not equivalent for the different classes of lipids, and this appears evident in samples where other classes than triglycerides do not represent only trace amounts. Nevertheless, it is difficult to draw a definitive conclusion on the efficiency of extraction of the different classes. A dedicated study should be performed considering the absolute quantity (and not the percentage profile) and also to perform the quantification of the different lipid classes as intact lipids and of the FAMEs after transesterification.

3.4. Green analytical evaluation

All the methods applied were evaluated for greenness and blueness using two specific tools (Fig. 7). To assess the greenness of the sample preparation methods, the PrepAGREE metric [31] was used. The software generates pictograms that display the score assigned to the method based on the 10 criteria of green sample preparation, and taking into account the importance of criteria by assigning them specific weight. The default weight was kept except for criteria 2 (use of safer solvents and reagents), 6 (maximizing sample throughput), and 7 (promote automation and integrated steps) to align with the previous evaluation [16,17] in comparison with the AOCS Ce 2b-11.

The MAED demonstrates excellent performance in maximizing sample throughput (Criteria 6). This criterion refers to the number of samples prepared in 1 h. MAED enables the preparation of 12 samples every 15 min, adding up to 48 per hour or even more if the system is equipped with a rotor with 24 positions (96 per hour), compared to 8 per hour with the official methods. This efficiency is the result of the integration of multiple steps in MAED, making it as automated as possible, as required by Criteria 7. Additionally, MAED leads to a reduction in power consumption (Criteria 8).

The final score obtained for all the methods is shown in Fig. 7A. It is evident that the lower score is assigned to the methods that use BF_3 for the derivatization, and the higher is assigned to the MAED approach. The two-step methods had an intermediate value if the derivatization is the HCl/MeOH, or much lower, also of the official one if the BF_3 is used. HCl/MeOH derivatization can be preferred over BF_3 for the transesterification of lipids to form FAME and there are several reasons for this preference, including safety, ease of use, effectiveness, and compatibility with different matrices. Specifically, methanolic HCl is less hazardous and easier to handle compared to BF_3, which is corrosive and requires special handling precautions. Methanolic HCl provides an effective and complete transesterification reaction for converting fatty acids into FAME and works efficiently for most lipid matrices, whereas BF_3 can lead to undesirable side reactions, such as the formation of artifacts in the presence of sensitive functional groups. Moreover, HCl/MeOH is more stable and easier to store.

The criteria 9 is based on the choice of the greenest possible post-sample preparation configuration for the analysis. In general, for a greener analytical approach, GC-FID is preferably used on the GC–MS, however, in addition to the sustainability impact, one should also consider the method's performance. In this regard, the proposed method, which utilized GC×GC-FID, is capable of providing detailed information while remaining more sustainable than MS. Through the utilization of GC×GC, the interpretation capabilities are improved, and the structured chemical pattern obtained from the 2D plot enables detailed characterization of FAMEs profile based on specific positions in the chromatogram assuring a reliable identification without the need to use an MS. Moreover, a noteworthy sustainable aspect of the proposed analytical methods is the utilization of a flow modulator instead of a cryogenic one.

On the other hand, we used the Blue applicability grade index (BAGI) [32] based on the blue principles of white analytical chemistry to evaluate the practicality of the methods. For a method to be considered practical, a final score above 60 is recommended. All the methods used achieved a score higher than 60 as shown in Figure 7B; however, the highest score was assigned to MAED, namely 75.0. The two crucial criteria that made the difference were criteria 5 and 6, namely the ability to process many samples within 1 h and the multi-step sample preparation merged into one single step. Regarding the two-step methods, they scored slightly over 60 (62.5) but less than the official methods (67.5). However, in addition to providing information on the FAMEs profile, the two-step methods also provide information on total fat; output that can be considered practical to many laboratories.

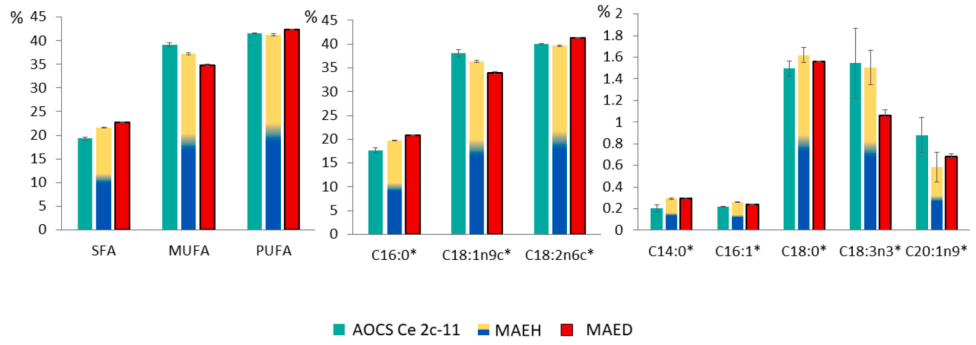

Fig. 6. a) Comparison of Official Method Ce 2c-11, two- and one-step MAE Percentage profile of Saturated, Monounsaturated, and Polyunsaturated fatty acids in Oat; b) Comparison of Official Method Ce 2c-11, two- and one-step MAE Percentage profile of FAME.

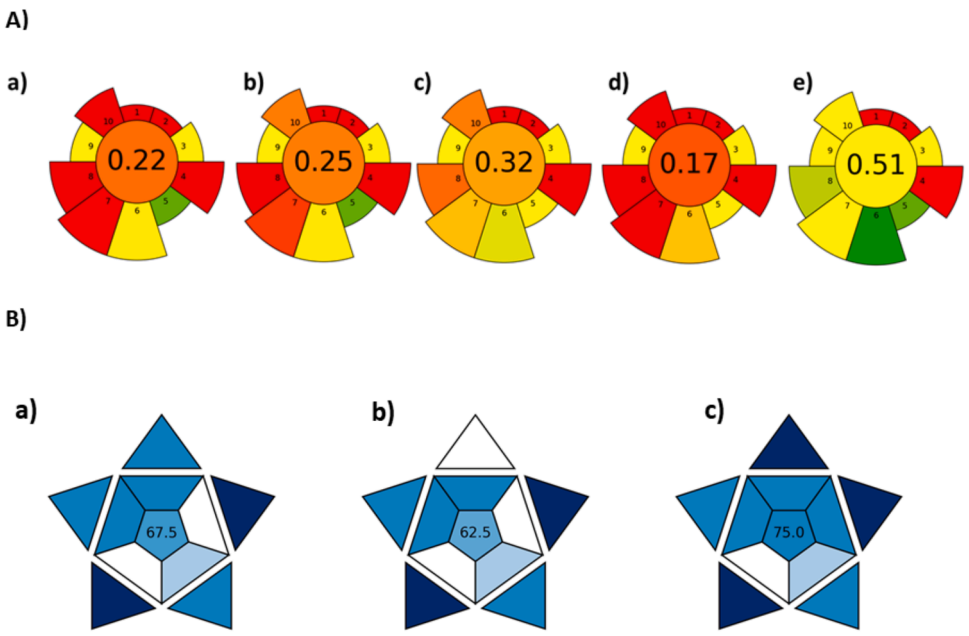

Fig. 7. A) Comparison of the greenest of the methods: a) AOCS Ce 2c-11; b) AOCS Ce 2b-11; c) MAEH-HCl/MeOH and MAE-HCl/MeOH; d) MAEH-BF_3 and MAE-BF_3; e) MAED. B) Comparison of the blueness of the methods: a) AOCS Ce 2c-11, AOCS Ce 2b-11; b) MAEH-HCl/MeOH, MAE-HCl/MeOH, MAEH-BF_3 and MAE-BF_3; c) MAED. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The microwave-assisted processes showed to be suitable for FAs analysis in foods, and comparable to Official methods both when performing the one-step method (MAED) and the two-steps methods (MAEH and MASE). It is evident that whether the total fat data is needed, the MAEH procedure should be preferred over the one-step methods, but otherwise no major differences were noticed in the FAs profile of the samples considered in the present study (except for oat) for which acid hydrolysis is required.

From the viewpoint of the derivatization step, it is worth stressing that the still overrated BF_3 derivatization procedure can be successfully replaced with the microwave-assisted HCl/MeOH derivatization, obtaining the same performance but improving the safety of the operator using less harmful reagents, being overall greener, and ensuring a higher throughput. To increase the greenness of the MAED method, in the future, more focus can be directed towards reducing solvent volumes, aiming to further enhance the method's environmental friendliness. Nevertheless, the representativeness of the sample must always kept in mind.

Moreover, further studies are necessary to design an optimal MADE procedure alternative to the official method involving preliminary acid hydrolysis (AOCS Ce 2c-11).

CRediT authorship contribution statement

Donatella Ferrara: Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Marco Beccaria:** Writing – review & editing, Visualization, Methodology, Data curation. **Chiara E. Cordero:** Writing – review & editing, Supervision, Funding acquisition. **Giorgia Purcaro:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2024.100124.

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