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A high throughput method for fatty acid profiling using simultaneous microwave-assisted extraction and derivatization followed by reversed fill/flush flow modulation comprehensive multidimensional gas chromatography

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ABSTRACT

The fatty acid composition of a food product provides information regarding the origin of the product and its overall quality, such as its nutritional value. This work proposes a fast and accurate method for preparing fatty acid methyl esters from a wide variety of food products by using a single-step microwave-assisted extraction and derivatization coupled to reversed fill/flush flow modulation comprehensive two-dimensional gas chromatography (GC×GC) – flame ionization detector (FID) to tentatively identify and quantify the individual fatty acid. The robustness of the GC×GC – FID platform was successfully assessed, as well as the reliability of the entire proposed procedure, assuring repeatability largely below 10%. The enhanced separation obtained by the use of GC×GC allowed for the identification of 81 FAMEs in a single run of 30 min. The fatty acid methyl esters profiles obtained with the proposed microwave-assisted extraction and derivatization were comparable with reference methods from the literature and the American Oil Chemistry Society. This method also proved to be a significant step towards a greener procedure than the reference one when evaluated based on the PrepAGREE metrics that have been recently proposed.

1. Introduction

The lipid content of food products, along with the fatty acid (FA) composition, is an essential parameter of its quality. Food products are routinely analyzed to determine their composition in saturated and unsaturated fatty acids to communicate their nutritional value, monitor their quality and origin, and confirm the absence of *trans*-FAs that may be generated by industrial processes such as hydrogenation or refining.

FAs are present mainly as triglycerides that represent, in general, the most abundant fraction in oils and fats (≥ 90%). FAs can be classified as saturated (SFAs), monounsaturated (MUFAs), or polyunsaturated (PUFAs). Then, the unsaturated fatty acids (UFAs) are further classified depending on the position of the double bond with respect to the terminal methyl group, and as *cis* or *trans* according to the isomeric configuration of the carboxylic chain due to the double bond.

Their analysis usually requires an initial extraction of the lipid fraction from the matrix components of the product. This extraction step is then followed by derivatization by transesterification to form the more volatile FA methyl esters (FAMEs) that can be analyzed by gas chromatography (GC), the preferred technique for this analysis.

Extraction and derivatization methods vary depending on the food matrix to be analyzed [1–4]. The extraction step usually includes solid- or liquid-liquid solvent extraction, where Soxhlet and Folch are the most common methods. These are sometimes used in combination with digestion steps, including microwave digestion. The derivatization step may be base or acid catalyzed, or use methanolic BF_3 or sodium methoxide as catalysts for the transesterification [5–7]. Although these procedures have proven to be efficient over the years, it can be noted that they are cumbersome and labor intensive. Moreover, they consume a rather large amount of solvents and can incur unexpected analyte losses due to the multiple-step manipulations. Consequently, single step extraction-

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derivatization techniques have gained interest [2,8,9]. Nevertheless, these methods are not yet predominant, and their application seems limited to only some kinds of samples.

Although GC is a very powerful separation analytical technique, FAMEs profiling can be challenging for some complex samples. The large variety of FAMEs present in a lipidic food sample, combined with their different proportions, may require the use of long capillary columns or even two different stationary phases (i.e. two GC runs) to obtain a more comprehensive characterization. Both alternatives are rather time-consuming. In this aspect, comprehensive two-dimensional GC (GC×GC) proved to be an interesting alternative to overcome the limitations in the separation of FAMEs in a shorter time [10–12]. Thanks to the increased chromatographic separation space and the orthogonal separation, FAMEs chromatograms present an ordered structure in the 2D-plot that can allow simpler identification for profiling compared to one dimension analysis, avoiding many possible coelutions among different FAME species thanks to the 2D-GC separation space.

Notwithstanding the advantages of using GC×GC for such analysis, its use for routine applications in food quality remains very limited. However, the separation power of GC×GC is certainly worth the investment. In this regard, incorporating less costly and environmentally sustainable modulators such as flow modulators and using simpler detectors such as FID (instead of MS, whenever possible) can improve its acceptance. Moreover, the possibility of a shorter time of analysis is certainly a beneficial characteristic.

On the one hand, it is clear that FAs profiling in dietary products is needed to ensure a healthy diet. On the other hand, simpler and greener methods of analysis are required. Hence, we present in this work the development of a new method for FAMEs profile analysis by the combination of a microwave-assisted single-step extraction-derivatization method with the powerful separation of GC×GC for analysis of the obtained FAMEs. This can lead to a simpler yet robust analytical method that could be easily applied to routine analysis.

2. Material and methods

2.1. Chemicals and reagents

All chemicals employed, both solvents (hexane, cyclohexane, methanol, chloroform and methyl acetate) and reference standards were from MilliporeSigma, the life science business of Merck KGaA (Darmstadt, Germany).

Pure standards solution of *n*-alkanes (from *n*-C7 to *n*-C30) for system evaluation, flow/pressure optimization and linear retention index (LRI) determination, prepared in hexane at the concentration of 600 mg/L; pure standards of Supelco C37 FAME Mix for system evaluation was prepared in hexane at the concentration 2 g/L. Fish oil from menhaden (MilliporeSigma) was used for analysis following the derivatization procedure below described.

Sodium methoxide (MilliporeSigma, #164,992) was weighted and solubilized in methanol at a concentration of 20 g/L. A solution of oxalic acid is prepared with 0.5 g in 15 mL diethyl ether. A 14% BF_3 / methanol solution (MilliporeSigma, #B1252), an acidic-methanolic solution 1.5 M (MilliporeSigma, #17,935) and a sodium methoxide solution (27 mg in 100 μL) are used as derivatization agents.

2.2. Samples

Different food samples and ready-to-eat products were bought in a local supermarket (Gembloux, Belgium). In particular the following products were tested: UHT milk, spreadable cheese (Philadelphia), cake (Madeleines), grissini, canned peas, chips, pizza, falafel, bacon, lasagne, hazelnut drink. All the sample were prepared in triplicate following to the microwave-assisted extraction and derivatization method (MAED) described below and the AOCS Official Method Ce 2b-11 [3].

2.3. Lipid extraction and derivatization methods for milk analysis

2.3.1. Bligh and Dyer extraction followed by acid-catalyzed derivatization

The method reported by Cruz-Hernandez et al. [1] was applied. Briefly, the milk sample was mixed with chloroform and methanol to obtain a chloroform:methanol:water mixture in proportions of 1:2:0.8 v/v. The sample was homogenized for two minutes before adding additional volumes of chloroform and water, yielding to the final proportions of 2:2:1.8 for the 3 solvents. The solution was homogenized and then centrifuged. Then, the lower chloroform layer was collected and concentrated to dryness using a rotary evaporator. The extracted fat was then treated to derivatize the FAs into FAMEs for GC analysis.

2.3.1.1. Base-catalyzed methylation. In agreement with Cruz-Hernandez et al. [1] methylation using sodium methoxide (27 mg in 100 μL) is rapid and complete within 10–15 min at 50 °C. The following procedure was applied: 2 mg of the total extracted milk fat was weighted in a 2 mL autosampler vial and dissolved in 1.7 mL of hexane and 40 μL of methyl acetate. Then, 100 μL sodium methoxide were added. After securely capping the vial, the solution was mixed and allowed to react for 15 min at room temperature with occasional mixing. The vial was cooled at −20 °C for 10 min, then 60 μL of oxalic acid (0.5 g in 15 mL diethyl ether) were added and mixed thoroughly. Finally, the vial was centrifuged to settle the sodium-oxalate precipitate. The upper phase was passed through a Pasteur pipette column containing a glass wool plug and a 2 cm bed of anhydrous Na_2SO_4 and collected directly into a 2 mL autosampler vial. All tests were performed in triplicate.

2.3.1.2. Acid-catalyzed methylation. Methylation using 5% HCl gas in anhydrous methanol (w/v) has the advantage of being clean, easily prepared and applicable to all common lipid structures, including non-esterified fatty acids. To 2-5 mg of sample, 1 mL of 5% HCl/methanol was added and heated at 80 °C for 30 min. FAMEs were then extracted with hexane and concentrated to 1 mL before the GC injection. All tests were performed in triplicate.

2.3.2. One step extraction and derivatization using BF_3

The method reported by Jariyasopit et al. [2] was applied. Briefly, 800 μL of acetone were added to 200 μL of cows' milk. The sample mixture was shaken at 1500 rpm for 3 min before being stored at −20 °C for 30 min. Then, it was centrifuged at 5000 g for 15 min and the supernatant was transferred to a Pyrex glass tube. The samples were mixed with 1 mL of 14% BF_3 in methanol and 1 mL of hexane was added afterwards. The samples were then placed in a water bath at 100 °C for 2 h. After cooling down to room temperature, the samples were mixed with 500 μL of Milli-Q water and vortexed briefly. Then, they were centrifuged at 650 g for 5 min and the upper phase, containing the FAMEs, was collected and stored until GC analysis. All tests were performed in triplicate.

2.4. Official method Ce 2b-11 by AOCS for other food samples

The Direct Methylation of Lipids in Foods by Alkali Hydrolysis (*Official Method Ce 2b-11 by AOCS*) was applied [3].

The appropriate amount (mg) of sample was precisely weighted according to the reference table reported in the method [3], and 5 mL of NaOH/methanol (20 g/L) was added. The solution was heated under reflux for 15 min. After this time, 5 mL BF_3 / methanol was added and the solution was kept under reflux for 2 additional minutes before adding 5 mL of hexane and removing from the heat source. All samples analyzed following this procedure were analyzed in triplicate.

2.5. One step microwave assisted extraction and derivatization (MAED)

An ETHOS X system equipped with an SR-12 eT TFM rotor was used (Milestone Srl, Bergamo, Italy). In this case, 0.5 g of sample

were weighted into the SR-12 eT TFM vessels, adding 10 mL of acidic-methanolic solution and 25 mL of cyclohexane before sealing the vessel and placing them inside the oven under continuous stirring. The samples were heated at 120 °C, testing different ramping programs. The maximum temperature was held for 15 or 30 min. The optimal conditions corresponded to a ramping up to 120 °C in 2 min and held for 15 min.

After the extraction and derivatization step, the system was cooled down and the vessels placed in ice for 15 min before opening them in order to exclude losses of the most volatile FAMEs (i.e., C_4 and C_6). If necessary, 2.5 g of NaCl were added to the solution and mixed, then centrifuged for 5 min to facilitate phase separation. The upper phase containing FAMEs was collected for further analysis. All tests were performed in triplicate.

2.6. GC×GC-FID instrumentation

All the samples were analyzed in a comprehensive multidimensional gas chromatographic system (GC×GC) consisting of a Triplus 100 multipurpose autosampler (Thermo Scientific from Interscience, Belgium) integrated with a Trace 1300 GC unit, coupled to FID. The system included an INSIGHT flow modulator (SepSolve Analytical Ltd, UK).

The ^{1}D column was a proprietary SepSolve 1D-FAMEs 20 m × 0.18 mm × 0.1 μm polar fused silica capillary column and the ^{2}D column was a SepSolve 2D-FAMEs 5 m × 0.25 mm × 0.1 μm non-polar fused silica capillary column (SepSolve Analytical Ltd, UK). The bleedline was 4.20 m × 0.1 mm uncoated capillary segment. The final optimized flow rates were as follows: first dimension flow was set at 0.5 mL/min; the auxiliary flow controller generating the second-dimension flow was set at 20 mL/min. Helium was used as carrier gas. Modulation period was set at 3 s, including 100 ms of reinjection time. The finally optimized oven temperature program was: 3 min at 40 °C, increase the temperature to 260 °C at 9 °C/min and hold it for 2 min. The injection was performed in split mode (1:10 ratio), injecting 0.5 μL at 250 °C. Detection was performed using a FID set at 270 °C (air flow: 350 mL/min, H_2: 35 mL/min; make-up gas: 20 mL/min). Data acquisition frequency was set to 100 Hz.

Data was acquired by Thermo Scientific Dionex Chromeleon 7 Chromatography Data System Version 7.3 (60,919) and processed by ChromSpace Version 1.5.1 by Markes International Limited.

3. Results and discussion

As aforementioned, the goal of the present work was to optimize a complete analytical protocol to maximize the sample throughput for the analysis of fatty acids in food for routine purposes. Indeed, fatty acid analysis is routinely performed to report on the saturated and unsaturated content for the nutritional label and to confirm the absence of *trans*-fatty acids.

To speed up the entire procedure, both the sample preparation and the GC separation were revised. For the latter traditional monodimensional (1D) separation, which requires long columns and often two analyses to obtain all the information needed, was replaced with a highly informative comprehensive two-dimensional (2D) GC separation equipped with a reversed fill/flush modulator. For the sample preparation step, the goal was to optimize a single-step extraction and derivatization method applicable to a broad range of food commodities, while avoiding the use of toxic reagents such as BF_3. The optimization of the two steps is discussed in detail below.

3.1. Multidimensional comprehensive gas chromatography analysis

In this study a reversed fill/flush (RFF) differential flow modulator based on the design of Griffith *et al.* was used [13]. Such an RFF modulator allows for a better band re-injection, with a consequential improved ^{2}D peak width and symmetry compared to the forward fill/flush modulator (FFF). As aforementioned, one of the main advantages of GC×GC is the generation of 2D plot showing clear group chemical patterns, which can be coherently translated from cryogenic to flow modulation but with the advantage of a cheaper and less power-consuming platform [14].

Although the apolar×polar (normal phase) combination is the most employed column configuration in GC×GC, many examples of the use of the polar×apolar combination are reported for FAMEs analysis [10–12]. In fact, the latter combination generally allows for a better separation of the *cis* and *trans* isomers, although it remains challenging as it is highly dependent on the overall sample profile and the dynamic range of the compounds present. In this study, a polar×non-polar set was used with the goal to optimize a rapid separation method for routine analysis, meaning about 30 min run time, while guaranteeing a coverage of a wide FAMEs distribution starting from the four carbon FAME (C_4). These conditions required a careful optimization of the amount injected and the initial isotherm to properly separate the C_4 FAMEs from the tailing of the solvent. The injection of 0.5 μL of sample solution instead of 1 μL, along with a rather long isotherm (i.e. 3 min) allowed for a satisfactory separation of the compound from the solvent, but also for less overload of later eluted compounds, thus leading to a better separation of critical pairs such as the C18:1 *cis* and *trans (*Supplementary Figure S1*)*.

The temperature program was thoughtfully optimized using the Supelco C37 FAMEs mixture and then verified with both fish oil and a milk sample. These samples were chosen for being complex samples covering a wide variety of FAMEs lengths (C_4–C_{24} for milk) and double bond distribution. The optimization was performed aiming at the best compromise between throughput and resolution of critical pairs, paying attention to limit the wraparound. The optimal temperature rate was set at 9 °C/min with a modulation time of 3 s (100 ms flushing).

The final separations obtained for the fish oil and milk sample are presented in Fig. 1A and B, the compound identification refers to Table 1, where also the comparison between the FAMEs profile according to the sample preparation method is reported (this is further discussed in the following section). The FAMEs profile of the two samples was tentatively identified based on the LRI calculated referring to the saturated FAMEs and on their position on the 2D plot. It should be noted that the LRIs were used as a rough approximation, as the stationary phase of the ^{1}D column used is a proprietary phase and only the polarity is known. While the milk sample was a sample bought at the supermarket and no reference separation was available for it, the fish oil sample was a reference standard of menhaden oil (MilliporeSigma), widely used for optimization purposes in the field of GC×GC [11,15,16]. A total of 64 FAMEs were tentatively identified in the fish oil, which is comparable with a cryogenic separation [11] and superior to the previously reported FFF flow modulation GC×GC (i.e., 52 peaks) [16] (although not the same column set was used). For the milk sample, a total of 42 FAMEs were separated and tentatively identified, for a total considering both samples of 79 different FAMEs.

The repeatability and stability of the GC×GC system were proven by injecting several times the FAMEs C37 standard mixture over a period of 2 months, obtaining an average ($n = 6$) relative standard deviation (RSD) of 0.02% for ^1t_R, 1.26% for ^2t_R and 1.2% for the peak areas. Moreover, a limit of quantification (LOQ) of 0.007% was estimated based on 10-times the S/N ratio.

3.2. Microwave-assisted extraction and derivatization

Most of the applications using microwave-assisted technology that have been reported are focused on the extraction of organic contaminants [17,18]. Although the potential of this technique has been proven in the extraction of lipids from different matrices [19], it remains a field of application that is not yet widely explored [20]. Only very few papers reported the use of microwave to assist the fatty acid derivatization step and most of them relied on the use of BF_3 as derivatization reagent [21–24].

Table 1
List of FAMEs tentatively identified based on the LRI compared to the literature and the 2D position in cow's milk and fish oil. Percentage profile obtained using both the MAED method herein proposed, and the respective reference method are also reported. (Complete table with all the samples reported in Supplementary Table S1.).

		LRI	LRI Exp		Fish oil		Milk	
Peak number	Compounds	Lib	Fish oil	Milk	MW	REF	MW	REF
1	**C4:0**	400		400			1.54±0.19	1.83±0.26
2	**C6:0**	600		600			1.89±0.04	1.55±0.23
3	**C8:0**	800		800			1.38±0.18	1.10±0.18
4	**C10:0**	1000		1000			3.69±0.18	2.89±0.34
5	**C11:0**	1100		1100			0.09±0.01	0.05±0.01
6	**C12:0**	1200	1200	1200	0.09±0.00	0.08±0.00	4.17±0.15	3.59±0.37
7	**C13:0 iso**	1253	1256	1257	0.01±0.00	0.01±0.00	0.02±0.00	0.02±0.00
8	**C13:0 anteiso**							
9	**C13:0**	1300	1300	1300	0.05±0.00	0.04±0.00	0.08±0.01	0.10±0.01
10	**C14:0 iso**	1358	1358	1355	0.07±0.00	0.06±0.00	0.07±0.00	0.07±0.01
11	**C14:0**	1400	1400	1400	7.99±0.07	7.94±0.02	12.19±0.60	11.7 ± 0.76
12	**C14:1**	1429	1429		0.03±0.00	0.02±0.01		
13	**C14:1n5**	1436	1436	1441	0.02±0.00	0.03±0.00	1.16±0.11	0.92±0.07
14	**C14:1**		1446		*0.06±0.00*	*0.04±0.00*		
15	**C15:0 iso**	1452	1455	1456	*0.36±0.00*	*0.32±0.00*	0.18±0.02	0.18±0.00
16	**C15:0 anteiso**	1467	1472	1469	0.07±0.01	0.12±0.01	0.37±0.02	0.38±0.02
17	**C15:0**	1500	1500	1500	0.65±0.01	0.63±0.01	1.03±0.09	0.10±0.04
18	**C15:1n5**	1539	1536	1535	0.04±0.00	0.04±0.00	0.04±0.00	0.00±0.00
19	**C16:0 iso**	1554	1553	1554	0.12±0.00	0.12±0.01	0.19±0.03	0.19±0.00
20	**C16:0**	1600	1600	1600	17.01±0.09	16.72±0.02	33.89±0.96	35.88±0.65
21	**C16:1n9**	1626–45	1634	1624	12.22±0.08	12.22±0.02	1.85±0.06	1.88±0.03
22	**C16:2n4**	1657	1679		0.28±0.00	0.30±0.00		
23	**C16:2**		1707		1.23±0.00	1.25±0.01		
24	**C16:3n3**	1733	1748		1.46±0.01	1.47±0.02		
25	**C16:3**		1788		0.14±0.01	0.15±0.01		
26	**C16:4**		1816		0.71±0.01	0.73±0.01		
27	**C17:0 iso**	1624	1653	1657	0.29±0.05	0.27±0.00	0.28±0.07	0.25±0.01
28	**C17:0 anteiso**	1653	1668	1672	0.14±0.00	0.12±0.01	0.39±0.09	0.36±0.02
29	**C17:0**	1700	1700	1700	0.59±0.01	0.61±0.01	0.42±0.01	0.46±0.01
30	**C17:1n7t**	1730	1730		*0.06±0.00*	*0.04±0.00*		
31	**C17:1n7c**	1743	1754	1751	0.29±0.00	0.25±0.09	0.24±0.01	0.23±0.02
32	**C17:2**		1789		0.04±0.01	0.04±0.00		
33	**C17:3n3**	1825	1832		0.08±0.00	0.05±0.00		
34	**C18:0 iso**	1767	1753		0.13±0.00	0.12±0.00		
35	**C18:0**	1800	1800	1800	3.10±0.01	3.00±0.03	8.77±0.16	9.17±0.51
36	**C18:1n9t**	1819		1816			1.32±0.02	1.37±0.07
37	**C18:1n9c**	1819	1829	1816	8.49±0.00	8.40±0.04	19.62±0.24	20.40±1.01
38	**C18:2n6t**	1874	1880	1873	1.61±0.01	1.64±0.01	1.91±0.04	1.93±0.12
39	**C18:2n6c**	1891	1900	1887	0.27±0.00	0.27±0.00	0.77±0.00	0.54±0.02
40	**C18:3n6+3n4**	1896/1890	1923		0.31±0.01	0.33±0.00		
41	**C18:3n6**	1874–1896		1893			0.04±0.01	0.03±0.01
42	**C18:3n3**	1928	1952	1928	2.07±0.01	2.05±0.01	0.45±0.00	0.40±0.11
43	**C18:3**		1971		0.10±0.00	0.12±0.01		
44	**C18:4**		1994		3.04±0.01	3.11±0.01		
45	**C19:0**	1900	1900	1900	0.08±0.00	0.08±0.00	0.10±0.00	0.26±0.09
46	**C19:1**		1924		0.11±0.00	0.12±0.00		
47	**C19:3n6**	1992	2022		0.03±0.00	0.02±0.00		
48	**C19:3n3**	2032	2058		0.03±0.00	0.03±0.00		
49	**C19:4**		2047		0.05±0.01	0.06±0.00		
50	**C19:5**		2114		0.06±0.01	0.06±0.01		
51	**C20:0**	2000	2000	2000	0.22±0.00	0.24±0.03	0.21±0.00	0.58±0.22
52	**C20:1**	2020	2022	2011	1.08±0.01	1.05±0.01	0.13±0.00	0.12±0.01
53	**C20:2n4**	2049	2057	2054	0.1 ± 0.00	0.09±0.00	0.04±0.01	0.04±0.01
54	**C20:2n6**	2068	2070		0.25±0.00	0.24±0.00		
55	**C20:3n6**	2096	2107	2081	0.25±0.00	0.24±0.00	0.14±0.00	0.10±0.00
56	**C20:3n3**	2134		2111			0.00±0.00	0.00±0.00
57	**C20:3n3+4n6**	2134	2132		1.24±0.01	1.10±0.01		
58	**C20:4n3**	2181	2184		1.52±0.00	1.48±0.00		
59	**C20:4n6**	2115		2100			0.16±0.00	0.14±0.00
60	**C20:4**		2160		*0.04±0.00*	*0.10±0.00*		
61	**C20:5n3**	2181	2218	2145	13.16±0.08	13.42±0.04	0.05±0.00	0.09±0.01
62	**C21:0**	2100	2094	2100	0.03±0.00	0.03±0.00	0.04±0.00	0.00±0.00
63	**C21:5n3**	2291	2312		0.65±0.01	0.66±0.00		
64	**C22:0**	2200	2200	2200	*0.15±0.00*	*0.14±0.00*	*0.23±0.02*	*0.00±0.00*
65	**C22:1n9**	2219	2223		0.31±0.00	0.30±0.00		
66	**C22:2n6**		2224	2247	0.05±0.00	0.05±0.00	*0.09±0.00*	*0.00±0.00*
67	**C22:3**			2262			*0.09±0.00*	*0.00±0.00*
68	**C22:4**		2308	2300	*0.11±0.00*	*0.21±0.00*	*0.08±0.02*	*0.02±0.02*
69	**C22:4**		2326		0.22±0.01	0.22±0.00		
70	**C22:4**		2345		0.53±0.01	0.54±0.01		
71	**C22:4**		2354		0.13±0.00	0.10±0.00		
72	**C22:5**			2319			0.01±0.02	0.00±0.00
73	**C22:5n6+5n3**		2384		2.42±0.02	2.45±0.01		
74	**C22:6n3c**	2416	2406	2362	12.75±0.09	13.21±0.06	0.11±0.01	0.11±0.01
75	**C23:0**	2300					0.14±0.01	0.00±0.00
76	**C23:1**							
77	**C24:0**	2400	2400		0.42±0.00	0.39±0.00		
78	**C24:1n9**	2420						
79	**C24:3**		2514		0.09±0.00	0.07±0.00		

Note: the significantly different values are indicated in italics.

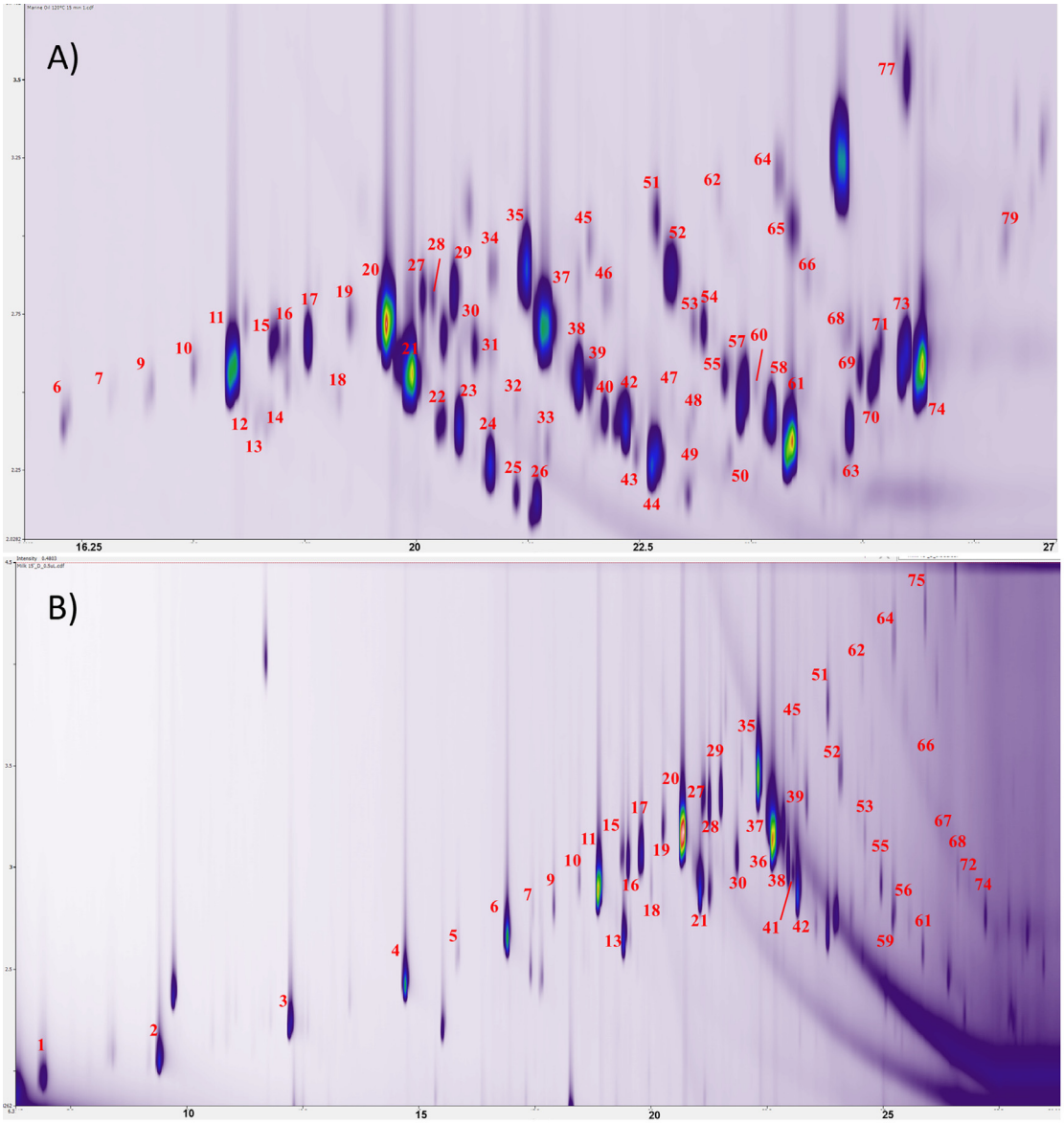

Fig. 1. 2D-GC FAME profile of A) menhaden fish oil and B) cow milk. Peak tentative identification as reported in Table 1.

The goal of this work was to propose a MAED method that can be used for a large variety of food commodities, maximizing the throughput. The choice of the derivatization reagent to use in the MAED procedure was based on different considerations related to efficiency, yield, throughput, and green and sustainability aspects. Base-catalyzed derivatization is a very rapid procedure that requires mild heating conditions. Nevertheless, it may fail in derivatizing non-esterified fatty acids and phospholipids, and esters may undergo saponification if the reaction is not properly stopped [20]. Thus, basic catalysis is preferred for the direct derivatization of edible oils as the presence of non-esterified fatty acid is ideally negligible [25].

Therefore, acid-catalyzed derivatization was chosen as it can react with both non-esterified and esterified FAs [25]. Although methanolic BF_3 is one of the most used reagents because of its high esterifying efficiency, it is highly toxic, expensive, and relatively unstable during storage, easily leading to the formation of artifacts and loss of unsaturated fatty acids (degradation) if not properly handled [26–28]. On the other hand, methanolic HCl is also a general purpose esterifying reagent. The inconvenient in preparation and stability during storage reported by some authors [20,29] can be easily overpassed by using commercially available ready-to-use methanolic HCl solution, which can be bought in convenient low volume format to minimize storage-related alterations. Therefore, the use of methanolic HCl was preferred over methanolic BF_3.

The comparison of the MAED method optimized using HCl in methanol was compared with the results obtained using extraction following different protocols and different derivatization reagents, as described below.

3.2.1. *Comparison of the MAED method with extraction followed by acid-based derivatization*

The first experiments were carried out using a milk sample, as it covers a broad range of fatty acids including very short C-chain (i.e., C_4), a large variety of *cis*- and *trans* isomers and double bond distribution. The first test was performed by comparing the single-step MAED method with an extraction of fats followed by the methanolic HCl derivatization step. The latter protocol was based on a method presented by some researchers from the Food and Drug Administration (FDA) for the analysis of FAMEs in dairy fats [1]. The method proposed a first extraction of the

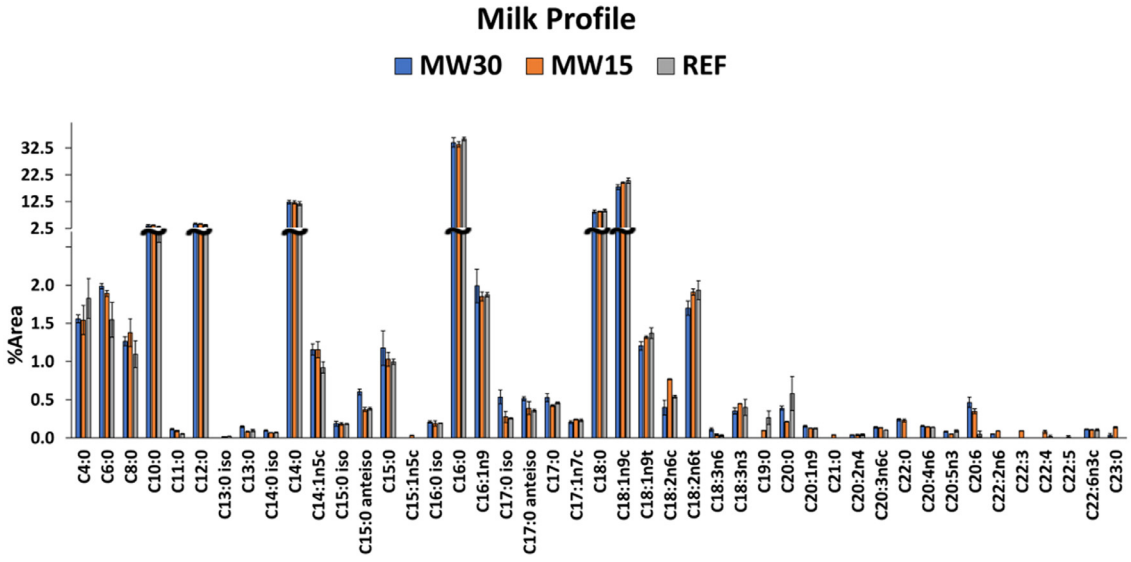

Fig. 2. Comparison of the FAMEs percentage profile of milk analyzed using the reference method (gray bars) [1] and the MAED at 120 °C for 30 min (blue bars) and 15 min (orange bars). Error bars indicate the standard deviation of three replicates.

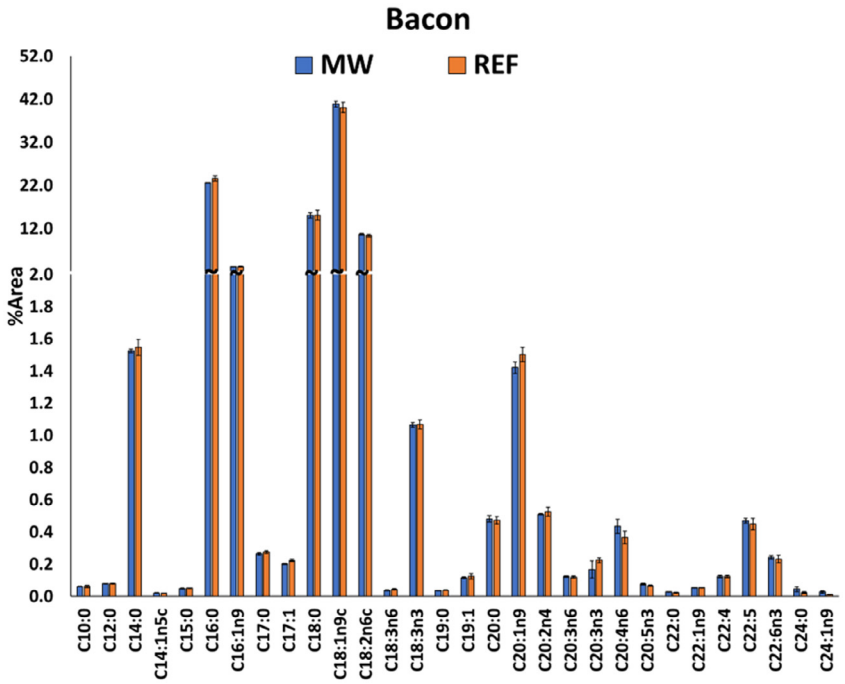

Fig. 3. Comparison of the FAMEs percentage profile of a sample of bacon analyzed using the reference method (blue bars) [3] and the MAED at 120 °C for 15 min (orange bars). Error bars indicate the standard deviation of three replicates.

lipid fraction using the Bligh and Dyer method [7] followed by either base-catalyzed methylation (using sodium methoxide) or acid-catalyzed methylation (using acidic methanol solution). For this work, only the methanolic HCl derivatization is discussed as a preliminary comparison of the two proposed procedures gave very similar results for the milk sample. The MAED method was tested at 80 °C and 120 °C for 30 min and 15 min of treatment. The use of the lower temperature (80 °C for 30 min) showed poor efficiency in both extraction and derivatization. Indeed, a loss of many FAMEs was observed along with a much dirtier chromatogram (data not reported). In the literature, the use of 80 °C for 1 h showed already low recoveries in fresh leaf tissue, a very low fat matrix [30], thus suggesting that a much longer time would possibly be necessary to reach satisfactory results. Nevertheless, it was tested to evaluate if the efficiency of the microwave might speed up the reaction time. Considering that the main goal was the throughput improvement, it was preferred the use of a higher temperature (120 °C) instead of testing a longer treatment time, as long as no artifact's formation was proven. Fig. 2 shows the percentage FAMEs profile obtained using the reference method [1] and the MAED method both at 120 °C for 30 and 15 min. No significant differences were observed between the two times tested and the reference method.

An attempt was made to reduce the amount of solvent, reducing to half the amount of cyclohexane, but the partition between the two phases changed, altering the FAMEs profile compared to the reference method. Therefore, considering that a minimum amount of solvent is needed to guarantee a reliable temperature reading of the instrument and that the catalyst amount needs to be far in excess to guarantee a broad application, the final conditions used were 10 mL of methanolic HCl solution with 25 mL of cyclohexane at 120 °C for 15 min. The repeatability of the entire procedure was assessed as 7% on average for all

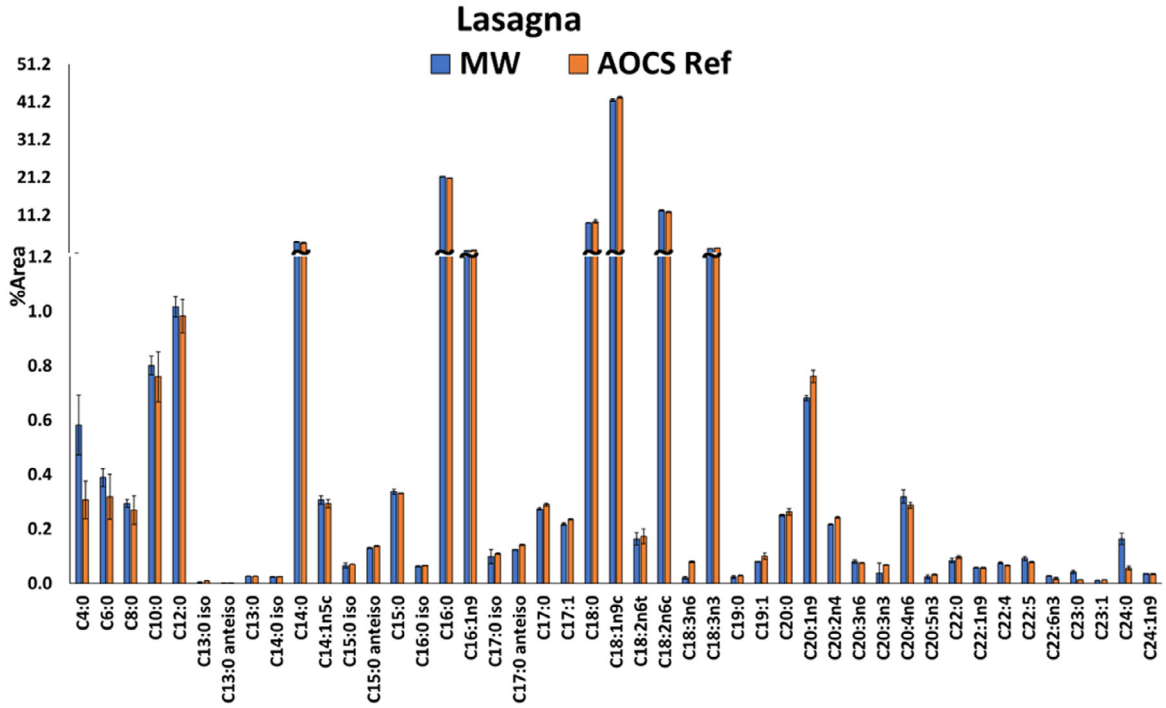

Fig. 4. Comparison of the FAMEs percentage profile of a sample of lasagna analyzed using the reference method (blue bars) [3] and the MAED at 120 °C for 15 min (orange bars). Error bars indicate the standard deviation of three replicates.

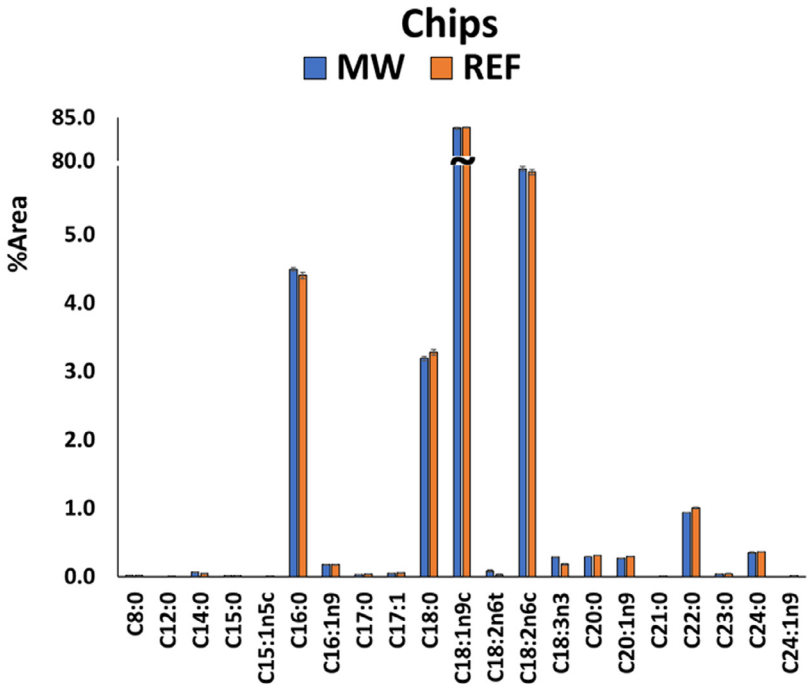

Fig. 5. Comparison of the FAMEs percentage profile of a sample of chips analyzed using the reference method (blue bars) [3] and the MAED at 120 °C for 15 min (orange bars). Error bars indicate the standard deviation of three replicates.

the FAMEs (median 5%). The pretreated milk sample was fortified with the Supelco C37 FAMEs standard mixture and the difference in peak volume corresponded to the added value within a 5% difference, which is well within the experimental variability.

The chromatographic separation obtained for the milk sample is reported in Fig. 1B and the complete comparison between the two methods (i.e., the reference method and the MAED one at 120 °C for 15 min) is reported in Table 1, along with the tentative identification of all the FAMEs.

3.2.2. *Comparison of the MAED method with the AOCS Ce 2b-11 method*

Considering that the goal was to obtain a general-purpose rapid method, a further comparison was performed with the Official Method Ce 2b-11 by the American Oil Chemical Society (AOCS), which applies to a wide variety of food commodities [3]. Different kinds of food samples were analyzed in triplicate with both methods in order to validate the comparison. Fig. 3–6 shows the bar plot comparisons of some of the samples analyzed with the two methods, namely the MAED one (blueish color) and the reference one (orangish color). The other samples are re-

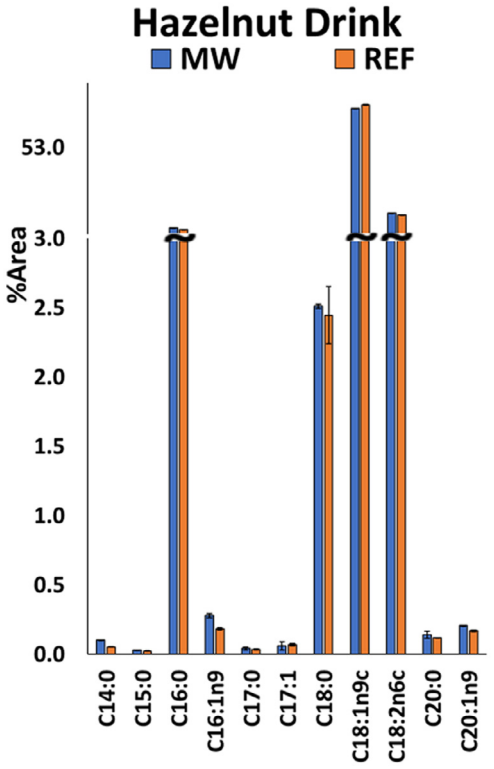

Fig. 6. Comparison of the FAMEs percentage profile of a sample of hazelnut drink analyzed using the reference method (blue bars) [3] and the MAED at 120 °C for 15 min (orange bars). Error bars indicate the standard deviation of three replicates.

ported in Supplementary Figure S2-S7. Also, the raw data for all the samples are reported in Supplementary Table S1. Error bars correspond to the standard deviation ($n = 3$). A *t*-test with Holm-Bonferroni correction was applied to compare the results statistically. In most cases, no significant differences (p-value > 0.05) were observed. Few exceptions refer to components present in low amounts (< 0.7%) and generally the response observed using the MAED procedure is greater than the reference method, suggesting a superior extraction performance. Exceptions to these findings were C18:3n6 in Lasagna and 18:1n9c in Cake.

In some matrices, the separation between $C_{18:1}$ *cis* and *trans* isomers (whether the *trans* was present) could not be observed because the *cis* isomer was present at a much higher concentration than the *trans* one and it was chromatographically overloaded. In these specific cases, the presence can be confirmed by repeating the injection with a dilute sample or a higher split ratio, as the separation efficiency was proven as shown in Supplementary Figure S1.

As an additional validation, the amount of SFA was calculated and compared with the nutritional label of each food sample (Fig. 7). The results of the two methods were in excellent agreement with the value reported on the nutritional label, except for a major discrepancy for peas and a slight one for the hazelnut drink. These differences are likely due to batch differences or to the use of a different method to obtain the nutritional label value.

3.2.3. Additional comparison for edible oil analysis

A final comparison was made with the official method for the characterization of the FAMEs profile in edible oil that consists in a rapid derivatization with KOH/methanol [31]. Although the proposed MAED approach is not comparable in terms of rapidity, the comparison was performed to ensure the reliability of the MAED method. This can be useful in the case where the analysis of edible oil samples would be needed along with samples of different nature. In such a case, it can certainly be convenient to use the same method, avoiding the preparation of dedicated reagents for only a few samples. The procedure was compared by analyzing 4 different edible oils, namely olive oils, sunflower oil, palm oil, and peanut oil, obtaining very comparable results (Fig. 8).

3.3. Green analytical considerations

The proposed MAED method coupled with the GC×GC-FID allowed for integrating multiple steps into a single one, promoting automation, minimizing energy consumption, using less toxic solvents, and being suitable for a wide variety of samples. Such aspects have been evaluated in comparison to the main reference method, i.e., *AOCS Official Method Ce 2b-11* [3], using the PrepAGREE metric [32,33]. The metric assigns a score for each of the 10 criteria of the green sample preparation approach, returning a pictogram to illustrate the final score given to the procedure. The default weights (as suggested in [33]) for each criterion were used, except for criteria 2 (use of safer solvent and reagents), 6

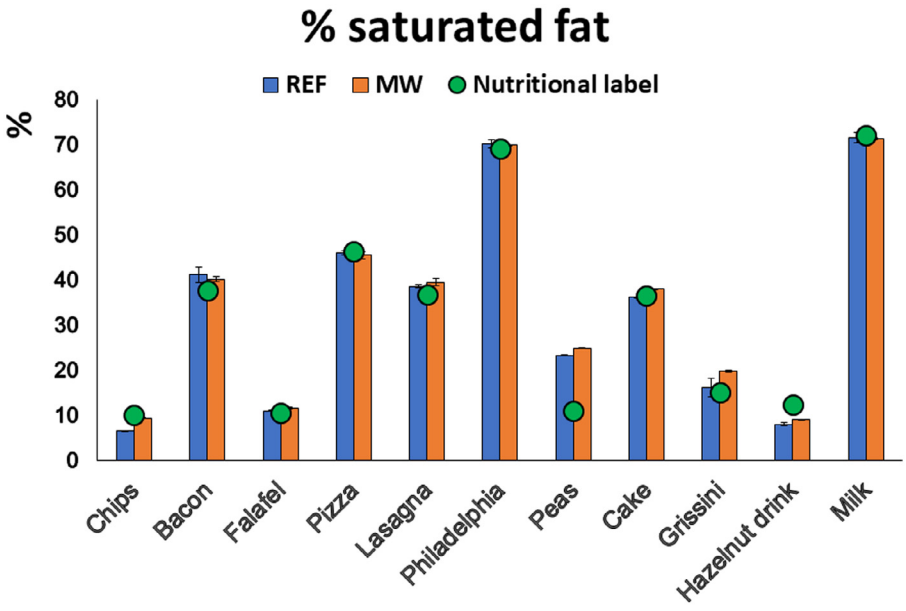

Fig. 7. Comparison of the% saturated fat calculated from the sum of SFA obtained using the reference method (blue bars) [3], the MAED at 120 °C for 15 min (orange bars), and compared to the data reported in the label (green spot). Error bars indicate the standard deviation of three replicates.

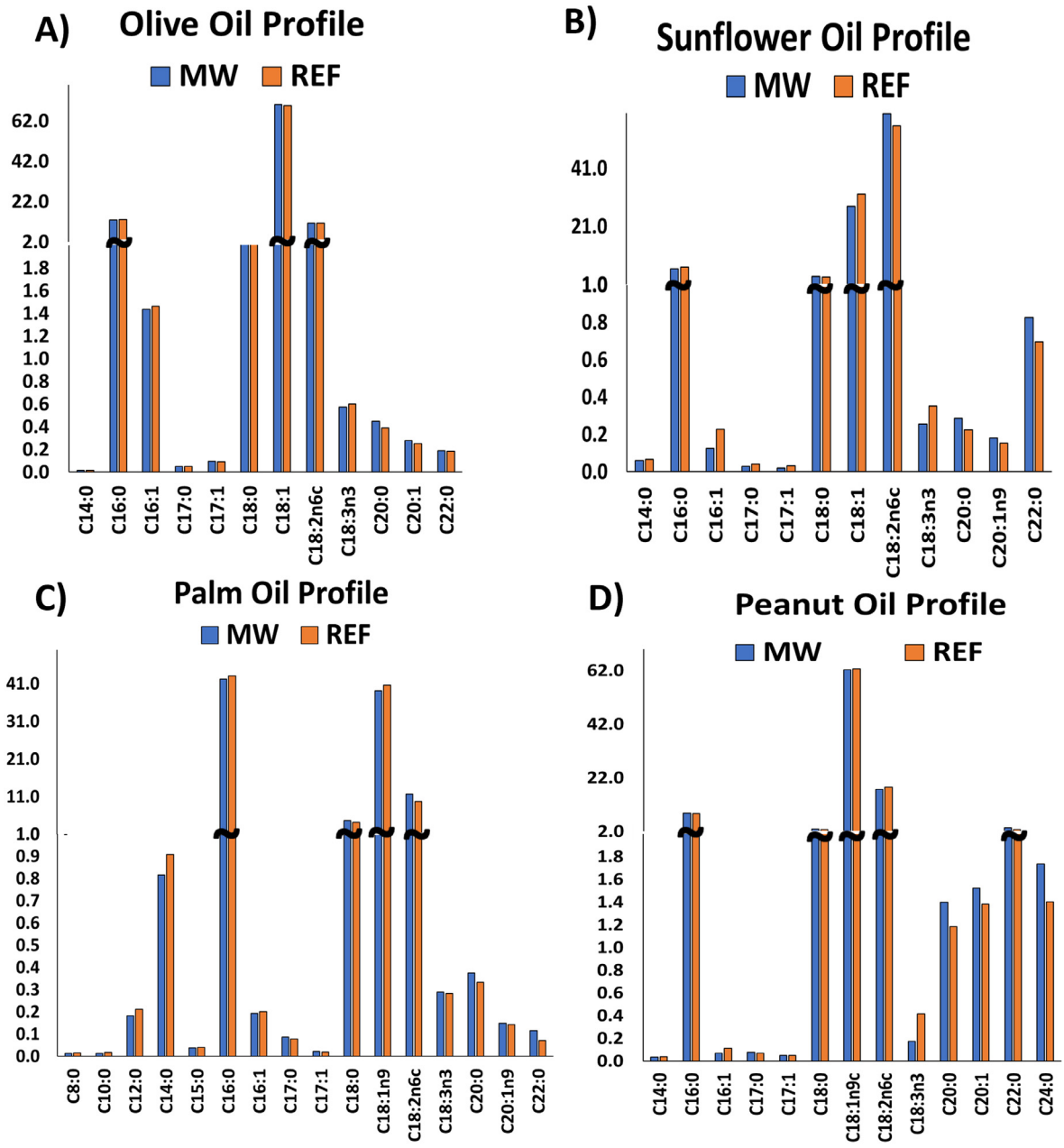

Fig. 8. Comparison of the% saturated fat calculated from the sum of SFA obtained using the reference method (blue bars) [3], the MAED at 120 °C for 15 min (orange bars). Error bars indicate the standard deviation of three replicates.

(maximize sample throughput) and 7 (integrate step and promote automation). The reasons for these modifications are herein explained:

I) Criterion 2: weight of 1 instead of 5 was used. Although still a rather high amount of solvent has been used in the MAED procedure, it used less toxic reagents than the reference method. This criterion is not able to capture this difference as it is mainly based on the volume used, while it is better reflected in criterion 10 (ensure safe procedure for the operator) as the number of hazards.

II) Criteria 6 and 7 were given a weight of 5 instead of 3 and 2, respectively, because the method's main goal was to maximize the throughput and integrate the extraction and derivatization steps. Therefore, these two criteria are the most relevant for our purpose. The main advantage of the proposed method regards the sample throughput. For instance, considering only the sample preparation, the MAED procedure allows to prepare simultaneously 12 samples every 15 min, so 48 per hour (or even more if the recently introduced rotor with 15 or 44 positions is used), while the AOCS method allows preparing 2 samples per hour as no automation is possible.

The final comparison of the two methods is reported in Fig. 9.

Unfortunately, in this metric the important role of GC×GC to increase the throughput by reducing the number of analyses required to obtain a holistic result cannot be included. As well as the enhance interpretation capability provided by this technique and the higher level of information. Actually, no metric considers the fundamental role of the analytical technique chosen for the final determination. The reduction of energy consumption may be the main goal but this should not be at the expense of the level of information provided by the analytical method. For instance, GC-FID is preferred over GC–MS in the green analytical approaches. It is indeed our opinion that the most accessible method that can provide the required information should be pre-

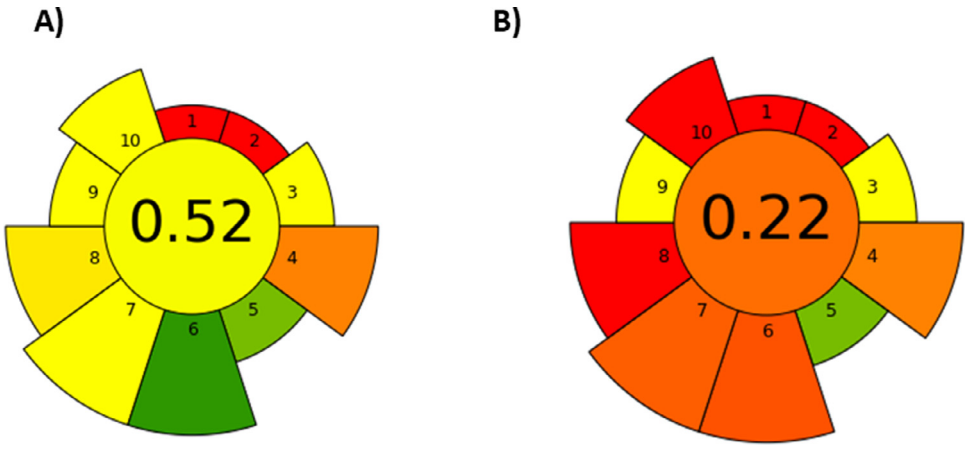

Fig. 9. Comparison of the greenest of the A MAED-GC×GC-FID method and B) the AOCS Official Method Ce 2b-11 official method.

ferred, but if a method can answer multiple questions at the same time, it should be favored even if it may seem less sustainable at first. The use of multidimensional chromatographic techniques falls within this situation. Leaving aside the discussion of the main advantages of comprehensive multidimensional techniques, and in this particular case of GC×GC, which are already widely reported [34,35], we wish to stress that the use of GC×GC significantly contributed to the improvement of the method throughput. Thanks to the use of GC×GC, the use of long chromatographic columns could be avoided, and a tentative yet reliable identification could be achieved without the need of an MS. In fact, thanks to the highly structured chemical pattern obtained on the 2D plot, it is possible to characterize with great detail any FAME profile based on the specific position in the chromatogram. Moreover, it is important to stress that the analytical determination (i.e., GC×GC-FID) and the sample preparation step (i.e., MAED) should be considered a whole method. It should be considered that a platform such as GC×GC can help overcoming the limitations of a simplified sample preparation step. In fact, the chromatographic system's highest sensitivity and orthogonal separation power allow for a more accurate and selective determination of the compounds of interest. Finally, a particular sustainable aspect of the proposed analytical platform is the use of a flow-modulator instead of a cryogenic one, which is more energetically sustainable.

4. Conclusions

FAs are routinely analyzed on food products to report some basic information about the nutritional content. Considering the health benefits and risks associated with the FA profile of dietary products, having more detailed information is certainly useful. Although the current methods of analysis provide reliable results, they are cumbersome and laborious. As a result, a complete FA profile is not always obtained. In this work we developed and validated a greener yet robust method for the analysis of FAs in food products. Indeed, using a single-step MAED combined with a fast GC×GC analysis allowed for a complete FA profiling in a short time (~30 min), with minimal sample and solvent consumption, and for multiple matrices. The results were comparable to those obtained by the methods currently in use.

It is noteworthy that the use of a flow modulator for the GC×GC analysis of the FAMEs made the analytical technique even greener and less costly. Moreover, the great separation power of GC×GC sufficed for the tentative identification of all FA without the need for MS hyphenation, simplifying the method even further and contributing to the acceptance of this technology for routine applications.

To sum up, the proposed method can be easily applied for routine analysis of FA profiling of food products of any kind, thus constituting a more sustainable and simpler yet reliable alternative to the traditional methods currently in use. Therefore, its application will contribute to the obtention of more detailed information about the nutritional value of food products.

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.

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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.2022.100039.

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