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A time efficient adaptation of GC-FID method for the analysis of PBMC lipid composition

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Abstract

Peripheral Blood Mononuclear Cells (PBMC) comprise of lymphocytes and monocytes which are involved in the pathogenesis of chronic inflammatory diseases. A method for estimation of PBMC lipid composition was modified from an existing method for plasma to support our fish oil intervention study in mild-moderate asthmatics. This modification involved an hour long direct transesterification reaction using methanol and acetyl chloride followed by a modified GC-FID method. The identification of PBMC fatty acid composition was achieved by relative comparison with a 37 Fatty Acid component standard. The method was found to be reproducible and accurate with a within batch and between batch Coefficient of variation (CV) of <10%. The modified method was adapted for PBMC total lipid estimation and was considered suitable to detect changes in PBMC composition following fish oil supplementation. This method is quick, scalable, and cost effective for large scale population and intervention studies.

Keywords: Fatty acids, PBMC, fish oil, Gas chromatography

Introduction

Fish oils comprise of two major omega 3 polyunsaturated fatty acids (PUFAs) namely Eicosapentaenoic Acid (EPA, 20:5) and Docosahexaenoic Acid (DHA, 22:6), these PUFAs are important structural components of cell membranes. In fish oil supplementation studies, an incorporation of EPA and DHA in total cell lipids and phospholipids, which occurs mostly at the expense of omega 6 PUFAs (primarily Arachidonic Acid, AA, 20:4), is expected.

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The extraction of fatty acids from cells and tissues is generally based on the original methods of Folch (1957) or Bligh & Dyer (1959), which have been widely applied and used on different types of cells (Damsgaard et al. 2008; Garcia-Larsen 2011; Grindel et al. 2013). Similarly, there are standard methods for derivatisation of lipids (acid-catalysed) (Lepage and Roy 1986; Morrison and Smith 1964, Matsumot et al. 2013) in a quantitative manner (Blau and Halket 1993; Christie 2003). Masood et al. (2005) proposed a method for measurement of fatty acids in plasma samples which is principally derived from Lepage & Roy (1986) with advantages of speed, reproducibility, lower toxicity and cost. While previous investigations have concentrated upon the use of plasma samples for the analysis of fatty acid composition of cells, the focus of this paper is the use of PBMC.

Materials and Methods

PBMC extraction

PBMC were extracted (within 2-4 hours of blood collection) from fresh blood as per manufacturers guidelines using Ficoll Paque PREMIUM (Catalogue number 28-4039-56 AA, GE Healthcare, UK). The diluted anticoagulant-treated blood was layered on the Ficoll-Paque PREMIUM solution (13.5 mL) (9mL blood diluted in balanced salt solution), followed by centrifugation at 400 x g for 30 min at 18–20°C. Following centrifugation, the upper layer containing plasma and platelets was removed using a sterile Pasteur pipette, leaving the layer of mononuclear cells undisturbed at the interface. Excess Ficoll-Paque PREMIUM was washed 27 mL of balanced salt solution. The extracted PBMC were suspended 1:1 in phosphate buffer saline. Any excess buffer was evaporated under nitrogen and sample stored at -20ºC until further analysis.

Masood et al. (2005) method modification

The Masood et al. (2005) method for derivatisation of fatty acid methyl esters (FAME) was scaled up. PMBC (100µl) was added to 3.6 ml of the stock solution (200µl of acetyl chloride and 3.4 ml of methanol) (catalogue number 00990 Fluka and

34860 respectively, Sigma-Aldrich, UK) and heated at 100°C for 60 min. After the methylation and cooling on ice, analytical grade n-hexane (1.5 ml) (catalogue number: 139386 Sigma-Aldrich, UK) was added to the tube and vortxed; upper organic phase was collected with a Pasteur pipette. This extraction procedure was repeated and hexane phases were combined for absolute recovery; finally nitrogen-gas was passed through to reduce the volume to 20-30μl. FAME samples (1μl) were loaded into a GC-FID (HP 5890 Series) for analysis. The samples were run on multi-purpose HP-5 column 30 m, 0.32 mm, 0.10 μm (catalogue number 19091J-313 Agilent, UK) followed by FID analysis. Helium/nitrogen was used as carrier gas (80bars), and both gases were found to be efficient for this analysis (<10% CV using either gas). The carrier gas pressure was lower than with the original method (Massod et al. 2005) however it corresponded to maximum pressure for the analyser used (HP-8590 series, Agilent). It was not possible to use hydrogen as carrier gas due to the health and safety regulations of the institution. The temperature programme for GC was modified to achieve a clearer resolution of the peaks with a starting temperature of 160°C and a ramp of 10°C/minute to achieve a final temperature of 270°C in 11 minutes. The run was held at 270°C for 5 minutes; hence the total run time was approximately 18 minutes. Purge valve was switched off for 0.3 min in the beginning to ensure most of the FAME sample was put on column and a split-ratio was adjusted to 15:1 to prevent column-overloading.

A set of standards (37 component FAME mix (catalogue number 47885-U Supelco, UK), reference oils: Menhaden fish oil (catalogue number: 47116, Sigma-Aldrich, UK), sunflower oil (Flora, UK) and olive oil (Napolina Ltd., UK) were analysed using the original and the modified GC-FID parameters. In the absence of a standard reference graph, or known retention times for the 37 component standard mix on a HP-5 column the elution order for the FAME were determined by comparison with elution on an equity 1 column (catalogue number 28039-U Sigma-Aldrich, UK) with a similar specification. Furthermore, to accurately identify the order of elution of the fatty acids on HP-5, standard FAME, pure methyl esters DHA (catalogue number 47570-U, Supelco, UK), Linoleic Acid (LA, 18:2, n-6)) and Alpha Linoleic Acid (ALA, 18:3, n-3) (catalogue numbers L1876 and L2626 respectively, Sigma-Aldrich, U.K) were tested individually, subsequently their retention times used as a reference for comparisons.

Validation of the modified method

The esterification and modified GC-FID method was validated to confirm the performance specifications when compared to the original method. In addition, the validation aimed to evaluate if the method was suitable for use in routine analysis. Parameters such as accuracy, precision, interference, and analytical specificity were assessed. There were limitations as to what could be achieved as part of the validation due to constraints on available materials and costs. The 37-component FAME mix was used as a reference standard and a measure of quality control during the experiments. All samples were analysed in duplicate to control for intra-assay variations. Within assay and between assay precision was determined by analysis of replicates of the 37-component FAME mix within a day and between two different days. Mean standard deviation and CV were recorded for each test. There may be error due to both the imprecision and inaccuracy of the method, therefore the combination of the two errors, or the total analytical error (TEa), determines the quality of the test result (Westgard, 2004). The TEa may be derived from biological variation or derived from manufacturers' specifications. For analytical techniques a mean CV is routinely set at 20% to account for TEa (Sittampalam et al, 2010). Since there was no manufacturers' guidance about TEa for FAME derived from PBMC on HP-5 column, an acceptable CV of 20% was set as standard to allow for imprecision, bias, manual error and total error of the tests. This CV was set as the highest error limit for each assay, which reflected the realistic performance expectations of the modified method based on the biological variation.

To determine accuracy, the modified method was compared to Masood et al, (2005) method. A range of standards including the 37-component FAME mix and in house esterified reference oil standards (fish, sunflower and olive oil) were analysed using GC-FID. The test material (PBMC) was analysed using the two GC-FID methods. The interference of a method is the ability of the method to measure the analyte of interest to the exclusion of other components. The method development was aimed to determine the relative percentages of omega 3 and omega 6 PUFAs of interest (AA, EPA and DHA). The modified temperature program used for the GC-FID allowed better resolution of the PUFAs of interest. Only those peaks were identified in samples which corresponded to the ones with the same retention time in the 37 component FAME mix as standard. FAME derived from PBMC showed some unidentified peaks as they did not correspond to any of the FAME in the standard and these peaks were subsequently excluded from analysis.

Data analysis

The data was analysed by the Clarity Lite software (Clarity v.2.4.1.77, DataApex Ltd, 2005). A standard calibration GC-FID graph was generated with the 37- component FAME mix and the resolution was compared to that of HP-5 for identification of FAME. The solvent peak was removed, area under peaks calculated and the proportion of each peak area to the sum of the area of all peaks calculated. This proportion represented the percentage of each fatty acid in the total fatty acids analysed (total PBMC lipid content). Unpaired t-test tests using SPSS (SPSS, version 19; SPSS; Chicago, IL) were used to compare mean data obtained for standards and PBMC total lipids when analysed using the two methods.

Results and Discussion

Assay Performance of Standards

A total of 30 FAME out of the 37 FAME listed by the manufacturer for the 37-component FAME mix were identified using the modified method and the area covered by these peaks was estimated to be 70% of the total peak area of the GC trace. A representative GC-FID profile of 37 component FAME mix on HP-5 using the Masood et al. (2005) method is presented in Figure1. Due to co-elution a number of peaks, although a number of peaks were identified, the area under the curve for all the fatty acids was not integrated. The within batch precision calculated by observing the mean CV of all the FAME identified on the same day was <5% (in five different runs). A between batch precision was calculated by measuring CV over two different days, which was less than 20%. Intra assay CV for saturated (Lauric Acid, Myristic Acid, Pentadecanoic Acid, Palmitic Acid, Heptadecanoic Acid, Stearic Acid), monounsaturated (Palmitoleic Acid, Oleic Acid), and polyunsaturated (LA, ALA, AA, EPA and DHA) fatty acid esters using the modified method were calculated as 10%, 5% and 6% respectively. Although Masood et al. (2005) have reported CV of approximately 5%, our CVs are slightly higher possibly due to manual injection methods, different

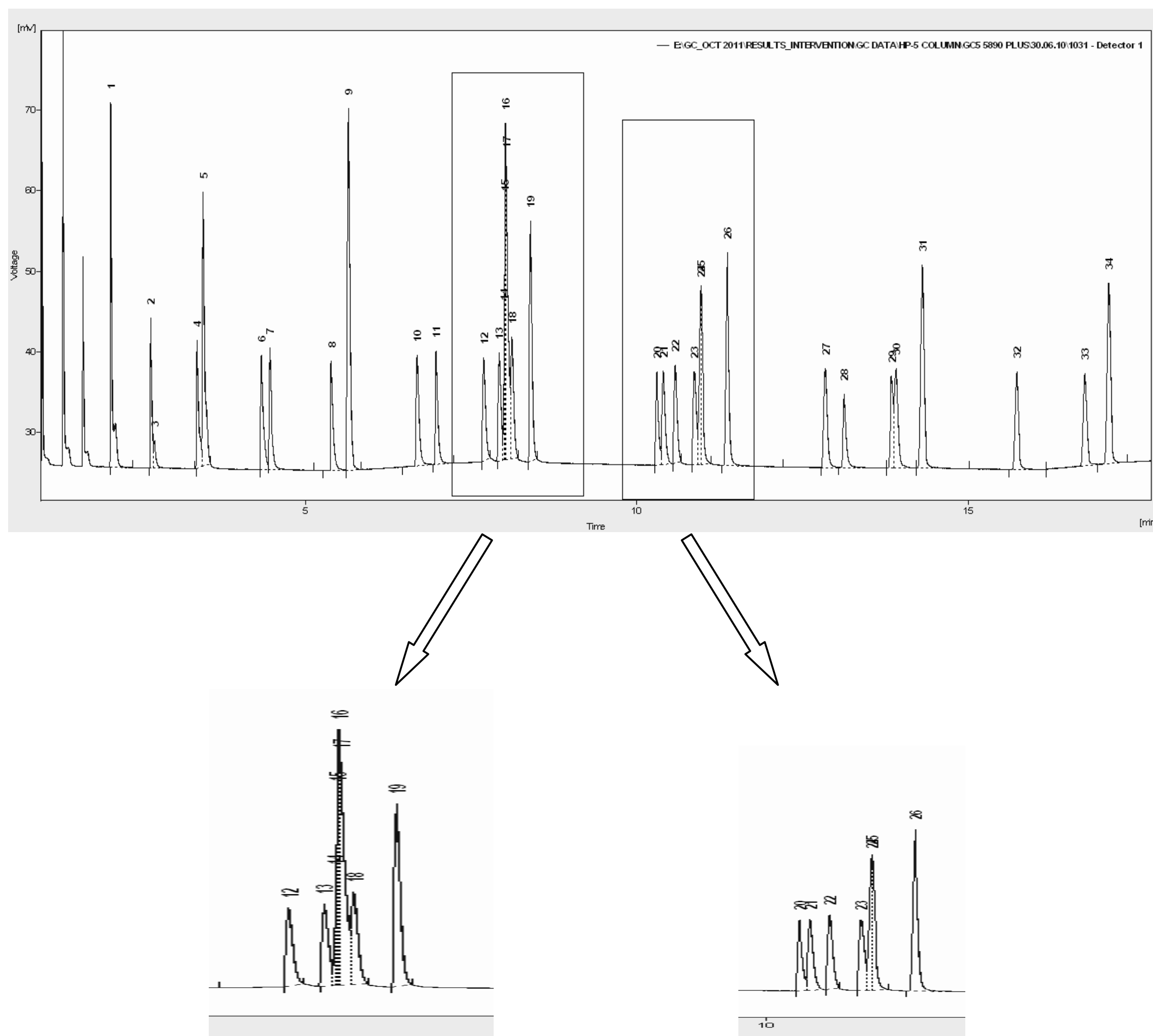

Figure 1. A GC-FID graph showing a profile of 37 component FAME mix on HP-5 using the modified method.
Key: 3. Lauric Acid, 4. Tridecanoic Acid, 5. Myristic Acid, 6. Myristoleic Acid, 7. Pentadecanoic Acid, 8. cis-10-Pentadecenoic Acid, 9. Palmitoleic Acid 10. Palmitic Acid, 11. Heptadecanoic Acid, ***12. cis-10-Heptadecenoic Acid, 13. Linoleic Acid, 14 α-Linolenic Acid, 15. Linolelaidic Acid, 16. γ-Linolenic Acid, 17. Oleic Acid, 18. Elaidic Acid, 19. Stearic Acid, 20. Arachidonic Acid, 21. Eicosapentaenoic Acid, 22. Eicosatrienoic Acid, 23. Eicosadienoic Acid 24. Eicosenoic Acid, 25 Eicosatrienoic Acid, 26. Arachidic Acid***, 27. Heneicosanoic Acid, 28. Docosahexaenoic Acid, 29. Docosadienoic Acid, 30. Erucic Acid, 31. Behenic Acid, 32. Tricosanoic Acid, 33. Nervonic Acid, 34. Lignoceric Acid **(Bold indicates fatty acids in sub panel)**

column and temperature parameters. The differences are however statistically not significant. The inter-assay CV for all fatty acids identified using the two methods was <10%. Table 1 shows the percent of fatty acids in the 37 component FAME mix relative to all fatty acids in the chromatogram based on the manufacturers guidance using the two methods, based on averages over two runs.

For Menhaden fish oil (approximately 80% of peaks were reported by the manufacturer, and remaining 20% were suggested to be derived from unidentified n-3 fatty acids. Using the modified method 79% of the peaks were comparable to the manufacturer's specifications; EPA and DHA were the two main components in the menhaden fish oil corresponding to 16% and 19% of the total respectively and the mean CV between two runs was calculated as less than 4%. Using the Masood et al. (2005) method, approximately 70% of the peaks in the GC trace were identified with a CV of less than 5%. EPA and DHA were identified as being 14% and 12% of the total respectively providing 80-120% recovery of these fatty acids in the esterified fish oil standard. Similarly, olive oil and sunflower oil provided 80-120% recovery and a mean CV of <9% using the two methods. There were no differences between the means for the two methods for all standard and reference oils when analysed using unpaired t-test ($p>0.05$).

PBMC total lipid composition

PBMC total lipids expressed as percentage of a fatty acid relative to all fatty acids in a chromatogram are shown in Table 2. For PBMC samples, the two methods showed comparable results, with the within and between batch precision <5% (run on the same day) and < 20% (run on different days)

respectively. Intra assay CV for saturated, monounsaturated and PUFA esters using the modified method were calculated as 10%, 5% and 6% respectively. Masood et al. have reported CV of approximately 5%, our CVs are slightly higher possibly due to manual injection methods, different column and different temperature parameters. A representative GC-FID graph showing total fatty acid profile for PBMC on HP-5 column using the modified method is shown in Figure 2.

Table 1: Percentage fatty acid composition in a 37 component FAME mix using the two methods, mean % shows the% of each fatty acid in the 37 component FAME mix relative to total fatty acid content. SFA= saturated fatty acids, MUFA= mono unsaturated fatty acids and PUFA=polyunsaturated fatty acids

	Manufactures guidance (% by weight)	Percentage (%) of fatty acids in a 37 component FAME mix relative to all fatty acids in a chromatogram			
Fatty Acid		**Modified method**		**Masood et al. (2005) method**	
		Mean	CV%	Mean	CV%
SFA					
Lauric Acid	4	3.8	9.1	3.3	5.1
Myristic Acid	4	4.6	17.1	4.1	8.7
Pentadecanoic Acid	2	2.7	23.1	2.4	17.4
Palmitic Acid	6	6.7	2.1	7.0	9.8
Heptadecanoic Acid	2	3.4	0.9	2.5	3.8
Stearic Acid	4	3.8	7.4	5.1	0.8
MUFA					
Palmitoleic Acid	2	3.1	7.1	2.0	11.4
Oleic Acid	4	3.9	5.3	5.4	9.2
PUFA					
LA	2	2.7	12.8	1.9	14.4
ALA	2	1.7	20.2	3.4	0.29
AA	2	3.4	6.5	2.1	7.3
EPA	2	2.2	9.4	2.4	10.1
DHA	2	1.9	3.6	1.81	7.3
Mean ± SD		3.4 ± 1.30		3.3 ± 1.61	

There were no differences between the means for the two methods when analysed using unpaired t-test (p>0.05).

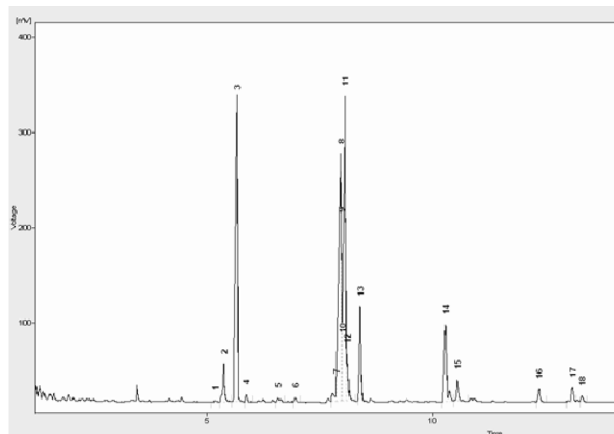

Figure 2: A representative GC-FID graph showing a total fatty acid profile for PBMC on HP-5 column using the modified method.

Key : 2. Myristic Acid, 3. Palmitic Acid, 5. Pentadecanoic Acid, 6. Heptadecanoic acid, 7. Linoleic Acid, 8. α-Linolenic Acid, 11. Oleic Acid, 13. Stearic Acid, 14. Arachidonic Acid, 15. Eicosapentaenoic Acid, 17. Docosahexaenoic Acid

Table 2: PBMC total lipids expressed as percentage of a fatty acid relative to all fatty acids in a chromatogram.

	Percentage (%) of fatty acids in a 37 component FAME mix relative to all fatty acids in a chromatogram			
Fatty Acid	**Modified method**		**Masood et al. (2005) method**	
	Mean	CV%	Mean	CV%
SFA				
Myristic Acid	9.1	3.7	9.3	0.3
Pentadecanoic Acid	2.3	5.3	-	-
Plamitic Acid	16.7	2.0	17.8	2.3
Heptadecanoic Acid	0.6	0.3	0.3	1.5
Stearic Acid	6.5	0.9	10.7	2.3
MUFA				
Palmitoleic Acid	3.3	3.9	-	
Oleic Acid	16.2	1.1	15.6	0.3
PUFA				
LA	8.5	1.0	10.0	0.6
ALA	0.9	1.2	0.6	3.3
AA	4.6	2.7	7.0	0.6
EPA	1.6	2.3	1.6	4.1
DHA	0.8	0.5	1.0	2.6
Mean ±SD	5.92 ± 5.7		7.3 ± 6.3	

There were no differences between the means for the two methods when analysed using unpaired t-test (p>0.05).

Conclusions

Approximately 70% of peaks were identified using the two methods. Several low molecular weight fatty acids (Butyric, Capric, and Lauric Acid) could not be identified by either method possibly due to the limitations of the column/temperature program. The modified method provided the following relative composition for PBMC total lipid: Palmitic Acid (17%), Stearic Acid (7%), Oleic Acid (16%), LA (9%), AA (5%), EPA (1.6%) and DHA (0.8%) (Table 1). Based on students' unpaired-t test there was no significant difference between the mean fatty acid percentages identified by the two methods (p>0.05). Small Loss of EPA, DHA and AA can occur due to thermal degradation (>180°C) of GC column resulting in a number of small minor peaks that separate from the main peaks and could account for the small peak area for DHA and AA in our findings compared to literature (Ackman 2006; Aued-Pimentel et al. 2010, Fournier et al. 2006).

The modified method was fast and efficient for PBMC total lipid analysis, requiring less than 4 hours for isolating PBMC samples followed by esterification/analysis by GC-FID. The cost per sample for processing including all reagents and materials required for isolation and analysis of PBMC using GC-FID was potentially lower than the original method due to the use of cost effective N_2 as carrier gas. Overall, the adaptation and modification outlined here provides a fast, economical and reproducible method for analysis of EPA, DHA and AA from PBMC.

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