Detection of Myristic and Palmitic Acid in Butter By Hydrolysis And Acid Methylation

N S Tapak1, S Senin1, A H Mohamed1, N N Dzulkifli1, Z M Zain2 and N M Ahmad3

1School Of Chemistry and Environment, Universiti Teknologi MARA Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000 Negeri Sembilan, Malaysia
2Electrochemical Material and Sensor Research Group, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor. Malaysia

*Corresponding author: normonica@uitm.edu.my

Abstract. Myristic acid and palmitic acid in butter was successfully detected by Gas Chromatography Flame Ionization Detector (GC-FID) after hydrolysis and methylated to fatty acid methyl esters (FAMES). Optimization of initial oven temperature and carrier gas flow rate were determined earlier, and it was found that 50°C and 2.0 mL/min were chosen as optimum initial oven temperature and carrier gas flow rate respectively. Two different calibration curves were constructed using methyl myristate and methyl palmitate. Both were found linear at 20 - 100 ppm with correlation coefficient ($R^2$) 0.9972 and 0.9933 respectively. Methyl stearate were used as an internal standard (IS). Limit of detection (LOD) where the lowest concentration that can be detected by the instrument was found at 16 ppm. In order to determine the concentration of FAMES in butter, fatty acids were hydrolyzed using lipase enzymes and were converted to FAMES by acid catalyst methylation method. Butter with Brand A, B and C was found to contain 24 ppm, 21 ppm and 30 ppm Methyl Myristate. Methyl Palmitate was only found in butter Brand B and not detected for another two brands.

1. Introduction
Butter is a general food of the public regardless of age as it is often used as a seasoning for many dishes such as stews, pasta, and rice. This is due to the desirable flavours possessed by butter itself that almost judged to be "natural" by public. If stored at low temperatures, it is suitable as a smear on other foods such as bread, biscuit, and pastry [1]. Therefore, it is considered as an important and quick add-on menu by a family because it can be used directly as one of the breakfast components and oil substitutes for various menu dishes [2]. Nevertheless, consuming foods that high in Fatty acid (FA) such as butter affect long-term health including the risk of cardiovascular and coronary heart diseases [3]. Therefore, reliable data and methods need to be established for the detection of FA in butter for the authorities related to food, dieticians and consumers. High level of unsaturated fatty acids (FAs) such as monounsaturated fatty acids (MUFA), polyunsaturated fatty acid (PUFA) and cis or trans leads to cardiovascular diseases, colon cancer, and various diseases such as obesity, hypertension and arthritis [4,5]. Therefore, accurately quantification method must be applied to measure the concentration of fatty acids (FAs) with high sensitivity and precision.
Gas chromatography (GC) equipped with flame ionization detector (FID) was found as the most common techniques for determination of FA composition [6,7,8]. To make it compatible with GC requirement, samples need to be volatiles prior to analysis which normally involve derivatization methylation process. A common approach involved the use of several catalysts such as in acid catalyst, base catalyst or acid-base catalyst [8, 9,10] to obtain a fatty acid methyl ester (FAMEs) which is more volatilize and are compatible to GC-FID. However, it was found that base catalyst method produces by-products and impurities that will lead to arise interferences to GC analysis [10] whilst acid-base catalyst is not able to fully methylates the FAs under mild condition [11]. To overcome the matters, acid catalyst method provides a complete methylation for all types of commonly used fatty acids [10]. To quantify FAMEs, GC couple with flame ionization detector (GC-FID) was found as alternative instruments to analysed FAMEs due to a volatility and compatibility of compound [12]. Moreover, this instrument able to detect all classes of FAMEs with higher resolution [13].

Since butter contains triacylglycerol in addition to fatty acids, a considerable hydrolysis step using the lipase enzyme should be considered to obtain the actual FA content prior to methylation process. In this research, three butter samples were hydrolysed using lipase enzyme before methylated using acid catalyst method to form a respective fatty acid methyl ester (FAMEs). Using a chromatogram of GC-FID, the observed retention time was compared with the standard FA to confirm their presence in the butter followed by quantification through an internal calibration curve.

2. Research Method

2.1. Samples, reagent and chemical
Three butter samples were purchased from a supermarket in Kuala Pilah, Malaysia. Analytical grade of methyl stearate (C18:0), methyl palmitate (C16:0) and methyl myristate (C14:0) were purchased from Sigma chemicals. HPLC grade of Diethyl ether and methanol (CH$_3$OH) were purchased from Fisher Scientific. Arabic gum, bovine serum albumin, lipase enzyme, hydrochloric acid (HCl), sodium hydrogen carbonate (NaHCO$_3$), and anhydrous sodium sulphate (Na$_2$SO$_4$) for hydrolysis and methylation proses were purchased from Merck (analytical grade). Deionised water from Purite was used throughout the analysis.

2.2. Instrumentation
Data management and analysis were performed using gas chromatography (GC) equipped with Flame Ionization Detector (FID) and HP5 capillary column (30 m x 250 μm x 0.25 μm) brand Agilent Technologies 7820A. The condition of GC-FID was followed [7, 14] with slight modification. In brief, nitrogen and hydrogen were used as a carrier gas with the initial oven temperature was set at 50 °C for 8 minutes at 2.0 mL/min gas flow rate. The injection port of GC-FID was 250 °C with 40:1 split ratio at 250 °C detector temperature. The retention time of methyl myristate, methyl palmitate and methyl stearate are expected to appear at 15.060, 19.613 and 24.102 minute, respectively.

2.3. Preparation of stock solution and working solution.
Methyl myristate (C14:0) and methyl palmitate (C16:0) was used as a standard solution. Accurate amount of 0.1 g of individual fatty acid methyl ester (FAME) was dissolved with 100 mL of diethyl ether to prepare 1000 ppm of individual FAME. Then, mixture of working solution containing methyl myristate (C14:0) and methyl palmitate (C16:0) were diluted to 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm for calibration purposes. Methyl stearate (C18:0) at 60 ppm was used as internal standard (IS).

2.4. Hydrolysis of triglycerides
The hydrolysis process was carried out using method from [15] with slight modification. In brief, 5 mL of butter emulsion was prepared by mixing 0.5 mL of butter sample with 4.5 mL Arabic gum at 10 % (w/v). The hydrolysis process was performed for 30 minutes at 400 rpm in a beaker with constant
temperature of 37 °C and pH 8.0. Subsequently, in the same beaker that containing 5 mL of butter emulsion, 10 mg/mL lipase enzyme, 10 mL distilled water, and 50 µL of bovine serum albumin 12.5 % (w/v) were added into the reaction beaker.

2.5. Methylation

Methylation process were carried out using method from [10] with slight modification. Briefly, 5 mL of hydrolysed samples was measured approximately. Then, the samples were transferred to a 10 mL test tube containing 1 mL of 4% (v/v) HCl/MeOH that was prepared earlier by diluting 4 mL of concentrated HCl with methanol in 100 mL volumetric flask. The mixture was placed in water bath and gradually heated for 30 minutes at 60-70 °C. Next, 3.0 mL of 2% (w/v) NaHCO$_3$ was used to neutralise the reaction mixture. Then, the sample was extracted with 5 mL diethyl ether. The upper layer which is the organic layer of diethyl ether was dried using anhydrous Na$_2$SO$_4$ then it was concentrated under nitrogen gas. 1 mL of internal standard (IS) of methyl stearate at 60 ppm were added to the sample before the injection.

2.6. Identification and quantification

To identify the FAMEs samples, GC-FID equipped with HP5 capillary column (30 m x 250 μm x 0.25 μm) brand Agilent Technologies 7820A will be used. Nitrogen and hydrogen were used as a carrier gas. Initial oven temperature was set at 50 °C for 8 minutes. Then, 5-fold of serial dilution of working mixture of methyl myristate and methyl palmitate were injected and measured. Then, 5-points of standard mixture will be used to plot a calibration curve [6]. Graph of peak ratio (peak area of interest/peak area for IS) against concentration was plotted as a standard calibration curve using fives working mixture consist of 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm.

The linearity of peak ratio obtained from the standard curve was used to fit in regression equation of $y = mx + c$ where $m$ is the slope and $c$ are the intercept [16]. Limit of detection (LOD) which is the lowest concentration detected by the instrument were determined by injecting the different concentration that consist of 12 ppm, 14 ppm, 16 ppm, and 18 ppm mixture of methyl myristate, methyl palmitate and methyl stearate at same GC condition. The concentration of the methyl myristate and methyl palmitate in three butter sample were determined in triplicate by linear regression equation of $y = mx + c$ obtained from the calibration curve where $y$ is the peak ratio and $x$ is the concentration of analyte [16].

3. Result and Discussion

3.1. Peak identification of methyl myristate, methyl palmitate, and methyl stearate

Sixty (60) ppm methyl myristate, methyl palmitate, and methyl stearate were used for identification of mixture of FAMEs using retention time. Figure 1 illustrates the chromatogram of individual FAMEs which are methyl myristate, methyl palmitate, and methyl stearate respectively which is summarized in table 1. Based on this study, the retention time of methyl myristate, methyl palmitate and methyl stearate were 5.419 minutes, 5.938 minutes and 6.433 minutes respectively. Regarding to the retention time of the three FAMES, methyl myristate was eluted first because it has the lowest boiling point compared to methyl palmitate and methyl stearate. This was contrary from the previous study where the retention time of methyl myristate, methyl palmitate, and methyl stearate were found at 34.846 minutes, 39.571 minutes and 43.873 minutes respectively [17]. This difference may be due to the different used of solvent to prepare the stock solution. In the previous study the solvent used was heptane that have a boiling point of 98 °C while the solvent used in this study was diethyl ether (DEE) that have a boiling point of 34.4 °C [18,19]. For the comparison purpose, the individual standard was mixed as a mixture standard FAMEs and injected to GC-FID. Retention time was compared to a previous individual FAMEs and was summarized table 2. It was observed that the retention time of FAMEs mixture was found similar between mixture and individual retention time indicates that none of the interferences occurred to a mixture standard.

Retention time is depending on to a boiling point of a compound. The lower of vapour pressure and volatility is contradict to the retention time. In this study, diethyl ether which have lower boiling point
and more volatile lead to a shorter retention time [19]. As methyl myristate, methyl palmitate, and methyl stearate have different values of boiling point which are 296.85 °C, 325.85 °C, and 351.85 °C respectively, the compound will elute with different retention time [20]. Methyl myristate was eluted first because it has the lower boiling point which means that it is more volatile than methyl palmitate and methyl stearate. Then, followed by methyl palmitate and methyl stearate due to its increasing boiling point and decreasing volatility. Another factor may cause by the polarity of analyte. As methyl myristate is less polar than methyl palmitate and methyl stearate, it will retain shorter in the column because it has lower affinity and less interaction in the non-polar HP5 capillary column. Therefore, methyl myristate was eluted first followed by methyl palmitate and methyl stearate.

**Figure 1.** GC-FID chromatogram of A) methyl myristate, B) methyl palmitate and C) methyl stearate and D) mixture of methyl myristate, methyl palmitate and methyl stearate. Flow rate: 2.0 mL/min, initial oven temperature: 50 °C.
Table 1. Retention time and peak area for individual FAMEs (60 ppm)

| FAMEs            | Boiling point (°C) | Retention time (min) | Peak area  |
|------------------|--------------------|-----------------------|------------|
| Methyl Myristate | 296.85             | 5.419                 | 179690     |
| Methyl Palmitate | 325.85             | 5.938                 | 166253     |
| Methyl Stearate  | 351.85             | 6.433                 | 202508     |

Table 2. Retention time and peak area of FAMEs mixture (60 ppm)

| Peak             | Retention time (min) | Peak area  |
|------------------|-----------------------|------------|
| Methyl Myristate | 5.407                 | 167411     |
| Methyl Palmitate | 5.933                 | 200797     |
| Methyl Stearate  | 6.430                 | 207239     |

3.2. Effect of initial oven temperature

Initial oven temperature is an importance parameter to measure because it will give an effect on the retention time of the solute. In this study, the separation based on initial oven temperature of 50 °C, 100 °C, 150 °C, and 200 °C were optimized. Figure 2A, 2B, 2C and 2D show the chromatograms for different initial oven temperature. Based on previous study, the higher the initial oven temperature resulting to shorter retention time but at too high temperature will trigger to co-elution of a compound and peak tailing [21]. An optimum initial oven temperature must be chosen to avoid the peak tailing or overlapping of an analyte.

Figure 2 demonstrates the graph of peak area against initial oven temperature. It was found the peak area of each FAMEs decreases from 50 °C to 200 °C. The optimum initial oven temperature at 50 °C will be used for subsequent analysis. This finding was almost similar to previous study which used 60 °C as an initial oven temperature even though different column size (30m x 0.53mm x 0.50 µm) and solvent (heptane) were reported [22].
3.3. Effect of flow rate

Flow rate is a parameter that determines the velocity of the mobile phase normally given in volume/time and have relationship with the quantification results [23,24]. Through this study, the carrier gas flow rate was set at 0.5 mL/min, 1.0 mL/min, 1.5 mL/min, 2.0 mL/min and 2.5 mL/min. Figure 3A, 3B, 3C, 3D and 3E shows the chromatogram for different flow rate at 50 °C initial oven temperature. Based on the analysis, the retention time are inversely proportional to the flow rate. This may be due to the interaction of the analyte with the stationary phase. In this study, 0.5 mL/min flow rate shows the longest retention time while 2.5 mL/min flow rate shows shortest retention time. An optimum flow rate must be selected to ensure a better separation and reduce the risk of peak broadening. Based on figure 3F, the optimum flow rate (2.0 mL/min) will be used in the subsequent experiments as the peak area at this flow rate is very significant and will likely give a high response to the construction of the calibration curve. This outcome was contrary to that of Gao et al. (2015) [25], who found that 2.2 mL/min was used as optimum flow rate. This discrepancy could be attributed to the uses of single carrier gas which was...
hydrogen instead of mixture of hydrogen and nitrogen gas while this study also used different types of capillary column which was BP70 capillary column.

Figure 3. GC-FID chromatogram for mixture of methyl myristate, methyl palmitate, and methyl stearate. Flow rate: A) 0.5 mL/min, B) 1.0 mL/min, C) 1.5 mL/min, D) 2.0 mL/min, E) 2.5 mL/min at initial oven temperature: 50 °C and F) optimization graph of flow rate (mL/min)
3.4. Calibration curve for standards FAMEs

Calibration curves measured the relationship between peak ratio (peak area of interest/peak area for IS) and the concentration of the components [13]. Graph of peak ratio against concentration were plotted as a calibration curve where peak area ratio as y-axis were calculated using formula below where R is peak ratio, $A_1$ is FAMEs peak area and $A_2$ is internal standard peak area.

$$R = \frac{A_1}{A_2}$$ (1)

Previous study has noted the importance of internal standard as it will rectify the error occurred throughout sample preparation or even during the sample injection [26]. In this study, methyl stearate was used as IS since it has the significant peak area compared methyl myristate and methyl palmitate. This is well agreed by [27] who reported methyl stearate as an internal standard in their study. Figure 4 (A) and (B) show the calibration curve of methyl myristate and methyl palmitate with correlation coefficient ($R^2$) of 0.9972 and 0.9933, respectively. It was found linear at 20 – 100 ppm using methyl stearate as an IS. This finding is contrary with previous study which reported a different linearity at 20 to 700 ppm with correlation coefficient of 0.9998 and 0.9999 respectively [28]. This is because, concentration greater than 100 ppm was not injected throughout this study to avoid column degradation for the good of the lifespan of the column.

![Figure 4](image)

**Figure 4.** Calibration curve of methyl myristate (A) and methyl palmitate (B) obtained from GC-FID.

3.5. Limit of detection (LOD)

Limit of detection (LOD) is a qualitative measurement which reported the smallest concentration of an analyte that can be detected by the method [13,23]. There were no signal or peak detected by the instrument at 12 and 14 ppm because they were too low to be detected by this GC-FID. However, the lowest concentration that can be detected by the instrument was found at 16 ppm. It was differed from previous study conducted by [28] that obtained lower LOD at 5 ppm. The result was contrary due to different column used in the study which was CP FFAP CB which has high polarity rather than HP5 capillary column that is non-polar. Besides that, the thickness of the column (0.32 µm and 0.25 µm) for CP FFAP CB and HP5 capillary column respectively are different with the present study.

3.6. Determination of methyl myristate and methyl palmitate in real sample

For the analysis of butter samples, the developed GC-FID method was profitably applied to simultaneous determination of methyl myristate and methyl palmitate. The concentration of the methyl myristate and methyl palmitate were determined by linear regression equation $y = mx + c$ obtained from the calibration curve where x is the concentration of analyte. Figure 5 show the chromatogram of sample
in Brand A, B and C. Based on the study, the concentration of methyl myristate in Brand A, B and C was found to be 24 ppm, 21 ppm and 30 ppm respectively. However, only Brand B contained methyl palmitate with concentration of 20 ppm. Some problems may arise from the derivatization process which typically involved incomplete conversion of FA to FAMES, alteration of FA composition, overlap of methyl ester peaks and damage to the chromatographic column due to traces of esterifying reagents [29]. Most of the problems has been avoided in the present research which may be due to the hydrolysis step that has been taken before methylation. However, limitation was seen at the figure 6(A), (B) and (C) where there are significant unidentified and unquantified peaks in each sample that could belongs to the unconverted fatty acids. The presence of such unconverted components may reduce the actual concentration of calculated myristic acid and palmitic acid which should be resolved in future study. To date, another lipid samples such as flaxseed oil [30] rat and human faeces along with fermentation fluids [31] and human milk [32] have been reported so far, quantified using GC-FID method.

![Figure 5. chromatogram of A) sample Brand A, B) Sample B and C) Sample C](image)

### 4. Conclusion
A GC-FID method was successfully designed to determine the concentration of myristic acid and palmitic acid in butter samples by hydrolysis process using lipase enzyme and acid catalyst methylation method to methylate the fatty acids into fatty acids methyl esters (FAMES). Initial oven temperature and flow rate were found optimized at 50 °C and 2.0 mL/min respectively which were used to construct the calibration curves. Calibration curve of methyl myristate and methyl palmitate was found linear from 20 to 100 ppm where the correlation coefficient (R2) was 0.9972 and 0.9933 respectively with LOD at 16 ppm. Quantification using calibration curve reveal the concentration of Methyl Myristate and Methyl Palmitate in the butter samples. Therefore, hydrolysis, acid methylation process and condition of GC-FID of this study will be a great platform to analyse all FAMES in butter samples. In future, FAMES profiling and quantification should be carried out by mass spectroscopy detector for a comparison study.
References

[1] Bobe G, Zimmerman S, Hammond E G, Freeman A E, Porter P A, Luhman C M, and Beitz D C 2007 Butter composition and texture from cows with different milk fatty acid compositions fed fish oil or roasted soybean in J. Dairy Sci. 90(6) p 2596–2603.

[2] Fíndík O and Andiç S 2017 Some chemical and microbiological properties of the butter and the butter oil produced from the same raw material LWT-Food Science and Technology 86 p 233–239.

[3] Morenga L T and Montez J M 2017 Health effects of saturated and trans-fatty acid intake in children and adolescents: Systematic review and meta-analysis Plos One 12(11) p 1-20.

[4] Bystrická Z and Ďuračková Z 2016 Gas chromatography determination of fatty acids in the human erythrocyte membranes – A review Prostaglandins Leukotrienes and Essential Fatty Acids 115 p 35–40.

[5] Ganguly R, and Pierce G N 2015 The toxicity of dietary trans fats Food. Chem. Toxicol. 78 p 170–176.

[6] Adjepong M, Valentinik P, Pickens C A, Li W, Appaw W and Fenton J 2017 Quantification of fattycid and mineral levels of selected seeds, nuts, and oils Ghana Journal of Food Composition and Analysis 55(11) p 43–49.

[7] Salimon J, Omar T A and Salih N 2017 An accurate and reliable method for identification and quantification of fatty acids and trans fatty acids in food fats samples using gas chromatography Arabian Journal of Chemistry 10 p 1875–1882.

[8] Abuégri D A, McElhenney W H and Willian K R 2012 Comparison of transesterification methods for fatty acid analysis in higher fungi: application to mushrooms Food Anal. Methods. 5 p 1159–1166.

[9] Carvalho A P and Malcata F X 2005 Preparation of Fatty Acid Methyl Esters for Gas-Chromatographic Analysis of Marine Lipids: Insight Studies J. Agric. Food Chem. 53(13) p 5049–5059.

[10] Igarashi M, Tsuzuki T, Kambe T and Miyazawa T 2004 Recommended Methods of Fatty Acid Methylester Preparation for Conjugated Dienes and Trienes in Food and Biological Samples J Nutr. Sci. Vitamino 50(2) p 121–128.

[11] Salimon J, Omar T A, and Salih N 2014 Comparison of two derivatization methods for the analysis of fatty acids and trans fatty acids in bakery products using gas chromatography The Scientific World Journal p 1-10.

[12] Jones A 2016 GC / FID Analysis of Fatty Acid Methyl Esters without Correction Factors Using the Polyarc Reactor Application Activated Research Company p 1–6.

[13] Aparicio-Ruíz R, García-González D L, Morales M T, Lobo-Prieto A and Romero I 2018 Comparison of two analytical methods validated for the determination of volatile compounds in virgin olive oil: GC-FID vs GC-MS Talanta 187 p 133–141.

[14] Costa N, Cruz R, Graça P, Breda J and Casal S 2016 Trans fatty acids in the Portuguese food market Food Control 64 p 128–134.

[15] Ghattas N, Abidi F, Galai S, Marzouki M N, and Salah A Ben 2014 Monoolein production by triglycerides hydrolysis using immobilized Rhizopus oryzae lipase Int. J. Biol. Macromol. p 68 1–6.

[16] Ponphaiboos J, Limmatvapirat S, Chaidedgumjorn A, and Limmatvapirat C 2018 Optimization and comparison of GC-FID and HPLC-ELSD methods for determination of lauric acid, mono-, di-, and trilaurins in modified coconut oil J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1099 p 110–116.

[17] Dixit S and Das M 2012 Fatty acid composition including trans-fatty acids in edible oils and fats: probable intake in indian population J. Food Sci. p 188-199.

[18] Mohon M, Associate R, Calder J, Student G and Wang W 2016 Cold start idles emissions from a modern Tier-4 turbo-charged diesel engine fueled with diesel-biodiesel, diesel-biodiesel-ethanol, and diesel-biodiesel-diethyl ether blends Appl. Energy 80 p 52–65.
[19] Hage D S and Carr J D 2011 *Analytical Chemistry and Quantitative Analysis. (1st ed.).* United States: Daniel Kaveney p 507-536.

[20] Hashimoto N, Nomura H, Suzuki M, Matsumoto T and Nishida H 2015 Evaporation characteristics of a palm methyl ester droplet at high ambient temperatures *Fuel* 143 p 202–210.

[21] Gómez-Cortés P, Rodríguez-Pino V, Juárez M, and Fuente M A De 2017 Optimization of milk odd and branched-chain fatty acids analysis by gas chromatography using an extremely polar stationary phase *Food Chem.* 231 p 11–18.

[22] Yurchenko S, Sats A, Poikalainen V, and Karus A 2016 Method for determination of fatty acids in bovine colostrum using GC-FID *Food Chem.* 212 p 117–122.

[23] Vékey K, Telekes A, and Vertes A 2008 *Medical Applications of Mass Spectrometry. (1st ed.). (Amsterdam)* Elsevier B.V p 61-89.

[24] Zuas O and Budiman H 2016 Estimating precision and accuracy of GC-TCD method for carbon dioxide, propane and carbon monoxide determination at different flow rate of carrier gas *Hem. Ind* 70(4) p 451–459.

[25] Gao F, Yang S, and Birch J 2015 Physicochemical characteristics, fatty acid positional distribution and triglyceride composition in oil extracted from carrot seeds using supercritical CO$_2$ *J. Food Compost. Anal.* 45 p 1-26.

[26] Laura A, Sobrado A, and Mariella L F 2016 Comparison of gas chromatography-combustion-mass spectrometry and gas chromatography-flame ionization detector for the determination of fatty acid methyl esters in biodiesel *J. Chromatogr. A* 1457 p 1-27.

[27] Potier S, Bernard M M, Schikorski D, Buatois B, Duriez O, Gabirot M, Leclaire S and Bonadonna F 2018 Preen oil chemical composition encodes individuality, seasonal variation and kinship in black kites Milvus migrans *Journal of Avian Biology* 49(7).

[28] Mannion D T, Furey A, and Kilca wley K N 2016 Comparison and validation of 2 analytical methods for the determination of free fatty acids in dairy products by gas chromatography with flame ionization detection *J. Dairy Sci.* 99(7) p 1–17.

[29] Simionato J I, Garcia J C, Santos G T D, Oliveira C C, Visentainer J V and Souza N E D 2010 Validation of the Determination of Fatty Acids in Milk by Gas Chromatography *J. Braz. Chem. Soc.* 21(3) p 520-524.

[30] Danish M and Nizami M 2019 Complete fatty acid analysis data of flaxseed oil using GC-FID method *Data in Brief* 23 p 1-6.

[31] Scortichini S, Boarelli M C, Silvi S and Fiorini D 2020 Development and validation of a GC-FID method for the analysis of short chain fatty acids in rat and human faeces and in fermentation fluid *J. Chromatogr. B* 1143 121972

[32] Rydlewski A A, Manin L P, Pizzo J S, Silva P D, Silva R, Tavares C B G, Paula M D, Pereira O, Santos O O and Visentaine J V 2021 Lipid profile by direct infusion ESI-MS and fatty acid composition by GC-FID in human milk: association with nutritional status of donors *J. Food Compost. Anal.* Journal pre proof.