ISSN 1061-9348, Journal of Analytical Chemistry, 2015, Vol. 70, No. 10, pp. 1218–1224. © Pleiades Publishing, Ltd., 2015.

ARTICLES

Rapid Microwave Assisted Preparation of Fatty Acid Methyl Esters for the Analysis of Fatty Acid Profiles in Foods[1]

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Received June 03, 2014; in final form, February 09, 2015

Abstract—A microwave (**MW**) assisted rapid high through-put method for the preparation of fatty acid methyl esters (**FAME**) for the analysis of fatty acid profiles in a selection of foods was evaluated by comparing fatty acid profiles with those resulting from conventional FAME preparation. The microwave method gave fatty acid profiles in close agreement with those arising from conventional methods and the protocol gave acceptable recoveries (98–102%) and repeatability (RSDs for replicate analyses 0.56–5.2%). In comparison to conventional methods the MW assisted method was simple, rapid and universally effective across foods ranging from dairy products to ready meals. EU regulation (No. 1169/2011) requiring declaration of the saturated, monounsaturated and polyunsaturated content of foods is placing pressure on processors and contract laboratories to analyse fatty acid profiles using relatively low through-put conventional methods. MW assisted preparation of FAMEs offers analysts a high-through-put, rapid and universal method to help overcome this potentially costly and labour intensive regulatory hurdle.

Keywords: FAMEs analysis, fatty acid analysis, fatty acid profiling, fatty acid GC, fat analysis GC

DOI: 10.1134/S1061934815100044

Preparation of fatty acid methyl esters and their separation and quantification using gas chromatography is by far the most commonly used method for the quantification of fatty acids in foods and other natural matrices [1–3]. Fatty acid methyl esters are usually prepared by firstly saponifying esterified fatty acids to free fatty acids and then re-esterifying them to form methyl esters. The procedure was first established by James and Martin [4] and subsequently optimised by themselves and other authors [5, 6] with the aim of reducing the polarity of the free fatty acids thus making them easier to separate and quantify for the then newly developed gas-liquid chromatography systems. The saponification step is usually carried out by heat treatment of the foodstuff in the presence of a strong alkali such as potassium hydroxide. The proceeding esterification condenses the carboxyl group of the free fatty acid and the hydroxyl group of an alcohol. This step is usually carried out in the presence of a catalyst (such as boron triflouride) which promotes the reactivity of the oxygen atom on the carboxyl group by protonating it. An alcohol (usually methanol) then combines with the protonated acid to yield an ester with the loss of water and the catalyst is removed with the water. The methyl esters are then extracted into an organic solvent for separation and quantification by gas chromatography. Both the saponification and esterification steps of FAME preparation involve often lengthy incubation, and the subsequent removal of the often toxic catalysts that add to the labour and time required to carry out a procedure which is used routinely in many food analytics laboratories. BF_3 is a commonly used acid catalyst for methylation but it is harmful, and the use of boron and fluorine is often restricted due to environmental laws. In addition, the methanolic BF3 reagent has a limited shelf life [7]. Therefore there is a desire amongst analysts to simplify and speed up the procedure, and indeed some simplified rapid methods have been developed [8, 9]. However many of these still require the use of a catalyst and cannot be automated. Indeed further impetus has been added to the search for a rapid and simple method for FAME preparation by the publication of EU Regulation No. 1169/2011 which will come into effect in December 2014, which will require food producers to display saturated, monounsaturated, polyunsaturated, and cholesterol contents on their foodstuffs [10]. The legislation requires food producers to give more detailed information on many of the macronutrients present in their foodstuffs. For carbohydrates, protein and salt, analysis is straight-forward and rapid, whilst analysis of fatty acid profiles requires up to a full day work-up followed by gas chromatographic analysis of fatty acid methyl ester derivatives. The through-put limiting steps in FAME preparation are usually con-

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Table 1. Fatty acid methyl ester derivatization methods used in the present study and the products to which they were applied

Product	Reagents	Reference
Milk and butter	Sodium hydrocarbonate followed by boron triflouride	[18]
Infant formula	Saponification: 0.5% sodium methylate in methanol. Methylation: boron triflouride in methanol	[19]
Cooked chicken breast slices, cooked ham slices, uncooked sausages	Saponification: 5 M KOH in methanol : water (50 : 50, v/v). Methylation: 2 M trimethylsilyldiazomethane in *n*-hexane	[20]
Spaghetti Bolognese, lasagne, chicken curry	Saponification: methanolic NaOH at 100°C for 5 min. Esterification: BF_3–methanol reagent	[21]

sidered to be the saponification and esterification steps in which the energy required for the saponification is supplied by heat and the lowering of the activation energy for the esterification by the catalyst. It is well known however that microwaves can be used as an energy source for many chemical reactions and could therefore be used to supply the energy for the transesterification of fatty acids for GC analysis [11]. Indeed there is intense interest in the use of microwave to assist in the preparation of FAME's for biodiesel production [12–16]. Methods for the MW assisted preparation of FAME's for analysis of fatty acid profiles have been published as far back as 1998 [17], however the technique has received little attention, possibly due to concerns over microwave induced losses of very volatile compounds. This is despite the fact that recent interest in microwave assisted synthesis has led to the development of sophisticated, high through-put equipment capable of providing the energy to drive many reactions which previously could only take place via a catalytic route.

In the present study we examine the use of one such system to aid in the preparation of fatty acid methyl esters in a range of foodstuffs. FAME's were also prepared for these foods using conventional catalytic methods and fatty acid profiles prepared using two methods are compared in order to evaluate the efficacy, accuracy and ease of use of the microwave assisted method.

EXPERIMENTAL

Reagents. Potassium hydroxide, acetyl chloride, methanol, salt, pentane and Supelco 37 FAME standard mix were purchased from Sigma-Aldrich (Ireland). Three samples of all products for FAME analysis were purchased from a local supermarket on three separate occasions.

Conventional preparation of FAME's. As outlined in the discussion analysts have been using FAME derivitization to analyse fatty acids in foods since the late 1950's and a wide variety of procedures have been used in different foodstuffs. Therefore since the aim of the present study was to compare MW assisted FAME preparation with conventional methods on a wide range of foods, a selection of conventional methods for FAME preparation was used depending on the foodstuff under investigation as no universal method appeared to be available. Table 1 lists the conventional methods used and the reagents involved in the derivitization, detailed descriptions of the methods are available from the cited references.

Fatty acids were expressed as percent of total fat content of each of the foodstuffs. Total fat content was determined by extracting 1 g of sample with 30 ml of chloroform-methanol (2 : 1) containing 0.005% (v/v) butylated hydroxy toluene shaking for two hours following an overnight extraction at 4°C. The final extract was dried over sodium sulphate and it's weight determined.

Microwave assisted preparation of FAME's. Microwave assisted FAME preparation was carried out using a MARS 6 Express 40 position Microwave Reaction System (CEM Corporation, Matthews, NC, USA). Reactions took place in PFA 55 ml reaction vessels. For FAME preparation 1 g of wet / 0.5 g dry / 1 mL of liquid (e.g., milk) / 3 drops oil were added to the reaction vessel containing a 10 mm stir bar. To this 10 ml of potassium hydroxide (2.5%) in methanol was added and the reaction vessel was heated in the MARS 6 Express system to 90°C over 4 min and held at this temperature for 10 min. The reaction vessels were then removed from the spindle wheel and cooled on ice for 5 min or until they had reached room temperature before they were opened. The methylation was then carried out by adding 15 mL of 5% acetyl chloride in MeOH solution and heating to 120°C over 4 min and holding at this temperature for 2 min. The reaction tubes were removed again and cooled on ice to room temperature. To the cooled tubes 10 mL of pentane was added and the reaction tubes were mixed by placing over a heater stirrer for 2 min. Following this, 15 mL of a saturated salt solution (30%, w/v) was added, and the solution was mixed again as described above. After it had separated the top pentane layer was removed and aliquoted into amber GC vials (1.5 mL) containing sodium sulphate and stored at –18°C until required for analysis. For both microwave and conventional FAME preparation three replicate derivitisations were carried out on each product.

Gas chromatography-flame ionisation detector (GC-FID) analysis. Gas chromatography was carried

Table 2. Fatty acid profiles for a selection of dairy products as determined using microwave assisted or conventional (CON) derivatization to fatty acid methyl esters following by GC-FID separation and quantification

Fatty acid*	Milk		Infant formula		Butter	
	MW	CON	MW	CON	MW	CON
C4:0	2.47	3.5	ND**	ND	ND	ND
C6:0	1.98	2.23	0.07	0.05	ND	ND
C8:0	1.18	1.37	0.78	0.53	ND	ND
C10:0	3.18	3.03	0.59	0.59	2.94	3.77
C10:1	0.51	ND	ND	ND	ND	ND
C12:0	3.21	4.11	7.09	7.63	2.97	4.38
C14:0	10.97	11.62	3.42	3.08	10.73	12.73
C14:1	1.26	ND	3.42	3.08	ND	ND
C16:0	30.64	32.61	0.17	0.16	1.02	1.52
C18:0	8.81	8.73	24.2	26.30	35.48	37.48
C16:1	1.42	ND	0.33	0.18	3.05	2.05
C18:1	30.06	20.33	42.27	47.1	30.06	20.33
C18:2 UC	1.58	1.20	14.23	14.78	ND	ND
CLA total	0.31	0.39	0.55	0.45	ND	ND
cis-9, *trans*-11 C18:2	1.44	ND	1.68	ND	1.68	2.98
C18:3 total	0.61	0.41	1.11	1.64	0.85	1.54

* Fatty acids were expressed as a % of total fat content.

** ND – not detected.

out using a Clarus 580 Gas Chromatograph fitted with a flame ionisation detector. A Zebron ZB-5MS Capillary 60 m × 0.25 mm (i.d.) × 0.25 um (film thickness) column was used for the separation. The injection volume was 1 μL, at a temperature of 200°C. The oven was programmed to hold at 50°C for 5 min and was then heated at 5 grad/min to 240°C, and held for at this temperature for 20 min (run-time 63 min). The carrier gas was hydrogen at a flow of 1 mL/min, and the split ratio was set at 20. The FID was set at 260°C. Compounds were identified by comparing their linear retention indices with those of authentic standards from the Supelco 37 FAME. Fatty acids were expressed as a percent of total fat content of each of the foodstuffs

Gas chromatography-mass spectrometry (GC-MS). To confirm the identity of FAME's formed using MW assisted and conventional preparation GC-MS analyses was performed on a Varian 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph (Palo Alto, CA, USA) and fitted with an identical column to that used for GC-FID quantification of FAME's. Mass spectrometry conditions were as follows: source 230°C; acquisition performed in electron impact mode (70 eV) using 5 scans/s for the mass range 20–240. The temperature of the transfer line was held constant at 220°C. Compounds were identified by comparing their mass spectra and linear retention indices either with those of authentic standards from the Supelco 37 FAME mix or with published values in the NIST library [22]. A selection of samples from each of the three batches of food products purchased was used for the confirmation of the identity of the fatty acids presented in Tables 2–5.

RESULTS AND DISCUSSION

Reproducibility, speed and recovery. Relative standard deviations for replicate analyses of individual fatty acids across the range of products analysed using the microwave assisted method of FAME preparation ranged between 0.56–5.23% with a mean value of 2.22%. This compares favourably with RSD values ranging from 0.87 to 8.5% and a mean value of 3.9% for profiles determined using conventional methods. The apparent superior reproducibility of the MW assisted method may not be a function of the use of microwave catalysis as opposed to conventional catalysis per se but may have arisen as a result of the superior speed and simplicity of the method in comparison to the conventional method leaving less room for human error. For example using the microwave assisted method up to 40 samples can be derivatised to FAME's in 8 simple steps over the course of 1 to 1.5 h. In contrast preparation times for conventionally prepared samples ranged from 8 to 24 h with the number of steps involved ranging from 12 to 18. Conventional methods also employed lengthy incubations and toxic catalysts such as BF_3. Tricosanoic acid methyl ester (C23:0) was included as an internal standard to estimate levels of recovery for both MW and conventional FAME preparation methods and levels of recovery were acceptable and comparable for both methods of FAME preparation ranging from 98–102%. The iden-

Table 3. Fatty acid profiles for a selection of meat products as determined using microwave assisted or conventional derivitisation to fatty acid methyl esters following by GC-FID separation and quantification

Fatty acid	Cooked chicken breast		Cooked ham		Sausages	
	MW	CON	MW	CON	MW	CON
C10:0	0.48	0.5	ND	ND	ND	ND
C12:0	1.8	ND	0.06	ND	ND	ND
C14:0	2.52	2.72	0.92	0.98	1.37	1.38
C15:0	0.39	0.44	ND	ND	ND	ND
C16:0	22.5	23.8	21.06	21.59	23.43	23.43
C17:0	1.28	1.19	ND	ND	0.42	0.41
C18:0	13.4	14.64	12.59	12.5	12.98	13.01
C20:0	0.28	0.26	0.15	0.17	0.15	0.15
C16:1	2.2	2.44	3.06	3.01	2.2	2.44
C18:1	37.9	37.22	40.28	40.59	37.9	37.22
C20:1	0.34	0.33	1.52	1.59	0.22	0.24
C24:1	0.11	0.1	ND	ND	ND	ND
C18:2	12.25	13.16	15.49	15.2	16.21	16.92
C18:3	2.3	2.08	2.06	1.88	1.52	1.42
C18:4	0.4	0.38	0.52	0.58	ND	ND
C20:4	0.1	ND	0.92	0.93	0.49	0.47
C20:5	0.1	ND	0.38	0.41	ND	ND
C22:5n3	ND	ND	0.26	0.27	0.21	0.19
C22.6	ND	ND	0.31	0.31	0.09	0.08

Table 4. Fatty acid profiles for a selection of ready meals as determined using microwave assisted or conventional derivitisation to fatty acid methyl esters following by GC-FID separation and quantification

Fatty acid	Bolognaise		Chicken curry		Lasagne	
	MW	CON	MW	CON	MW	CON
C8:0	0.21	0.23	6.52	6.82	0.51	0.51
C10:0	0.22	0.24	4.22	4.57	0.41	0.37
C12:0	0.28	0.29	42.51	42.19	1.81	1.71
C14:0	1.85	1.9	12.47	12.42	5.17	5.47
C16:0	21.51	21.82	8.41	7.89	26.41	27.30
C18:0	9.32	9.01	2.88	2.87	15.42	15.22
C16:1	1.98	1.94	0.2	0.22	1.98	1.94
C18:1	54.75	53.81	15.4	15.63	54.75	53.81
C18:2	9.7	9.59	6.01	6.65	14.433	12.62
C18:3	0.69	0.72	0.88	0.84	2.29	2.39
C20:4	0.17	0.18	0.27	0.22	0.31	0.28
C20:5	0.12	0.13	ND	0.17	0.21	0.20
C22.6	0.29	0.27	ND	0.18	0.53	0.49

tities of all the methyl esters identified and quantified and presented in Tables 1–4 were confirmed by (1) comparison of the MS spectra of a selection of samples from each batch with those of authenticated standards in the Supelco 37 mix and spectra in the NIST library (2005) and (2) calculation of linear retention and comparison to literature values.

Dairy products. Table 2 presents fatty acid contents for three dairy products (full fat milk, butter and infant formula) as determined using either microwave assisted or conventional derivatization to their fatty acid methyl ester derivatives and separation and quantification by GC-FID. For milk samples fatty acid profiles for both MW assisted and conventionally prepared samples were quantitatively and qualitatively similar to those reported by Stoop et al. [23] and DePeters et al. [24] using a similar FAME preparation method to that used in the present study.

The major fatty acid in all milk samples was palmitic acid (C16:0) followed by oleic acid (cis C18:1) and

Table 5. Fatty acid profiles for a selection of peanut butter and olive oil as determined using microwave assisted or conventional derivatisztion to fatty acid methyl esters following by GC-FID separation and quantification

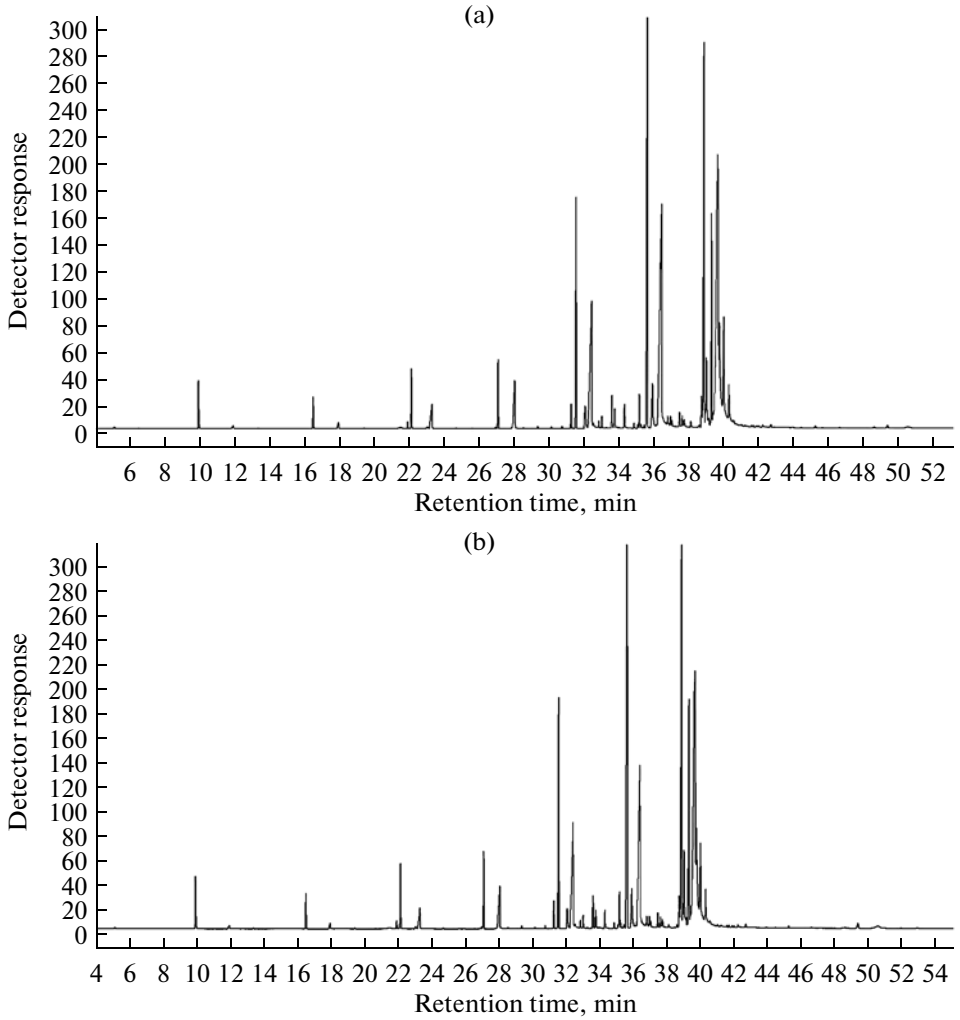
Fatty acid	Peanut butter		Olive oil	
	MW	CON	MW	CON
C14:0	0.32	0.38	0.05	ND
C16:0	9.95	10.18	11.90	11.70
cis-9 C16:1	1.77	1.69	0.79	0.85
C17:0	1.31	1.38	0.10	0.10
C17:1	0.25	0.29	0.10	0.10
C18:0	4.69	4.82	3.86	3.76
cis C18:1	47.16	48.31	75.98	75.98
cis C18:2	28.37	26.19	6.10	5.74
C18:3 total	ND	ND	0.80	0.76
C20:0	2.01	2.03	0.59	0.63
C20.1	0.69	0.72	ND	ND
C22:0	2.44	2.53	0.30	0.34
C22:1n9	0.35	0.37	ND	ND
C24:0	1.37	1.34	0.05	0.04

stearic acid (C18:0). There was also a high proportion of *trans*-oleic acid (8.13–8.81% of total fats). The fatty acid profile of MW and conventionally prepared FAME's of infant formula are in close accordance with those reported by a number of authors [25–28]. The major fats present in this case in descending order of abundance are oleic acid (cis C18:1), stearic acid (C18:0) and unconjugated linoelaidic acid (C18:2). It is worth noting that for both infant formula and butter samples butyric acid was not present, which is most likely due to heat induced losses in this fatty acid during heat processing. Figure a, b present GC-FID traces of fatty acid profiles derived from milk samples using either microwave assisted or conventional FAME preparation.

As is evident from the figure fatty acid profiles as measured using conventional and microwave assisted FAME preparation were qualitatively and quantitatively similar. For all dairy samples percentage differences between MW and conventionally prepared samples ranged from 4.3 to 8.1%. Whilst this may appear to be high, fatty acids with large percentage differences to conventionally prepared samples were those present in the least abundance and thus a small quantitative difference resulted in large percentage difference. In addition the mean percentage variation across all dairy samples was relatively low at 5.6%. It was also apparent that there is little difference in the levels of short chain fatty acids such as butyric and valeric acid between MW assisted and conventional prepared samples. This indicates that the MW assisted protocol did not induce higher losses of these highly volatile compounds than those prepared conventionally despite the high temperatures involved in the heating steps.

Meat products. Fatty acid contents for three meat products as determined using either microwave assisted or conventional derivatization to their fatty acid methyl esters derivatives and separation and quantification by GC-FID are presented in Table 3. For meat samples fatty acid profiles for both MW assisted and conventionally prepared samples were quantitatively and qualitatively similar to those reported by Santos et al. [27] using a similar conventional FAME preparation method to that used in the present study. Similarly fatty acid profiles for cooked chicken breast and breakfast sausages were qualitatively and quantitatively similar to those reported by Cortinas et al [28] and Juarez et al. [20] respectively. As expected in all meat samples, oleic acid was the most abundant fatty acid present, representing over 40% of the total fats present in cooked ham. Palmitic acid was the next most abundant fatty acid followed by linoleic acid. Similar to dairy samples there was relatively little variation in levels of reported fatty acids in MW assisted versus conventionally prepared samples. The percentage range in variation between conventionally prepared and MW assisted samples ranged from 4.4 to 7.76% with higher levels of variation being detected for samples present in relatively low amounts as noted for dairy samples. The mean difference to values determined using conventional FAME preparation across all meat samples was 5.7%.

Prepared ready meals. Hectic consumer lifestyles has led to a strong demand for pre-prepared ready meals. For example in the UK the market was worth £3.7 billion in 2013 and is forecast to grow by 28.4% to a total of £4.9 billion in 2017. Prepared ready meals represent a challenge for the determination of fatty acid levels consisting as they do of a mixture of fatty acids derived from a range of sources resulting in a very complex profile. Nonetheless the saturated, unsaturated, monounsaturated and polyunsaturated fat content of these products will have to be declared according to new EU regulations [10]. Table 4 presents the



Sample GC-FID chromatograms of fatty acid profiles of full fat milk samples as analyzed using either microwave assisted (a) or conventional (b) FAME preparation and separation and quantification by GC-FID.

fatty acid profiles of three of the most popular chilled ready meals currently on the market as determined using either conventional or MW assisted FAME preparation.

It is difficult to adequately compare profiles presented here to those reported by other authors as recipes and fatty acid composition vary hugely worldwide for these types of products. However the proportions of saturated/polyunsaturated, *trans*- and *cis*-fats are broadly in line with those reported in United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference [29]. In addition, levels of difference between profiles determined by MW assisted method were in close agreement with those determined using conventional FAME preparation (difference between MW and conventionally prepared FAME was between 4.7 and 7.6%).

Olive oil and peanut butter. Many analysts determine the fatty acid profiles of the two products above as a means of checking the accuracy of the fatty acid determination assay as they both have relatively standard compositions. In the present case the profiles reported are in keeping with those published by El-Rawas et al. [30] and Stefanoudaki et al. [31] for peanut butter and virgin oilve oil, respectively (Table 5).

It should be noted that the high fat content in these products necessitated that the FAME mixtures prepared by both the conventional and MW assisted method had to be diluted 20 fold prior to injection onto the GC to prevent overloading the column, peak broadening and a loss of resolution (as was the case for butter samples). In agreement with numerous studies by other authors [32], oleic acid (C18:1) was by the far the most abundant fatty acid rep in virgin olive (≈75%) regardless of the FAME preparation method. Oleic acid (≈47–48%) was also dominant in peanut butter samples, followed by linoleic acid (≈28%) and palmitic acid (≈9.9%). Again a relatively small percentage variation in fatty acid levels, as determined using conventional versus microwave assisted preparation, was

detected (4.7 and 6.0% difference for peanut butter and olive oil, respectively).

Fatty acid profiles for MW assisted and conventionally prepared FAME were qualitatively and quantitatively similar for a range of food products. The recovery and reproducibility for both MW and conventionally derivatized FAME's were acceptable and of a similar order. In comparison to the conventionally prepared method, the MW method is rapid, simple and does not require the use of toxic catalysts with the potential for artefact production. For example, using MW method up to 40 samples could be derivatized in under 2 h in comparison to up to 24 h using the conventional methods. The MW assisted method also has the advantage of being universally effective for samples ranging from meat to dairy to complex foods such as ready meals. It is also worth noting that while in the present study total fat content was determined using a solvent extraction technique, systems are available which can carry out this relatively cumbersome step rapidly and with a high through-put with the added advantage of being Association of Official Agricultural Chemists (AOAC) accredited for both milk [33] and meat products [34].

ACKNOWLEDGMENTS

The authors would like to acknowledge the assistance of Joseph Woods O'Rourke and Dr. Sabine Harrison in the preparation of MW assisted FAME's and GC-FID analysis of same.

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