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New Analytical Methods

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Development and Validation of a Novel Free Fatty Acid Butyl Ester Gas Chromatography Method for the Determination of Free Fatty Acids in Dairy Products.

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ABSTRACT: Accurate quantification of free fatty acids in dairy products is important for both product quality control and for legislative purposes. In this study a novel fatty acid butyl ester method was developed where extracted free fatty acids are converted to butyl esters prior to gas chromatography flame ionization detection. The method was comprehensively validated to establish linearity (20-700 mg/L, $R^2 > 0.9964$), limits of detection (5-8 mg/L), limits of quantification (15-20 mg/L), accuracy (1.6-5.4 % relative error), inter-day precision (4.4-5.3 % relative standard deviation) and intra-day precision (0.9-5.6 % relative standard deviation) for each individual free fatty acid. Seventeen dairy samples were analyzed covering diverse sample matrices, fat content and degrees of lipolysis. The method was compared to direct on-column injection and fatty acid methyl ester methods and overcomes limitations associated with these methods such as either column phase absorption or deterioration, accurate quantification of short chain free fatty acids and the underestimation of polyunsaturated FFA.

INTRODUCTION:

Free fatty acids (FFAs) are important components in dairy fat as they have a range of potential attributes; flavor development, anti-microbial activity, nutrition and functional characteristics[1,2]. The accurate quantification of FFAs in dairy products is also important for quality control, legislative purposes, authentication, food research and product development. Therefore it is important to have an

accurate, reliable and robust method that can be used to quantify FFAs in all dairy products. Some difficulties arise in relation to the quantification of FFAs in dairy products, which relate to the wide variation in fat content (~0.5 to >80 %), degree of lipolysis (0.5 to 82 %), and because of the differing solubility and volatility of FFAs based on chain length[1]. Therefore fat extraction techniques need to be able to take into account these differences and efficiently extract both water soluble short chain FFAs and organic soluble medium and long chain FFAs. A method should also avoid the use of an evaporation step to prevent losses of volatile short chain FFAs and remove or negate any water that may be present in the sample as it can interfere in the extraction of water soluble FFAs. The most common approach to quantify FFAs is the use of gas chromatography flame ionization detection (GC-FID), which is likely to remain the case as the equipment is relatively cheap, simple, robust, reproducible, widely available and arguably more accurate than mass selective detectors for quantification purposes. While there have been some recent advances in methodology, which have been reviewed[1], development in FFA determination in dairy products has been largely lacking for the past several decades.

The two most commonly utilized GC-FID methods to date, fatty acid methyl esterification (FAME) where the FFAs are esterified in the GC injector to methyl esters using tetramethylammonium hydroxide as a catalyst[3] and direct on-column injection where the isolated FFA extract is injected directly into the GC[4], were assessed for linearity, accuracy, limits of detection (LOD) and limits of quantification (LOQ) by Mannion et al.[5]. Prior to this study, these two methods have been in existence for the past 20+ years without a comprehensive assessment of their quantitative suitability for use with a diverse range of dairy samples matrices that are typically tested for in regulatory and food testing laboratories. The direct on-column approach had lower levels of LOD and LOQ, mainly because the FFAs are directly injected onto the GC as opposed to a split injection used in the FAME method. However, both methods had significant disadvantages. The direct injection on-column method results in accumulative deterioration of the column phase and irreversible FFA absorption[5], which adversely impacts method robustness and the quantification of some longer chain FFAs. In the FAME method,

butyric acid co-eluted with the injection solvent impacting on its quantification, especially at low concentrations. Artifact peaks, which are believed to be a result of by-products from the tetramethylammonium hydroxide (TMAH) reaction, were found to interfere with the quantification of other short-chain FFAs. Also, losses of polyunsaturated FFAs were observed because of the strong alkaline nature of the TMAH reagent[5].

This study was undertaken to create a new GC-FID method that overcomes existing limitations of the FAME and direct on-column methods, but that is also robust, accurate and with a low LOD and LOQ. We believe that combining aspects of both the FAME and direct on-column methods offers the best potential. The use of solid phase extraction to isolate the FFAs from glycerides (mono-, di and tri-glycerides) as used in the direct on-column method[4] provides a relatively pure FFA extract. This can be converted to butyl esters instead of methyl esters to potentially overcome issues with losses of short chain volatiles during evaporation steps, and with issues related to water solubility and co-elution with the injection peak, as these butyl esters are of higher molecular weight, more non-polar, less volatile and less water soluble. Butyl esters have been previously used to quantify short chain FFAs in dairy products[6,7] and for the determination of FFAs in milk fat[8] and cheese[8-11].

Very little detailed information on the analysis of FFAs in dairy products exists, especially in relation to method validation, accuracy, precision and robustness. The term “dairy” encompasses a very wide and diverse range of products and most methods described are limited in their application to specific dairy products. Therefore having a single quantifiable robust method would be a significant analytical advancement and of great benefit for both regulatory and food testing laboratories. This study covers the validation of a fatty acid butyl ester (FABE) method for the quantification of FFAs in a range of dairy products and compares it to the existing widely used FAME and direct on-column methods.

MATERIALS AND METHODS

Solvents, standards and samples were sourced as per Mannion et al.[5]. Infant formula, milk powder, and enzyme modified cheese (EMC) powder samples were stored under nitrogen in sealed containers at room temperature. Milk, yoghurt, butter, EMC paste and ice cream samples were transferred into sterile containers which were frozen at -18 °C until required. All cheese samples were vacuum packed and frozen at -18 °C. All samples were stored in dark conditions.

FABE analysis was carried out on a Varian CP3800 gas chromatograph (Aquilant, Dublin 22, Ireland) equipped with a CP8400 auto-sampler and flame ionization detector. The column was a CP FFAP CB capillary column (25 m x 250µm I.D., 0.32 µm phase thickness - Agilent Technologies, Ireland). A Varian 1079 programmed temperature vaporizer (PTV) injector was used and held at 250 °C for the entire run. A split of 1:50 was used. The inlet liner was a split/splitless wool packed liner with a volume of 250µl. An injection volume of 0.7µl was used, which was calculated to yield a vapor cloud of 208µl. The column oven was held at 40 °C for 2 min and raised to 240 °C at 7.5 °C/min, which was held for 5 min. The total runtime was 33.67 min. The FID was operated at 300 °C. The carrier gas was helium and was held at a constant flow of 1.2 mL/min.

A 7696A Sample Prep Workbench (Agilent Technologies, Little Island, Cork, Ireland) was employed in the preparation of all standards for calibration and validation studies, and for the derivitization reactions (butyl ester derivitization using BF_3 in butanol as a catalyst). This instrument has been applied by Mannion et al[5] in previous work for FAME analysis. It was configured with a 100 µl gas tight syringe in the back tower, and a 500 µl gas tight syringe in the front tower.

Lipid extraction and solid phase extraction were carried out as per the procedure outlined by Mannion et al.[5]. To briefly describe, lipids were extracted from the sample using heptane/diethyl ether (1:1 v/v). Solid phase extraction was carried out on this extract to isolate the FFAs from the rest of the lipid mixture, using 5 mL of 2% (v/v) formic acid in diethyl ether.

The FFA extract (60 µl) was transferred into a capped 2 mL amber GC vial containing a 400 µl glass insert. BF_3 in butanol reagent (60 µl) was added to the sealed vial containing the FFA extract. This was

vortexed for 10 seconds and then was heated at 80 °C for 1 h. After heating, 30 µl of deionized water and 30 µl of heptane were added and further vortexed for 1 min. Phase separation occurs between the aqueous and organic layer (butyl esters reside in organic layer). An aliquot of 80 µl of the organic layer was transferred into a capped 2 mL amber vial with a 400 µl glass insert containing 200 µl of saturated NaCl solution and vortexed for 1 min. After mixing, 60 µl of the upper layer containing the butyl esters was transferred into a capped 2 mL amber vial containing a 250 µl glass insert. A 0.7 µl aliquot of this solution was sampled for GC analysis.

The extraction procedures described (lipid extraction followed by solid phase extraction) were applied to six replicates (n=6) of each sample. Three of these replicates were spiked with 0.5 mg of each FFA (C4:0-C18:3), this was done by adding 0.5 mL of a solution containing each FFA at 1000 ppm concentration in heptane, with the exception of the EMC samples where 5 mL of this solution was added. These spiked samples were used for recovery determinations by comparing the difference in measurements against the un-spiked samples. The collected 5 mL extracts of 2 % formic acid/diethyl ether (FA/DE) from the solid phase extraction step were treated according to the derivitization protocol as described, and analyzed by GC-FID. For samples where the concentration was greater than the calibration range, the extract was diluted and re-analyzed until within range.

All standard mixtures, which were used for instrument calibration and method validation studies; were prepared as described by Mannion et al.[5]. These were prepared using the Nu-Chek certified FFA calibration mix, which contained FFAs C4:0-C18:3. The standards were collected in 2 % FA/DE solution, converted to butyl esters using the protocol described, using the sample prep workbench (Agilent Technologies Ltd, Ireland). The internal standard (ISTD) (C5:0, C11:0 and C17:0) was added during the dilution step. Five point calibration curves were established with a concentration range of 20-700 ppm for all FABEs with the ISTDs at a concentration of 200 ppm. Calibration curves were established based on the correction factors outlined in Mannion et al.[5].

For analysis of each dairy product, a batch consisted of sixteen samples (n=16). This comprised of three un-spiked samples and three spiked samples for recovery determinations; totaling six samples (n=6), with each sample derivitized in duplicate (n=12). Four standards were included with each sample batch (n=16) to monitor inter-day precision, as part of the method validation study. Intra-day precision was also monitored, where ten standards (n=10) were analyzed sequentially in one day.

RESULTS & DISCUSSION

Method Validation

The linearity was investigated where the FFAs were collected in 2 % FA/DE as per standards and derivitized to butyl esters. The linear range was established at 20-700 ppm. The calibration data obtained where line equation, correlation coefficient and linear range are illustrated in Table 1. Linearity was obtained over these concentration ranges where correlation coefficients of ≥0.996 were achieved for all butyl esters. LOD and LOQ were evaluated based on the signal to noise ratio. LOD was established at 3 times and LOQ at 10 times the signal to noise level (Table 1), which was 5 ppm (LOD) and 15 ppm (LOQ) for most FFAs. The exceptions were C4:0-C8:0, which were at 8 ppm and 20 ppm for LOD and LOQ respectively.

Precision was monitored over the course of the analysis by analyzing FFA standards (C4:0-C18:3) at 100 ppm concentration in 2% FA/DE, which were converted to butyl esters using the sample prep workbench (Table 1). Percentage (%) relative standard deviation (RSD) is used to express precision, which is calculated by the following formula:

$$\% \, RSD = \frac{SD}{x} \text{ x } 100$$

Where SD is the standard deviation and $\bar{x}$ is the mean. Intra-day repeatability was established by analyzing ten samples (n=10) in one day with 0.9-5.6 % RSD achieved across all butyl esters. The inter-day study was structured so that precision was monitored during the analysis of the dairy products. Four

FFA samples were analyzed in the same batch with each dairy product (n=72), which were analyzed over the course of approximately 1 month for the inter-day study. Excellent repeatability, 4.4-5.4 % RSD, was achieved across all esters.

The accuracy of the method was expressed as the relative error (RE) of each FFA across the concentration range 20-700 ppm prepared in 2 % FA/DE, with the acids subsequently converted to butyl esters prior to analysis. Table 1 shows the average error across this concentration range, a more detailed breakdown is shown in Table S-1 (Supporting Information). The RE is based on the true value of the butyl ester in solution compared with the value obtained from the analysis. The average values were obtained from the analysis of 9 calibration curves, along with the replicate RSD values. Across all the esters the highest error was 5.6-19.5 % at 20 ppm, the RE % is significantly reduced at the higher concentration range (100-700 ppm) yielding 0.2-5.0 % across all esters.

Application to Dairy Samples

A typical chromatogram of FABE analysis is shown in Figure 1 (Special reserve Cheddar cheese sample). The complete separation of C4:0-C18:3 butyl esters are evident. The results obtained from the FABE method for all samples are displayed in Table 2. The study by Mannion et al.[5] employed a direct on-column approach and FAME approach to analyze FFAs in these same samples. A summary of results obtained from these methods compared to the FABE method are displayed in Table 3. Detailed results are shown in Tables S-2 to S-4. Values reported are the average values obtained from six replicates (n=6). Recovery was determined by measuring the difference between samples spiked with 0.5 mg of each FFA (C4:0-C18:3), except for the EMCs where 5mg was added, and un-spiked samples.

For the cheese samples (Brie, processed cheese, light Cheddar, mild Cheddar, special reserve Cheddar and Blue Stilton) total FFA recoveries were >95 % with the exception of processed cheese where a total FFA recovery of 85 % was obtained. When reviewing the individual recoveries for processed cheese the

majority of the FFAs achieved >92 % recovery with the exception of C14:0, C16:0, C18:0, and C18:1, where a much lower recovery (>46 %) was achieved. The processed cheese sample showed some variation in the recoveries obtained between the direct on column (88 %), FAME (102 %) and FABE (88 %) methods. Also there was a higher variation in the measured ppm concentrations between the 3 methods; 1246 ppm for FABE, 1104 ppm for the direct on-column and 952 ppm for the FAME (Table 3) for processed cheese (RSD 13.4 %), when compared to the other cheeses analyzed (5.2-9.7 % RSD). Processed cheese is not a natural cheese and contains emulsifiers which may have adversely impacted fat separation and thus isolation of the FFAs from the lipid extract, either during the solvent extraction and/or solid phase extraction steps. For the solvent extraction, diethyl ether/heptane (1:1 v/v) was used. Increasing the ratio of the non-polar heptane to the more polar diethyl ether, might improve the recoveries of the longer chain FFAs, however this could potentially reduce the recoveries of the short chain acids. Despite the lower recoveries obtained for the longer chain acids, consistent results were achieved with an RSD 1-7 % across the range of FFAs. While complete recovery of FFAs greater than C14:0 was not achieved from the processed cheese sample, the method displayed analytical precision. FFA concentrations varied considerably across the different cheese types.

Infant formula, milk powder and yoghurt samples achieved total recoveries of >94 %, although the short chain FFAs were below the established limit of quantification (LOQ 20 ppm). Total FFA concentrations were relatively low in the powder and yoghurt samples, at 296 ppm and 217 ppm for milk powder and infant formula, respectively, and at 223 ppm for yoghurt. Concentrations of 661 ppm and 90 ppm were determined for ice cream and milk, respectively. However, the analysis of the milk and ice cream samples had a high RSD for some individual acids (45 % for C14:0 in ice cream and 23 % for C16:0 in milk) and poor recovery for others (133 % for C16:0 and 165 % for C18:0 in ice cream). As highlighted by Mannion et al.[5], the extraction method employed doesn't seem to be effective in obtaining complete recovery of all FFAs from these samples (ice cream and milk) nor does it seem capable of achieving consistent results. This is demonstrated in Table 3 where 51.4 % and 64.3 % RSD was obtained for milk

and ice cream respectively between the three methods. Despite what seems to be excellent total recoveries for the FABE method (109 % and 98 % for ice cream and milk respectively), the inconsistency of the analysis demonstrates that the extraction procedure is not suitable for the FFA analysis of these samples. This is most likely a result of the fact that the milk fat globule membrane is largely intact potentially inhibiting the solvent during fat extraction. The addition of a suitable solvent to disrupt the lipoprotein complex is necessary, such as the use of ethanol as described in the analysis of milk by De Jong and Badings[4].

For the butter samples high recoveries (90-106 %) and excellent RSD (1-6 %) values were obtained across all butyl esters. The FABE method seems accurate and precise with high recoveries, however when the results are compared (Table 3) to those achieved by the other methods, there was a significant difference. The FFA concentrations for butter by the direct on-column, FAME and FABE methods were 1492, 1295 and 1960 ppm, respectively. These differences could not be solely attributed to instrument variance or accumulative absorption of FFAs on the column phase. Further investigation highlighted the presence of glycerides in the FFA extract for this sample type. The solid phase extraction step describes the use of 10 mL of 20 % DE in hexane to remove glycerides from the column prior to collecting the FFAs in 5 mL of 2 % FA/DE. As butter has a very high fat content (~ 80 %) it seems that the washing step was insufficient in removing all the glycerides from the solid phase extraction cartridge. As an acidic catalyst BF_3 is capable of esterifying FFAs as well as trans-esterifying glycerides, thus if FFAs and glycerides are not completely separated, glycerides will inadvertently be converted to butyl esters and quantified as FFAs. To confirm the presence of glycerides in the butter extract after solid phase extraction, the use of TMAH was employed. TMAH can be used to form methyl esters of both FFAs and triglycerides in the same solution[3], by forming ammonium salts of FFAs in an aqueous layer and forming methyl esters of glycerides in a separate organic layer. The ammonium salts of FFAs are subsequently converted to methyl esters within the GC injector without any interference from glycerides. This derivitization procedure was carried out on the butter FFA extracts with the resulting

organic layer analyzed by GC-FID. Figure S-1 is a chromatogram obtained from the analysis of the organic layer after TMAH derivitization of a butter FFA extract. While this is only a qualitative determination, fatty acid peaks were evident and had to result from the presence of glycerides. A comparison of these peak areas against methylated FFA extracts (Figure S-2) confirms considerable responses, indicating the presence of glycerides. Thus these glycerides are converted to butyl esters resulting in the over estimation of FFAs in butter when using the FABE method. This was an unforeseen outcome of the analysis and has not previously been reported. Thus for FABE analyses of butter samples the solid phase extraction step needs to be modified to ensure complete removal of the glycerides prior to derivatization. This could be easily achieved by increasing the amount of solvent during the washing steps prior to collecting the FFAs in the 2 % FA/DE solution.

Out of all the samples analyzed in this study the EMCs had the highest FFA concentrations ranging from 44,712–131,622 ppm. Recovery determinations for the powder samples were greater (88 % and 93 % for butter powder and cream powder respectively) than for the paste samples (47-82 %). The recovery of longer chain FFAs (>C14:0) was lower in the EMC samples and was even more pronounced for the paste samples. The lowest total recovery was achieved for the natural blue cheese paste samples at 47 % with the cream paste and butter paste at 70 % and 82 %, respectively. It was difficult to form a reliable conclusion on the recovery determinations reported in Mannion et al[5], where some of the recovery determinations calculated were not dependable, e.g. -27 % (C18:1 from nat. blue cheese paste) and 177 % (C16:0 from cream paste). This was believed to be partly a result of the accumulative absorption of FFAs onto the column phase because of the extremely high concentrations of FFAs in these samples. Given that this issue was not experienced with butyl esters, it must be considered that the recovery losses were because of the practical aspects of the extraction method and not a result of issues with the instrument analysis. After a review of the area responses it seems overloading of the aminopropyl columns occurred with the spiked EMC samples because of the very high concentration of FFAs present. For recovery determinations where a known amount of FFAs (C4:0-C18:3) was added to

the sample, it was necessary to add a large amount of this FFA mix (5 mg of each FFA) to the EMCs, where only 0.5 mg of each FFA was sufficient for the other sample types in this study. This was necessary to ensure that sufficient measurable differences in area responses could be achieved between the spiked and un-spiked samples, especially when the measured concentration was outside the linear range of the analysis and a dilution of the sample extract was employed. A consequence of this however was that the capacity of the aminopropyl cartridges was exceeded resulting in a loss of FFAs. There are obvious solutions to this problem; a further reduction of the EMC sample volume (0.5 g was used in this study) and/or greater capacity aminopropyl columns.

The application of the FABE method yielded excellent RSD values for most samples, with the cheese samples (1-7 % RSD), EMCs (1-8 % RSD), infant milk formula (2 % RSD), milk powder (5 % RSD), butter (2 % RSD) and yoghurt (8 % RSD) all displaying excellent consistency between the replicates. As discussed, the analysis of ice cream and milk yielded poor results between the replicates. Although ice cream obtained 12 % RSD for the total FFA measurement, when the individual measurements of FFAs were studied higher RSD values were observed between the replicates. RSDs of 21 %, 26 % and 45 % were obtained for C10:0, C12:0 and for C14:0, respectively. Milk yielded 33 % RSD for the total FFA measurements, the individual FFA RSD measurements ranged from 6-37 % with 101 % RSD being obtained for C18:3 butyl ester replicates. For the remaining samples (Cheese, EMCs, powders, butter and yoghurt) the FABE method displayed consistency in its application. The lipid extraction, solid phase extraction, derivitizing to butyl esters using automation, and the analysis of the butyl esters were all performed with excellent precision (1-8 % RSD).

FABE verses Direct On-Column and FAME

The effect of the BF_3 solution on column integrity was a potential concern because of its acidic nature, because of experiences with the direct on-column method with formic acid[5]. Figure 2 displays chromatograms of two typical calibration standards at different stages of column lifetime (~120

analyses apart) using the FABE method. No retention time shift was evident. The only retention time shift experienced was after routine maintenance of the instrument (septa and liner replacement, column cutting) performed every 200 injections. The lack of any significant adverse impact of the BF_3 solution on the column phase was likely a result of the split ratio (1:50) and low injection volume (0.7 µl). The butyl ester method is significantly more robust than the direct on-column method. The issue of sample carryover was a major drawback of the direct on-column method, necessitating the employment of formic acid blanks between every injection, which exacerbated column degradation, retention time shifts and increased overall analysis times. It was evident from preliminary analysis butyl esters did not yield any sample carryover, even when handling samples with very high concentrations of FFAs. Figures S-3 and S-4 show the chromatogram of a blank injection post a 700 ppm FFA calibration mix after the direct on-column method and FABE method, respectively. FFA accumulative column phase absorption is clearly evident in the direct on-column method with no peaks present in the FABE method.

A main advantage of the direct on-column method over the FAME analysis was the resolution of all fatty acid peaks including C4:0[5]. Also no additional reagent is required for the FFA extract prior to analysis and therefore there are no resulting artefact peaks. For the FAME method methyl butyrate eluted very close to the solvent peak and there was an artefact peak (5 min RT) believed to be trimethylamine[5] that interfered with analysis at low concentrations (<20 ppm) (Figure 3). In the case of the FABE method (Figure 4) butyl butyrate elutes (8.8 min RT) after butanol (~7 min RT) despite the fact that butanol is in excess for the butyl derivitization step; thus, no interference is evident. BF_3 also created artefact peaks (8.6 min and 8.9 min RT), that eluted very close to butyl butyrate but did not interfere with quantification. For validation the LOD was reported at 3 times signal to noise and LOQ at 10 times signal to noise. At these thresholds LOD was established at 8 ppm and LOQ at 20 ppm for C4:0 for the FABE method. This was also compared against serial dilutions of a butyl ester sample and it was found that quantifiable results were achieved as low as 5 ppm (Figure S-5), despite these

responses being below the LOQ threshold (Table S-5). Thus, quantifiable results for the butyl esters can be obtained at low concentrations, in contrast to the FAME method where the C4:0 methyl ester could not be reliably quantified below 20 ppm.

FABE as an Alternative for Determination of FFAs in Dairy

The FABE method overcame the significant limitations of the direct on-column method relating to FFA accumulation onto the column phase and column phase degradation allowing for a more robust analysis with stable retention times and longer column life. The analysis of FFAs as esters seems to be a logical solution to issues of sample carry over and column degradation. A further advantage is the ability to incorporate automation into the method to reduce labor, human error and solvent usage. In relation to the direct comparison of FABE and FAME, butyl esters are much more suitable than methyl esters for the analysis of short chain fatty acids in dairy samples because of their decreased volatility, particularly evident in the determination of C4:0. Even when using higher split ratios and lower injection volumes the LOD and LOQ thresholds of the FABE method were similar to the FAME method. The application of the FABE method proved suitable for the majority of samples in this study. However, there remains scope to improve the extraction protocol, or add additional steps, to allow for analysis of samples such as milk and ice cream because of the integrity of the milk fat globule membrane. Also consideration needs to be given to the level of fat content, degree of lipolysis and sample matrix, as highlighted by the EMC, butter and processed cheese samples. Overall the FABE approach is robust, sensitive, accurate and precise and is a more suitable alternative to existing methods for determining the range of FFAs found in dairy products.

ASSOCIATED CONTENT

Supporting Information

Chromatographic plots of on-column, FAME and FABE analysis. Tables displaying some of the validation data for the FABE method, along with results of the various dairy samples comparing the direct on-column, FAME and FABE methods.

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through the contribution of authors †‡ and †* and reviewed by ‡. All authors have given approval to the final version of the manuscript. Authors †*/ and ‡ designed the experiment and author †‡ undertook the study.

Notes

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TABLES

Table 1. Validation data for the fatty acid butyl ester method. Displayed are the linear calibration, limits of detection (LOD), limits of quantification (LOQ), intra-day and inter-day relative standard deviation (RSD) and relative error results.

Butyl ester	line Equation	Linear Range (ppm)	Correlation Coefficient (R^2)	LOD[a] (ppm)	LOQ[a] (ppm)	Intra-Day[b] RSD (%)	Inter-Day[c] RSD (%)	Relative Error[d] (%)
C4:0	y = 0.9022x + 0.0139	20-700	0.9996	8	20	2.3	4.9	2.4
C6:0	y = 1.1366x - 0.0203	20-700	0.9995	8	20	1.9	5.1	2.2
C8:0	y = 1.3231x - 0.0643	20-700	0.9964	8	20	4.7	5.1	5.4
C10:0	y = 0.9868x - 0.0016	20-700	0.9999	5	15	1.8	4.4	1.6
C12:0	y = 1.143x - 0.0109	20-700	0.9998	5	15	1.9	4.8	3
C14:0	y = 1.1947x + 0.006	20-700	0.9978	5	15	5.6	5.1	2.4
C16:0	y = 1.0876x - 0.0205	20-700	0.9996	5	15	2.3	4.9	3.4
C18:0	y = 1.1067x - 0.0021	20-700	0.9999	5	15	2.0	5.2	3.9
C18:1	y = 1.068x - 0.0064	20-700	0.9999	5	15	0.9	4.7	3.3
C18:2	y = 1.0478x - 0.007	20-700	0.9999	5	15	2.1	5.4	4.2
C18:3	y = 1.0616x - 0.0064	20-700	0.9999	5	15	1.3	5.3	4.3

[a]LOD stablished at 3 times signal to noise. LOQ established at 10 times signal to noise.

[b]Average values obtained from ten samples (n=10) analyzed in a single day.

[c]Average values obtained from seventy two samples (n=72) analyzed over 1 month.

[d]Average values obtained from across 20-700 ppm concentration range.

Table 2. Fatty acid butyl ester (FABE) analysis of dairy samples. Displayed are the average values of six samples (n=6). Included are the measured ppm, % recovery and % RSD values of the ppm replicates.

		Brie	Processed Cheese	Light Cheddar	Mild Cheddar	Special Reserve	Blue Stilton	Milk Powder	Infant Formula	Butter	Yoghurt	Ice Cream	Milk	Butter Powder	Nat.Cream Powder	Cream Paste	Butter Paste	Blue Cheese Paste
C4:0	ppm	38	39	35	64	86	158	12	5	16	12	11	4	3386	1942	6497	5552	5899
	Recovery[a]	96	97	97	95	97	99	92	94	106	96	100	97	97	98	112	110	51
	RSD (%)	4	1	4	1	1	2	5	4	5	10	7	6	8	10	11	13	5
C6:0	ppm	16	16	14	17	28	61	7	-	11	7	5	-	1524	967	2935	3514	3746
	Recovery[a]	100	98	101	103	100	104	99	98	103	99	100	99	98	105	87	88	52
	RSD (%)	5	5	2	2	3	2	13	-	6	12	9	-	8	12	10	2	4
C8:0	ppm	15	17	12	18	22	38	6	-	10	6	7	-	1533	1017	3337	2188	2205
	Recovery[a]	101	97	96	96	98	97	93	100	103	97	101	102	94	112	71	78	64
	RSD (%)	4	4	8	4	3	2	12	-	3	13	8	-	14	10	14	6	6
C10:0	ppm	37	37	34	44	60	81	10	-	54	5	8	-	3369	1904	7151	4245	4681
	Recovery[a]	99	96	98	99	99	101	91	95	102	97	100	99	98	95	93	103	56
	RSD (%)	2	2	3	1	3	3	9	-	2	10	21	-	6	9	10	5	5
C12:0	ppm	55	53	46	62	81	110	12	9	88	6	18	4	4569	2125	7108	4962	5256
	Recovery[a]	99	92	100	100	100	104	96	97	103	97	98	98	91	98	89	95	46
	RSD (%)	2	3	4	1	1	5	8	2	1	14	26	7	6	14	6	3	3
C14:0	ppm	140	139	98	170	213	305	26	10	193	9	30	8	9931	5457	17960	17389	14995
	Recovery[a]	97	76	97	103	94	115	97	97	94	96	98	96	75	102	70	76	56
	RSD (%)	2	11	4	1	2	5	10	2	2	9	45	4	15	11	13	8	5
C16:0	ppm	389	386	274	474	577	811	90	69	607	89	254	38	23364	15471	46728	33657	42694
	Recovery[a]	94	46	100	100	88	135	87	90	90	93	133	96	101	86	29	95	54
	RSD (%)	2	9	4	2	1	5	6	2	1	7	14	23	5	8	6	5	4
C18:0	ppm	161	129	114	181	205	288	49	33	250	61	270	27	4741	4296	9689	11411	11201
	Recovery[a]	96	85	103	97	93	106	94	98	101	95	165	95	79	82	55	65	36
	RSD (%)	3	8	3	2	1	5	4	3	1	10	9	18	5	10	6	1	3
C18:1	ppm	274	342	138	343	577	1265	61	49	608	12	47	10	12925	10551	26835	21449	19626
	Recovery[a]	93	61	97	100	97	170	88	90	106	96	103	94	68	72	44	55	28
	RSD (%)	3	7	3	1	1	4	9	2	1	14	21	37	4	10	4	1	2
C18:2	ppm	32	62	27	33	50	176	12	29	76	12	11	9	1308	698	2457	1610	2526
	Recovery[a]	95	95	98	121	94	105	96	92	100	95	97	95	82	86	58	67	38
	RSD (%)	4	3	9	5	3	4	8	3	2	6	8	-	5	13	4	2	2
C18:3	ppm	22	23	19	27	31	59	10	9	43	-	-	10	526	252	895	743	518
	Recovery[a]	96	96	98	96	95	95	101	103	101	100	101	93	87	89	63	71	40
	RSD (%)	3	7	6	3	2	3	13	2	2	-	-	101	5	12	10	8	2
Total	ppm	1183	1246	814	1436	1936	3355	296	217	1960	223	661	90	67206	44712	131622	106748	113377
	Recovery[a]	97	85	99	101	96	112	94	98	101	96	109	98	88	93	70	82	47
	RSD (%)	2	7	3	1	1	4	5	2	1	8	12	33	3	8	4	1	3

[a]Calculated from the addition of 0.5mg of each FFA (C4:0-C18:3) to sample (spiked sample) and measuring difference against un-spiked sample. For EMC samples 5mg of each FFA was added.

Table 3. Summary of total FFA measurements obtained from on-column, fatty acid methyl ester (FAME) and fatty acid butyl ester (FABE) analysis of dairy samples. Displayed are the average ppm values (n=6) and the % relative standard deviation (RSD) in the measurements between the three methods.

Sample	Direct On-Column[a]	FAME[a]	FABE	RSD (%)
Brie	1135	1067	1183	5.2
Processed Cheese	1104	952	1246	13.4
Light Cheddar	969	827	814	9.8
Mild Cheddar	1525	1627	1436	6.2
Special Reserve Cheddar	2289	2316	1936	9.7
Blue Stilton	3109	2856	3355	8.0
Milk Powder	337	333	296	7.1
Infant Formula	173	227	217	13.8
Butter	1492	1295	1960	21.6
Yoghurt	182	246	223	14.9
Ice Cream	323	176	661	64.3
Milk	290	286	90	51.4
Natural Butter Powder	73652	90949	67206	15.9
Natural Cream Powder	48680	49274	44712	5.2
Natural Cream paste	126615	114969	131622	6.9
Natural Butter paste	106079	98129	106748	4.6
Natural Blue Cheese paste	116085	104376	113377	5.5

[a]Results obtained from Mannion et al.[5].

FIGURES

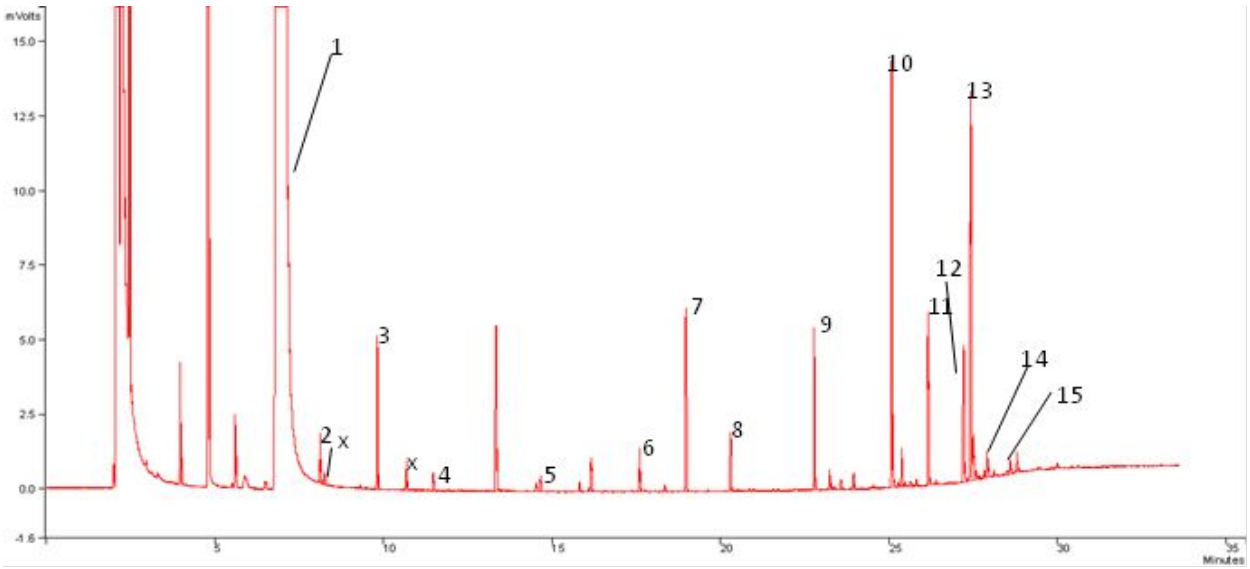

Figure 1. FABE chromatogram of special reserve Cheddar. Peaks are: 1 butanol; 2 butyl butyrate; x unknown; 3 butyl valerate[a]; 4 butyl caproate; 5 butyl caprylate; 6 butyl caprate; 7 butyl undecanoate[a]; 8 butyl laurate; 9 butyl myristate; 10 butyl palmitate; 11 butyl heptadecanoate[a]; 12 butyl stearate; 13 butyl oleate; 14 butyl linoleate; 15 butyl linolenate.

[a]Internal Standard

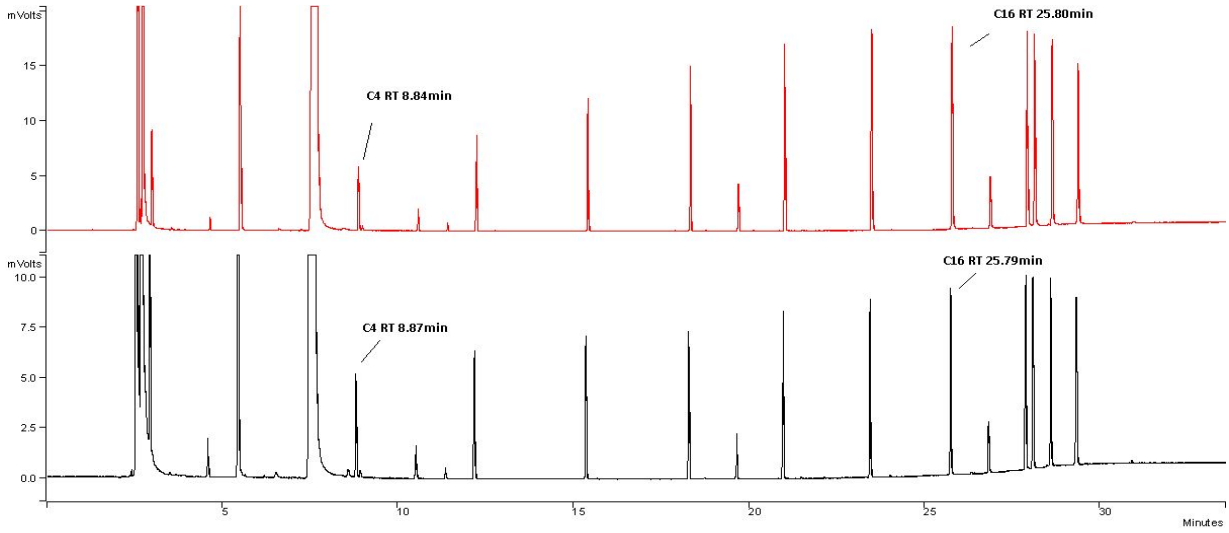

Figure 2. Two chromatograms of a typical butyl ester standard calibration mix analyzed at different stages in the column lifetime, approx. 120 analyses apart. Peak retention times remain stable.

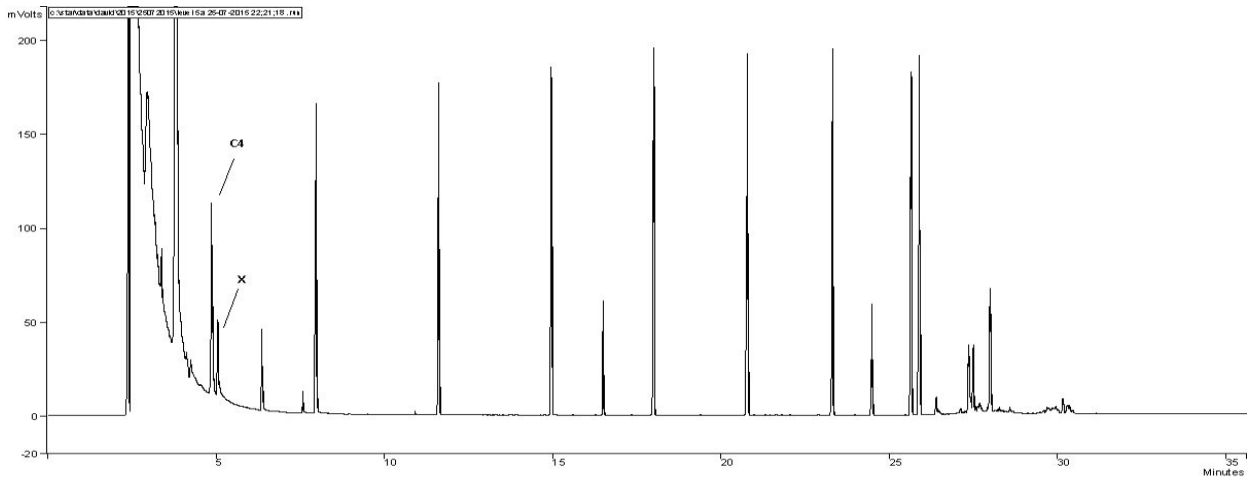

Figure 3. Chromatogram of a FAME calibration standard

[x]Unknown artefact (likely TMA)

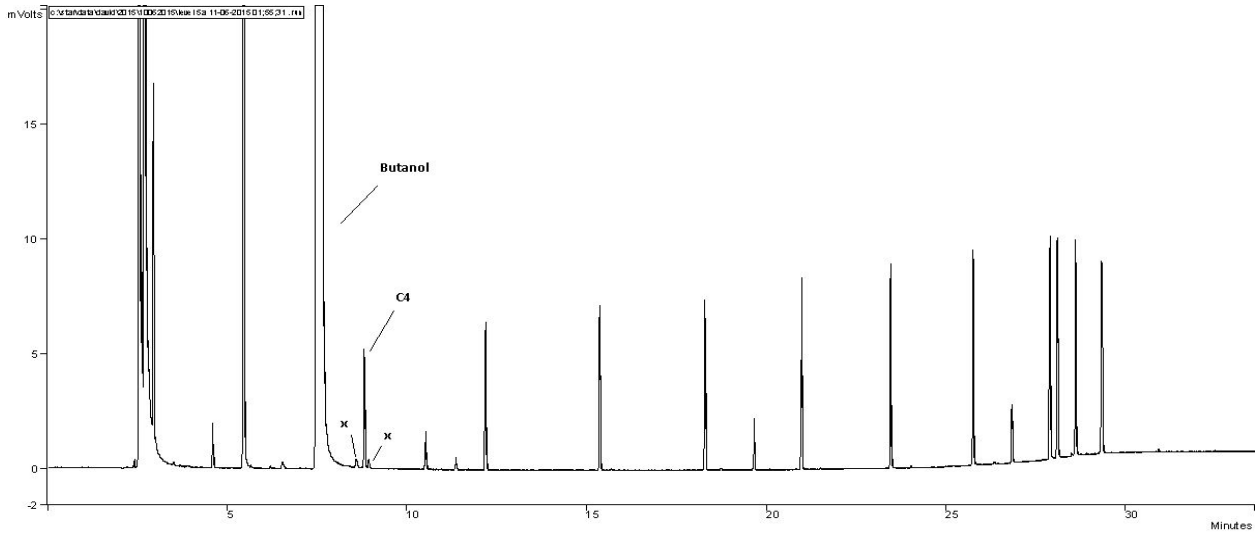

Figure 4. Chromatogram of a FABE calibration standard.

[x]Unknown

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