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## The use of gas chromatography to analyse compositional changes of fatty acids in rat liver tissue during pregnancy.

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<b>Corresponding Author:</b>	Caroline Childs University of Southampton Southampton, Hampshire UNITED KINGDOM
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	c.e.childs@soton.ac.uk
<b>Corresponding Author's Institution:</b>	University of Southampton
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Helena Lucy Fisk, BSc
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Helena Lucy Fisk, BSc Annette West Graham Burdge Philip Calder
<b>Order of Authors Secondary Information:</b>	
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## **The use of gas chromatography to analyse compositional changes of fatty acids in rat liver tissue during pregnancy.**

Fisk, Helena L; West, Annette L; Childs, Caroline E; Burdge, Graham C; Calder, Philip C.

Human Development & Health Academic Unit, Faculty of Medicine, University of Southampton, MP887 Southampton General Hospital, Tremona Road, Southampton SO16 6YD, UK. Email: [h.fisk@soton.ac.uk](mailto:h.fisk@soton.ac.uk); [a.west@soton.ac.uk](mailto:a.west@soton.ac.uk); [c.e.childs@soton.ac.uk](mailto:c.e.childs@soton.ac.uk); [g.c.burdge@soton.ac.uk](mailto:g.c.burdge@soton.ac.uk); [p.c.calder@soton.ac.uk](mailto:p.c.calder@soton.ac.uk).

**Corresponding author:** Fisk, Helena L, Human Development & Health Academic Unit, Faculty of Medicine, University of Southampton, MP887 Southampton General Hospital, Tremona Road, Southampton SO16 6YD, UK. Tel 02380 798664 Fax 02380 795255. Email [h.fisk@soton.ac.uk](mailto:h.fisk@soton.ac.uk).

**Keywords:** gas chromatography, fatty acid, pregnancy, cholesteryl ester, solid phase extraction, polyunsaturated fatty acids

### **Short abstract:**

Pregnancy leads to significant changes to the fatty acid composition of maternal tissues. Lipid profiles can be obtained via gas chromatography to allow identification and quantification of fatty acids in individual lipid classes among rats fed various high and low fat diets during pregnancy.

### **Long abstract:**

Gas chromatography (GC) is a highly sensitive method used to identify and quantify the fatty acid content of lipids from tissues, cells and plasma/serum, yielding results with high accuracy and high reproducibility. In metabolic and nutrition studies GC allows assessment of changes in fatty acid concentrations following interventions or during changes in physiological state such as pregnancy. Solid phase extraction (SPE) using aminopropyl silica cartridges allows separation of the major lipid classes including triacylglycerols, different phospholipids, and cholesteryl esters (CE). GC combined with SPE was used to analyse the changes in fatty acid composition of the CE fraction in the livers of virgin and pregnant rats that had been fed various high and low fat diets. There are significant diet x pregnancy interaction effects upon the omega-3 and omega-6 fatty acid content of liver CE, indicating that pregnant females have a different response to dietary manipulation than is seen among virgin females.

### **Introduction:**

Gas chromatography (GC) is a well-established technique used to identify and quantify the incorporation of fatty acids into lipid pools and cell membranes<sup>1,2</sup> during supplementation or physiological conditions such as obesity (and related diseases such as diabetes) or pregnancy<sup>3-5</sup>. It is also suitable for analysing the types and quantities of fats in foods. This is useful when characterising experimental diets, as well as ensuring that the food industry complies with regulations. For example, GC can be used to confirm the identity and quantity of fatty acids within a product such as a dietary supplement to ensure that labelling is

correct and regulations are adhered to<sup>6,7</sup>. Analysis of fatty acids can provide valuable insights into lipid metabolism in health and disease, the impact of dietary change, and the effect of changes in physiological state<sup>8</sup>. Use of GC to study samples during pregnancy has provided important information on changes in fatty acid and complex lipid homeostasis<sup>3</sup>.

In advance of the chromatographic separation, lipids are typically extracted from the sample using the solubility of lipids in solvent mixtures of chloroform and methanol. Sodium chloride is added to facilitate the separation of the mixture into aqueous and organic lipid containing phases<sup>9,10</sup>. Complex lipid classes of interest can be separated from the total lipid extract by solid phase extraction (SPE). This separation technique elutes lipid classes based upon their polarity or binding affinity. Triacylglycerols (TAG) and cholesteryl esters (CE) are eluted first as a combined fraction, further classes, phosphatidylcholine (PC), Phosphatidylethanolamine (PE) and non-esterified fatty acids (NEFA) are eluted by increasing the polarity of the eluting solvent. The separation of TAG from CE exploits the binding of TAG only to a fresh SPE cartridge, allowing CE to be eluted. TAG can then be eluted by increasing the polarity of the eluting solvent<sup>9,10</sup>. This method allows multiple samples to be separated simultaneously with a higher yield than is achieved with thin layer chromatography which means that relatively small sized samples (e.g. < 100 µl plasma or serum, < 100 mg tissue) can be analysed<sup>11,12</sup>.

GC is a well-established technique first described in the 1950s; it was suggested that the mobile phase in the then liquid-liquid systems could be replaced with vapour. It was initially used for petroleum analysis but rapidly expanded into other areas such as amino acid analysis and lipid biochemistry which is still of major interest. Advances in GC equipment and technology such as the development of capillary columns from the previously used packed columns has led to our current techniques in which fatty acids are able to be separated more efficiently at lower temperatures resulting in GC being routinely used to identify and quantify fatty acids in a wide range of investigations<sup>13</sup>.

GC requires fatty acids to be derivatised in order that they may become sufficiently volatile to be eluted at reasonable temperatures without thermal decomposition. This usually involves the substitution of a functional group containing hydrogen to form esters, thioesters or amides for analysis. Methyl esters are commonly studied derivatives which are produced by methylation. In this method the ester bonds in complex lipids are hydrolysed to release free fatty acids which are transmethylated to form fatty acid methyl esters (FAME). The resulting profile of FAME, determined by GC, is referred to as the fatty acid composition and may be easily compared between different experimental groups<sup>9,10</sup>. The technique allows both the proportions of individual fatty acids and their concentrations to be measured.

In addition to the use of GC for analysing fatty acids in nutrition studies and within the food industry, the technique can be used across a wide range of analytical fields. For example, environmental analyses using GC include measuring water contamination by insecticides and soil analyses measuring chlorobenzene content. In toxicology, GC has also been used to identify illegal substances in urine and blood samples of individuals; such as sports

performance enhancers<sup>12</sup> and the ability to separate complex mixtures of hydrocarbons makes this technique popular in the petroleum industry for petrochemical analysis<sup>12</sup>.

Pregnancy is associated with significant changes to the fatty acid composition of maternal tissues, specifically in the content of omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFA)<sup>(3)</sup>. In the current study, we exemplify the use of GC in the measurement of fatty acids by describing its use in the analysis of the fatty acid composition of liver tissue taken from virgin and pregnant rats fed low and high fat diets with different oil sources. The experimental diets provided here were a low fat soybean oil based diet, a high-fat soybean oil-based diet (130.9 g total fat/kg total fat) or a high-fat linseed oil-based diet (130.9 g total fat/kg diet), provided for 20 days. The full nutrient and fatty acid composition of these diets have been described previously<sup>14</sup>. The soybean oil diets are rich in linoleic acid (18:2n-6) and contain some  $\alpha$ -linolenic acid (18:3n-3) while the linseed oil diet is rich in  $\alpha$ -linolenic acid. These high-fat diets represent different rations of linoleic to  $\alpha$ -linolenic acids (ratios of 8:1 and 1:1, respectively). The method for isolation of individual lipid classes and analysis by GC is well established and validated, and has been published previously<sup>10</sup> but without the detailed technical description found herein.

## **Protocol:**

### **1. Animal procedures:**

1.1) All animal work should be carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986).

1.2) Mate Wistar rats aged 10 weeks old by monogamous breeding, and confirm pregnancy by the appearance of a vaginal plug. Record this as day 1 of gestation, and commence experimental diet. For virgin females, house each rat individually, and commence experimental diet.

1.3) After feeding the experimental diets for 20 days euthanize rats by CO<sub>2</sub> asphyxiation followed by cervical dislocation.

1.4) Use dissection forceps and scissors to expose the abdominal cavity and excise the liver by cutting the ligaments, which connect the liver to the diaphragm, anterior wall of the abdomen, stomach and duodenum. Wash the liver in PBS and freeze in liquid nitrogen before storing at -80 °C.

### **2. Preparation of a total lipid extract<sup>9</sup>**

2.1) Add molecular sieves (to fill 1/10<sup>th</sup> of solvent container) to all solvents to create 'dry' solvents. **Carry out all solvent work within a fume hood.**

2.2) Cut approximately 100 mg frozen liver and weigh. Place the tissue into a tube in an ice bucket and add 0.8 ml ice cold 0.9% NaCl. Homogenise the tissue.

2.3) Add internal standards dissolved in 1 ml/mg of 'CAUTION' dry chloroform: methanol (2:1, v/v) containing 'CAUTION' butylated hydroxytoluene (BHT; 50 mg/l) as anti-oxidant. For 100 mg of rat liver add 100 µg of CE standard (Cholesteryl heptadecanoate 17:0).

2.4) Add 5.0 ml dry chloroform: methanol (2:1, v/v) containing BHT (50mg/l).

2.5) Add 1.0 ml 1 M NaCl, mix thoroughly by vortexing until mixture looks uniform. Samples can be capped and stored at -20 °C at this stage for up to a week.

2.6) Centrifuge at 1000 x g for 10 minutes, low brake at room temperature.

2.7) Collect lower phase using glass Pasteur pipette, transfer to new screw cap glass tube and dry under nitrogen at 40 °C. Samples can be capped and stored at -20 °C at this stage for up to a week.

### 3. Separation of lipid classes by solid phase extraction (SPE)<sup>10</sup>

3.1) Connect the SPE tank to a vacuum pump and place **aminopropyl silica SPE cartridge** on the tank.

3.2) Place new screw-cap glass tube labelled TAG and CE in the tank rack under the column to collect first fraction.

3.3) Dissolve the total lipid extract in 1.0 ml dry chloroform and vortex mix.

3.4) Apply sample to the column using a glass Pasteur pipette and allow to drip through into the screw-cap tube under gravity. When no further drips fall, remove the remaining liquid by vacuum.

3.5) Elute the TAG and CE fraction under vacuum, wash the column with 2 x 1.0 ml washes of dry chloroform.

3.6) When all liquid is removed, dry the TAG and CE fraction under nitrogen at 40 °C. Samples can be capped and stored at -20°C at this stage for up to a week.

3.7) Place new screw-cap glass tube labelled PC into the tank tray under the column.

3.8) Elute the PC fraction under vacuum with the addition of 2 x 1.0 ml dry chloroform: methanol (60:40, v/v) until all liquid is removed from the column.

3.9) Remove and dry PC fraction under nitrogen at 40°C. Samples can be capped and stored at -20 °C at this stage for up to a week.

3.10) Place a new screw-cap glass tube labelled PE into the tank tray and elute PE fraction with the addition of 1.0 ml dry methanol under vacuum.

3.11) Remove and dry PE fraction under nitrogen at 40 °C. Samples can be capped and stored at -20 °C at this stage for up to a week.

3.12) Place new screw-cap glass tube labelled NEFA into the tank tray and elute NEFA fraction under vacuum by the addition of 2 x 1.0 ml washes of dry chloroform: methanol: glacial acetic acid 'CAUTION' (100:2:2, v/v/v).

3.13) Remove collected NEFA fraction and dry under nitrogen at 40 °C. Samples can be capped and stored at -20 °C at this stage for up to a week.

3.14) Place a new **aminopropyl silica SPE cartridge** on the SPE tank and place a screw-cap glass tube in the tank tray under the cartridge to collect waste.

3.15) Wash the column with 3 washes of dry hexane 'CAUTION' under vacuum and then a final 1.0ml wash under gravity. Do not allow the cartridge to become dry (turn the cartridge column channels to a closed position when hexane level is close to the cartridge matrix).

3.16) Replace waste tube with new screw-cap glass tube labelled CE.

3.17) Dissolve the dried TAG and CE fraction (prepared in step 2.6) in 1.0 ml of dry hexane and vortex. Apply this to the column using a glass Pasteur pipette and allow to drip through under gravity.

3.18) When no further drips fall remove the remaining liquid under vacuum.

3.19) Under vacuum, wash the column with 2 x 1.0 ml washes of dry hexane to elute CE and dry collected fraction under nitrogen at 40 °C. Samples can be capped and stored at -20 °C at this stage for up to a week.

3.20) Place new screw-cap glass tube labelled TAG in the tank tray and elute TAG with the addition of 2 x 1.0 ml washes of dry hexane: methanol: ethyl acetate 'CAUTION' (100:5:5) under vacuum.

3.21) Dry collected fraction under nitrogen at 40°C. Samples can be capped and stored at -20 °C at this stage for up to a week.

#### **4. Preparation of FAME from CE<sup>10</sup>**

4.1) Add 0.5 ml of dry toluene 'CAUTION' to the separated CE fraction (collected in step 3.19) and vortex mix.

4.2) Prepare methylation reagent (dry methanol with 2% (v/v) H<sub>2</sub>SO<sub>4</sub> 'CAUTION'), of which 1.0 ml is required per sample. Dispense volume of dry methanol into glass or suitable plastic container with lid and add the required amount of H<sub>2</sub>SO<sub>4</sub> dropwise then mix by inversion.

4.3) Add 1.0 ml of the methylating reagent to the samples dissolved in dry toluene, cap the tubes securely and mix gently.

4.4) Heat the samples for 2 hours at 50 °C.

4.5) After 2 hours remove tubes from heat. Once cool add 1.0 ml neutralising solution (0.25 M  $\text{KHCO}_3$  'CAUTION' 0.5M  $\text{K}_2\text{CO}_3$  'CAUTION').

4.6) Add 1.0ml dry hexane and vortex mix.

4.7) Centrifuge at 250 x g for 2 minutes, low brake at room temperature.

4.8) Collect upper phase which contains the FAME and transfer into a new non screw-cap disposable glass tube.

4.9) Dry the collected FAME under nitrogen at 40 °C. Samples can be capped and stored at -20 °C at this stage for up to a week.

## 5. Removal of free cholesterol contamination from CE FAME<sup>14</sup>

(Free Cholesterol can contaminate the sample; see (figure 1) for example chromatograph traces with and without free cholesterol removal).

5.1) Place a waste tube into SPE tank and place a **silica gel SPE cartridge** onto the tank.

5.2) Wash column with 3 x 1 ml washes of dry hexane under vacuum and 1 x 1 ml under gravity.

5.3) Remove waste washes and add new waste tube into tank.

5.4) Dissolve CE FAME in 1 ml dry hexane, vortex mix.

5.5) Apply to the column using a glass Pasteur pipette and allow to drip through under gravity.

5.6) Wash column with 3 x 1 ml washes of hexane under vacuum.

5.7) Remove waste washes and place new non screw cap tube into tank labelled CE FAME.

5.8) Elute the CE FAME with 2 x 1 ml dry hexane : diethyl ether 'CAUTION' (95:5 v/v) washes.

5.9) Dry under nitrogen at 40°C. Samples can be capped and stored at -20°C at this stage for up to a week.

## 6. Transfer of FAME into GC auto sampler vial

6.1) Add 75 µl dry hexane to sample, vortex mix and transfer to a GC auto sample vial.

6.2) Add a further 75 µl dry hexane to sample, vortex mix and transfer to the same GC auto sample vial. Samples can be capped and stored at -20 °C at this stage for up to a month.

## **7. Analysis using the Gas Chromatograph<sup>14</sup>**

7.1) Analyse FAME on a gas chromatograph. Example set up: 30 m x 0.25 µm x 0.25 mm BPX-70 fused silica capillary column with temperature protocol:

Initial temperature 115 °C, hold 2 min, ramp 10°C/min to 200 °C, hold 18.5 min, ramp 60°C/min to 245 °C, hold 4 min.

Column: Helium gas, flow rate 1.0, pressure 14.6 and velocity 29.

Injector: Temperature = 300 °C.

Detector: Hydrogen flow 40.0, air flow 184.0, make up gas Helium, flow 45.0, temperature = 300 °C.

7.2) Set split ratio as appropriate –(e.g.25:1 for CE FAME analysis).

7.3) Determine the area under each peak using appropriate software and identify FAME by comparison with standards. See (figure 2) for example chromatograms.

7.4) Use the area under the peak data to calculate the contribution of individual fatty acids as a percentage of total fatty acids.

7.5) Calculate absolute concentrations of fatty acids by dividing the area of internal standard by the amount added. Divide the area of each fatty acid by this result to obtain absolute concentrations of each fatty acid within the amount of tissue used.

### **Representative Results:**

The success of this method is dependent on following the protocol precisely and on using clean solvents and reagents in order to reduce ‘noise’ and contamination that can appear on a chromatogram. Contaminated samples are more challenging to analyse, lowering the accuracy of the area under the curve calculations. If the protocol is followed successfully a chromatogram with clear symmetrical, well defined peaks and with minimal background noise should be obtained as illustrated in (figure 3). If contamination has occurred the chromatogram will show additional peaks and exhibit non-symmetrical (skewed) peaks as illustrated in (figure 4). Contamination from free cholesterol will occur when running FAME derived from CE see (figure 1), unless the cholesterol is removed (as described in protocol step 5).

The use of a prepared calibration mix allows for the identification of the FAME in the sample. The calibration mix is run using the same instrument settings as the samples so that the chromatogram can be compared to the sample and peaks correctly identified based upon their retention times as illustrated in (figure 2).



Peak area is used to calculate the percentage of specific fatty acids within the total. Once data have been collected, it is useful to inspect them for outliers as shown in (figure 5). Outlier samples can then be further investigated and the extraction and/or analysis repeated if necessary.

Adding an internal standard to samples (as described in protocol step 2.3) allows the quantification of fatty acids within the sample by calculations using the area of known quantity of the internal standard peak relative to the area of the peak of interest, and adjusted for the original sample volume or weight. In (table 1), FAME within rat liver CE are described as a % of total fatty acids (g/100g total fatty acid) within liver CE. These data describe the CE fatty acids in the liver of virgin or pregnant rats fed for 20 days on one of three different diets. Statistical analysis using two-factor ANOVA (factors: diet, pregnant vs virgin) reveals significant effects of diet and pregnancy upon the proportion of several fatty acids, as well as significant diet x pregnancy interactions (table 1). As an illustration (figure 6) shows that the arachidonic acid (AA; 20:4n-6) content of liver CE in pregnant rats is more influenced by diet than that of virgin females.

#### **Tables and Figures:**

**Figure 1:** Comparative gas chromatograms of identical samples illustrating the importance of free cholesterol removal in rat liver tissue. The untreated sample shows evidence of free cholesterol contamination by the addition undesirable peaks as circled. These can occur anywhere throughout the sample potentially disrupting peaks of interest and affecting the accuracy of area under the curve calculations and the resulting quantification of those fatty acids. The chromatogram produced from the sample where free cholesterol was removed shows well defined, distinguishable peaks with no background interference which would produce results with high accuracy.

**Figure 2:** Example of the method used to identify sample CE FAME from rat liver tissue using a prepared calibration mix. A prepared calibration mix is run using the same instrument settings as the samples so that the chromatograms can be compared to identify fatty acids within the sample. The accompanying chromatogram for the calibration mix allows the FAME in the mix to be labelled. This labelled trace can then be compared to the chromatogram from the sample where easily identifiable large peaks can be compared by their retention times to those on the calibration mix then labelled appropriately. For example highlighted on the trace. is 16:0 to illustrate the comparison of retention times from the calibration mix above to the fatty acids in the sample trace below allowing correct labelling of fatty acids.

**Figure 3:** Good example of a gas chromatogram depicting cholesteryl ester fatty acids (as methyl esters) from rat liver tissue. Peaks of interest are easily distinguished as they are symmetrical, well defined and there is very little background noise. All peaks are uninterrupted and there are no sloping peaks allowing easy integration and accurate area under the curve calculation.

**Figure 4:** Example of a contaminated NEFA gas chromatogram obtained from human plasma. There are many unexpected peaks, as circled at the beginning of the sample, which can affect the integration of genuine peaks. The peaks are interrupted and undefined as seen towards the end of the sample and have become sloped as circled. This will affect the accuracy of the area under the curve calculations and the quantification of these fatty acids.

**Figure 5:** Identification of outlier data among fatty acid composition of liver CE in virgin rats fed a high-fat soybean oil diet (n=6). This illustrates how percentage data of analysed fatty acids can be used to determine ambiguous results. These data indicate that sample 125 may be an outlier, and requires further investigation. The five major fatty acids within CE (16:0, 18:0, 18:1n-9, 18:2n-6, 20:4n-6) are not shown.

**Figure 6:** Arachidonic acid content of liver CE among virgin and pregnant rats fed experimental diets. Values are means  $\pm$  SD, n = 6. Means without a common letter differ,  $P < 0.05$ . \* different from virgin females within matched dietary group,  $P < 0.05$ . This is a visual representation of the results from (table 1) showing the differences in liver CE content of virgin rats fed various diets compared to pregnant rats fed those same diets. Differences can be seen in each dietary group between the virgin and pregnant rats.

**Table 1:** Fatty acid composition of rat liver cholesteryl esters of virgin and pregnant rats fed experimental diets. Values are means (SD), n = 6. ND indicates not detected (mean  $< 0.1\%$ ). Means without a common letter within virgin or pregnant females differ,  $P < 0.05$ . \* different from virgin females within matched dietary group,  $P < 0.05$ . This table shows the mean values of n-3 and n-6 fatty acids present in the liver tissue of virgin and pregnant rats when fed low and varying high fat diets. The results were statistically analysed using analysis of variance (ANOVA) with a significance level of  $P < 0.05$  for differences between virgin and pregnant rats, the type of diet and diet x pregnancy interaction. ANOVA results suggest there are significant differences in the levels of arachidonic acid (20:4n-6) between virgin and pregnant rats within matched dietary groups.

## Discussion:

Gas chromatography is an accurate technique to use for fatty acid analysis, and its high reproducibility deems this technique suitable for clinical analyses. Appropriate GC columns must be used to enable identification of fatty acids of interest, with available columns having variations in the polarity of the stationary phase, column length and internal diameter. The use of a fused silica capillary column in this method of analysis provides good thermal stability and high reproducibility of retention times due to its high surface inertness and good resolution<sup>8</sup>.

Critical steps within this protocol include lower and upper phase collection steps (protocol steps 2.7 and 4.8). It is important that as much of the correct phase is collected as possible without contamination with any of the unwanted phase. The presence of contaminants in a sample will result in an undesirable chromatographic output, as illustrated in (figure 2). When collecting CE during SPE it is imperative that the cartridge columns are kept saturated with solvent (protocol step 3.15) following the washes with hexane to ensure the sample

penetrates the column to allow successful separation of CE from TAG. The further separation of CE to remove free cholesterol is important to avoid contamination appearing on chromatograph traces as illustrated in (figure 1). It is also important to ensure steps requiring removal of liquid under vacuum are followed precisely so all liquid is completely removed from the column to ensure a good yield.

The main limitation of GC is that complex lipids such as phospholipids and triacylglycerols need to be saponified prior to derivitisation to form FAME prior to analysis, so information on the specific structures of these lipids and typical combinations of fatty acids is lost<sup>10</sup>. All steps in this protocol must be carried out in a fume hood due to the use of solvents which limits the suitability of surroundings this method can be performed in. This method can also be time consuming for analysing a small number of samples, typically taking two working days to get from sample of interest to data output, unless fully automated procedures are used. However, when processing larger numbers of samples, each stage can be done in batches to maximise time effectiveness of this technique and availability of equipment to other users. Certain techniques within the protocol require practice and manual dexterity such as upper and lower phase extractions (protocol steps 2.7 and 4.8) which could be a problem for people with problematic joints or who are prone to negative effects of repetitive movements.

Steps within this method can be easily added or removed to facilitate the collection of different fractions for a wide range of samples; for example, PE collection may not be required when analysing plasma, but is of interest in cell and tissue samples<sup>10</sup>. This method can also be modified for the analysis of cells of total lipid extracts, where the SPE steps can be omitted if desired. One such variation is that described for red blood cells which omits the total lipid extraction step as well as SPE steps<sup>15</sup>. In contrast to the current protocol, the method used in this study uses 250 µl of a methylating reagent (14% boron trifluoride) which is added directly to red cells along with 250 µl of hexane and heated for 10 minutes at 100°C. The neutralisation step is omitted and instead water and hexane are added; the sample is then centrifuged and the hexane upper phase collected and transferred directly into a GC auto sampler vial without drying under nitrogen and re-dissolving in hexane.

GC is versatile method and provides reliable results with a range of available modifications<sup>15</sup> and can be used to analyse a wide variety of samples. It has advantages over mass spectrometry (MS) when analysing n-6 and n-3 fatty acid metabolism as it is able to distinguish between structurally similar fatty acids as it uses retention time for labelling as opposed to atomic mass. MS is able to identify fatty acids within a sample but unable to distinguish double bond positions in stereoisomers and therefore unable to tell certain fatty acids apart. If required, both methods can be used in tandem by GC-MS<sup>16</sup>. This technique is employed during investigation into lipid metabolism and function in the field of lipidomics. The use of GC in tandem with MS has led to great advances as it allows manipulation of fatty acid identification by use of different stationary phases in GC to discriminate between fatty acids that MS alone cannot. GC can also be used in combination with electron impact mass spectrometry (EI-MS) which allows for the identification of fatty acids when they are combined with alternative chemical derivatives such as picolinyl esters. This widens the use of the technique and continues to improve lipid profiling as a research method in a range of

areas such as physiology, clinical biomarker detection and pathology as well as lipid biochemistry<sup>17</sup>.

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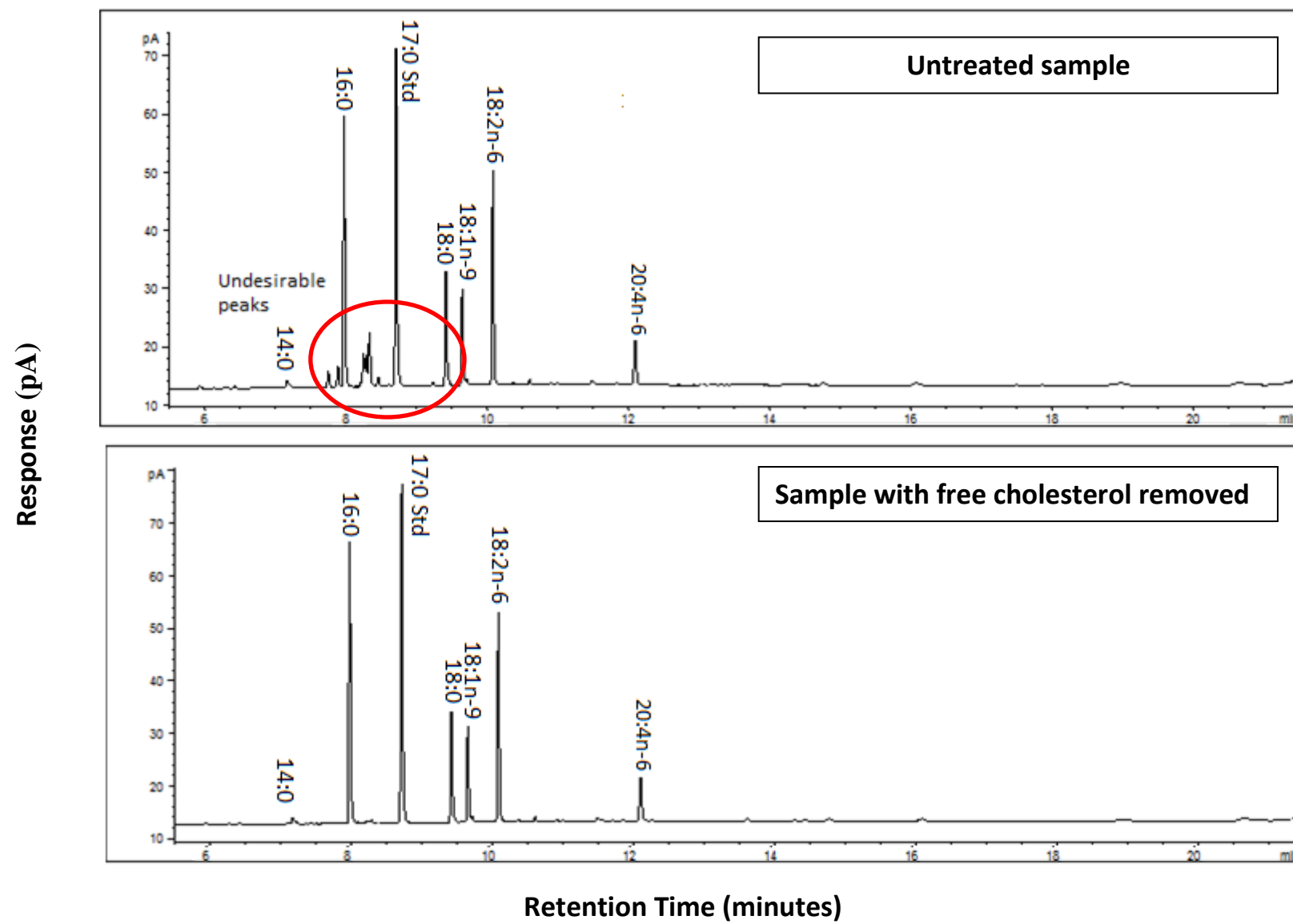
### Disclosures:

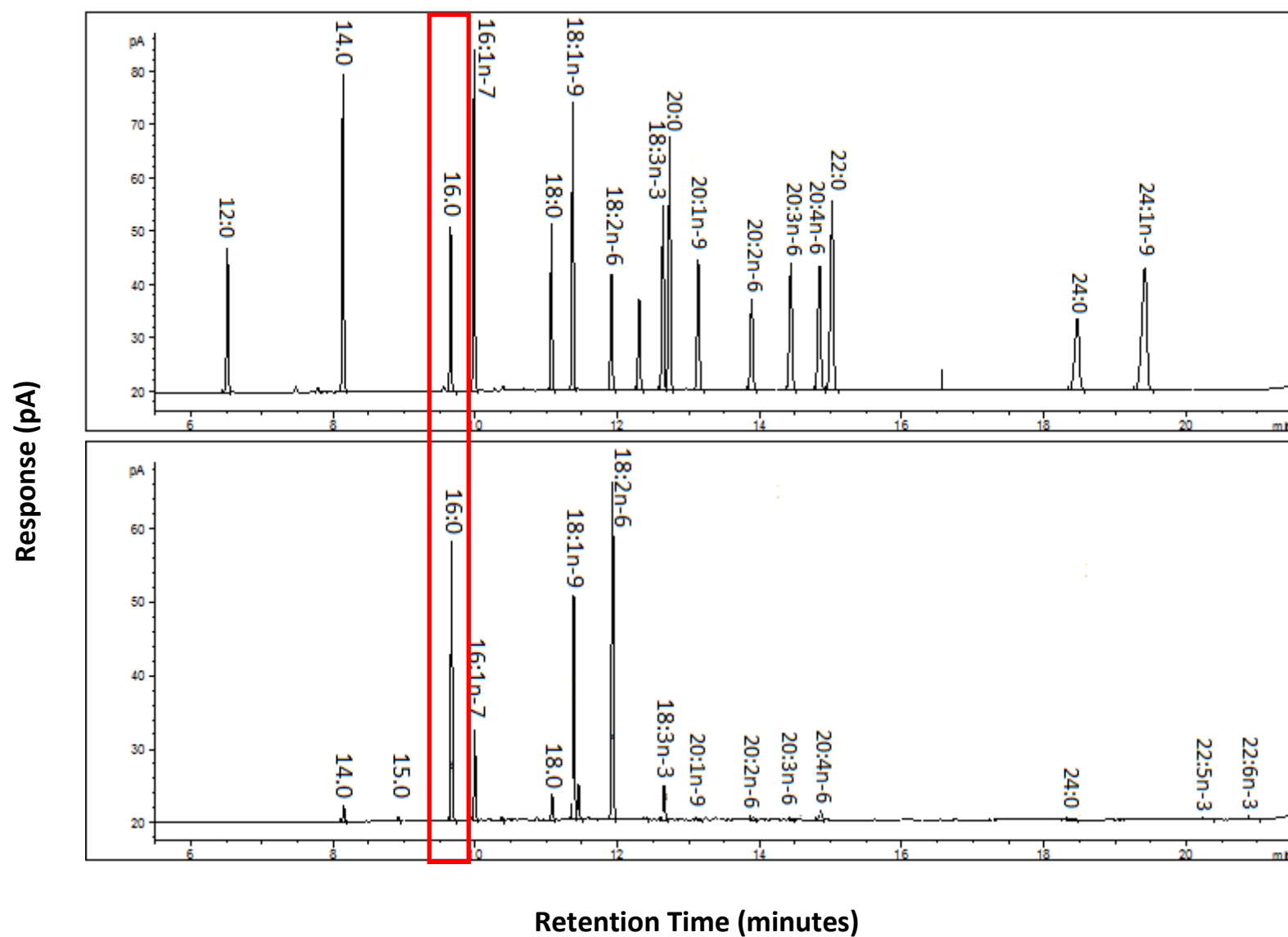
The authors declare that they have no competing financial interests.

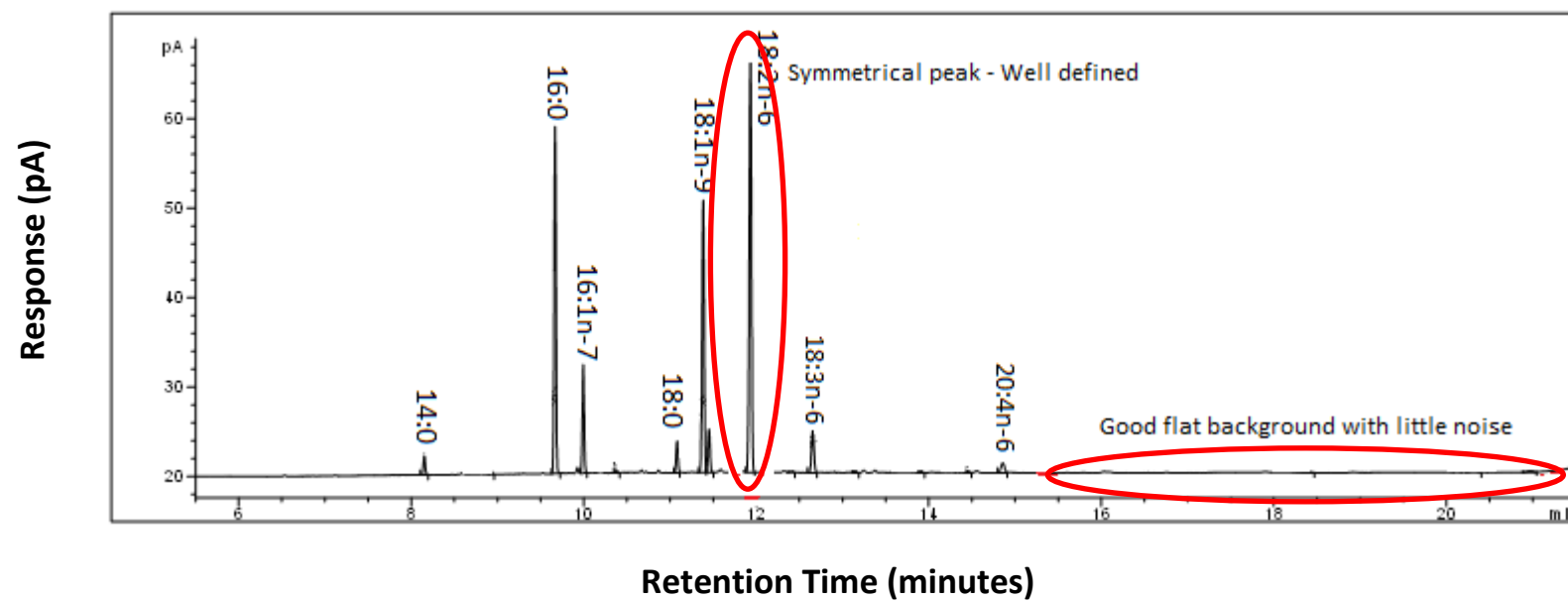
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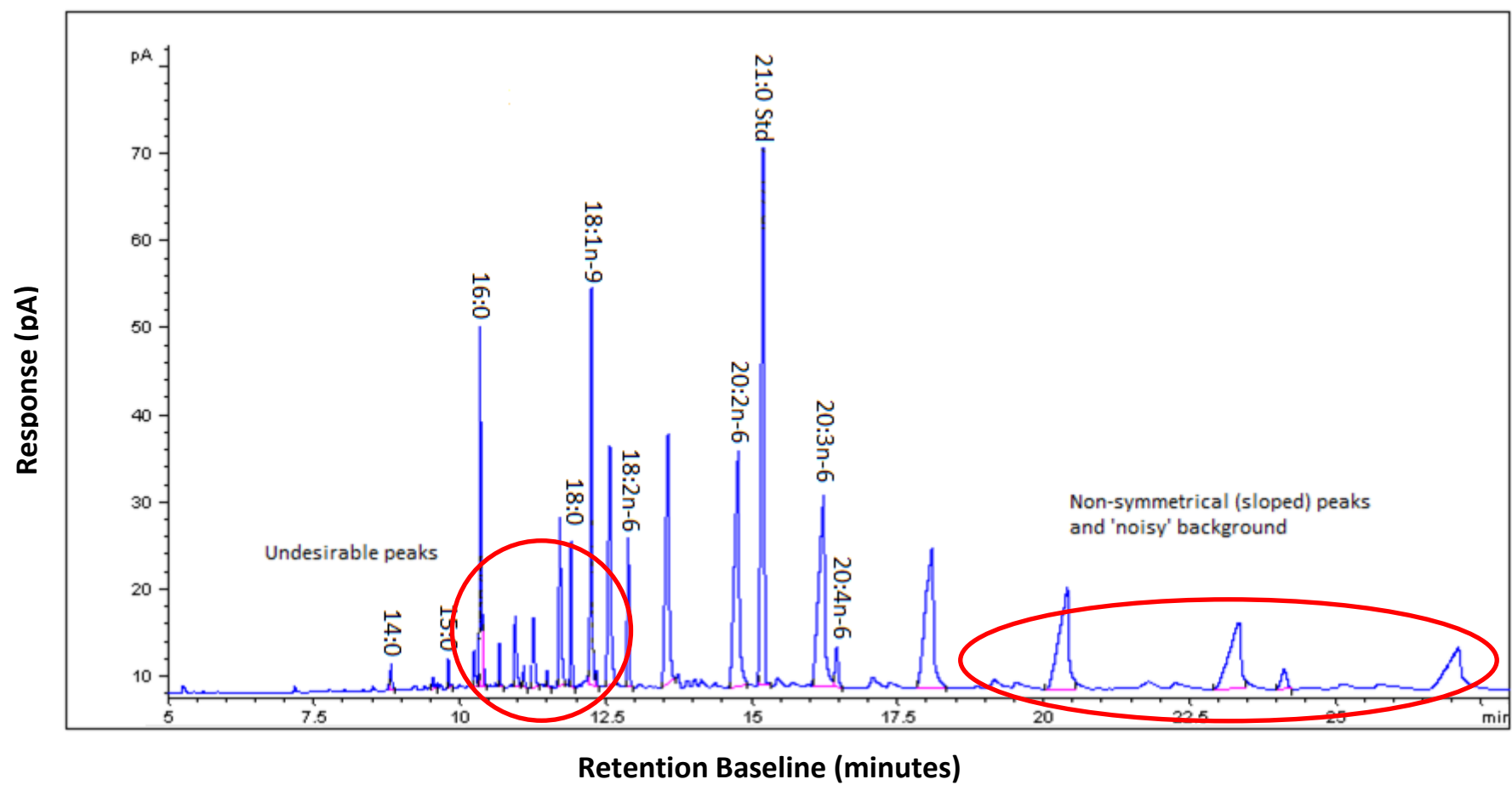






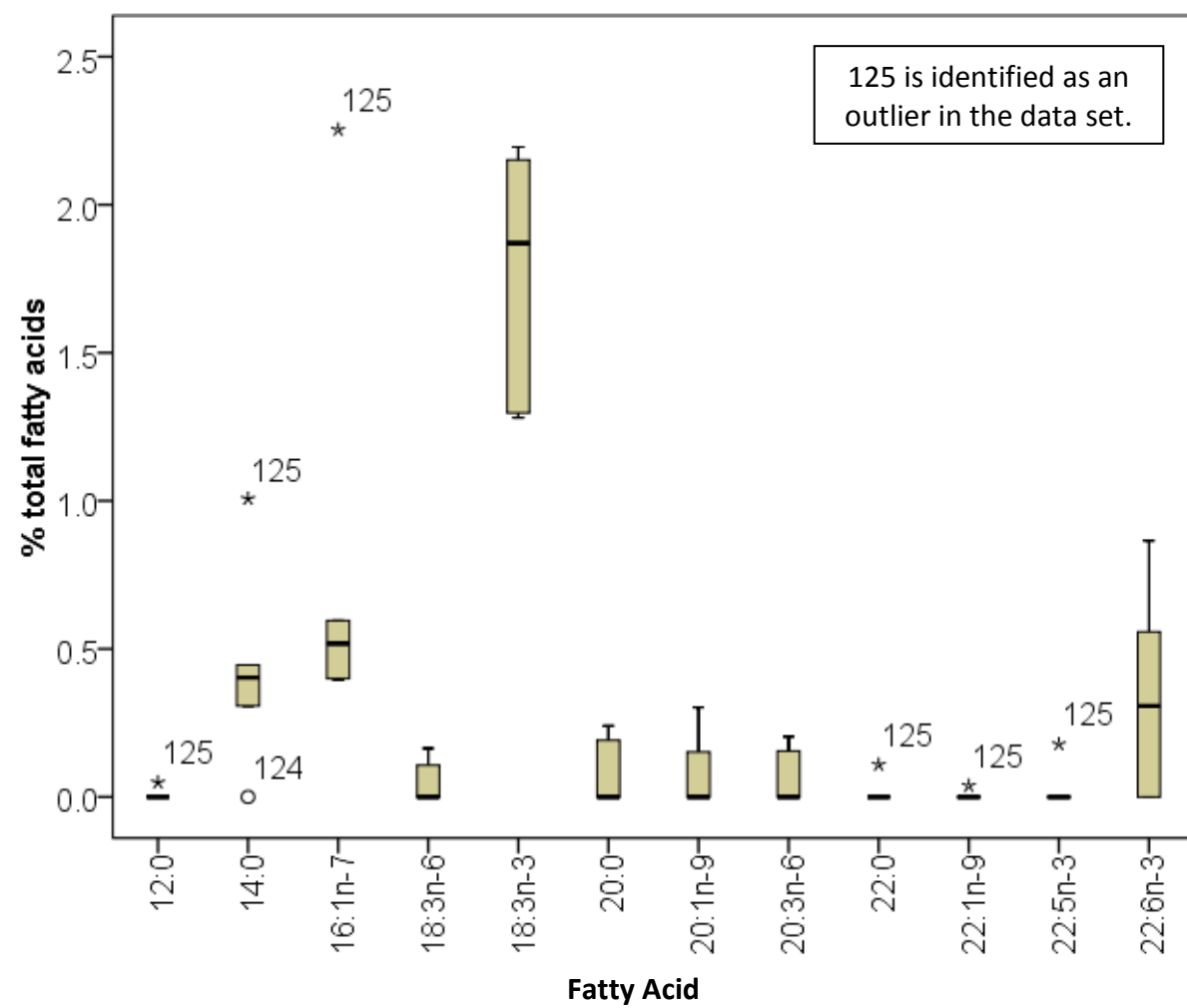
\*Figure 4

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\*Figure 5

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\*Figure 6

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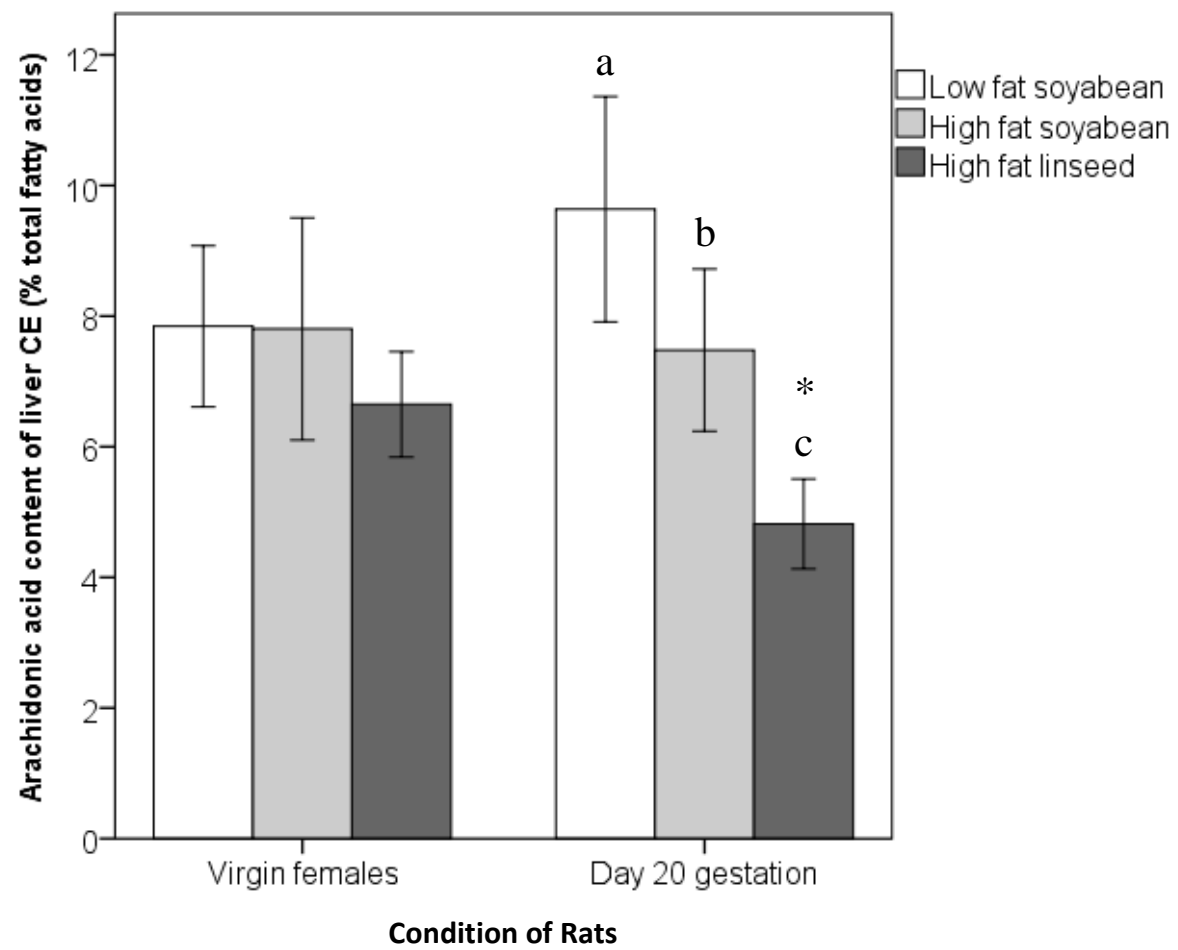


Table 1  
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	Virgin Female			Day 20 gestation			ANOVA <i>P</i>		
	Low fat soybean oil	High fat soybean oil	High fat linseed oil	Low fat soybean oil	High fat soybean oil	High fat linseed oil	Diet	Pregnancy	Diet x Pregnancy
<i>Liver cholesteryl ester (g/100 g total fatty acids)</i>									
14:0	0.6 <sup>a</sup> (0.2)	0.3 <sup>b</sup> (0.2)	0.2 <sup>b</sup> (0.1)	0.3 <sup>*</sup> (0.2)	0.5 (0.3)	0.3 (0.2)	0.059	0.840	0.018
16:0	43.7 <sup>a</sup> (3.2)	46.7 <sup>a</sup> (4.9)	23.4 <sup>b</sup> (2.2)	43.0 <sup>a</sup> (3.4)	35.1 <sup>*b</sup> (3.1)	37.7 <sup>*b</sup> (2.1)	<0.001	0.545	<0.001
18:0	21.1 <sup>a</sup> (3.5)	13.8 <sup>b</sup> (3.2)	22.1 <sup>a</sup> (3.7)	15.5 <sup>*</sup> (2.6)	15.3 (2.0)	16.6 <sup>*</sup> (3.3)	0.002	0.005	0.015
20:0	0.3 (0.3)	ND	0.1 (0.1)	0.4 (0.4)	0.4 <sup>*</sup> (0.3)	0.2 (0.2)	0.132	0.022	0.348
22:0	ND	ND	ND	ND	0.1 (0.2)	ND	0.415	0.341	0.415
24:0	ND	ND	ND	0.4 <sup>*a</sup> (0.1)	0.1 <sup>b</sup> (0.2)	0.1 <sup>b</sup> (0.1)	<0.001	<0.001	<0.001
18:3n-3	1.0 <sup>a</sup> (0.2)	1.9 <sup>a</sup> (0.4)	12.4 <sup>b</sup> (2.6)	1.0 <sup>a</sup> (0.3)	1.8 <sup>a</sup> (0.1)	10.6 <sup>b</sup> (1.1)	<0.001	0.117	0.124
20:5n-3	0.2 <sup>a</sup> (0.2)	ND <sup>a</sup>	4.1 <sup>b</sup> (0.6)	0.2 <sup>a</sup> (0.5)	ND <sup>a</sup>	2.0 <sup>*b</sup> (0.6)	<0.001	<0.001	<0.001
22:5n-3	0.2 <sup>a</sup> (0.2)	ND <sup>a</sup>	1.1 <sup>b</sup> (0.3)	0.2 (0.3)	0.2 <sup>*</sup> (0.2)	0.6 <sup>*</sup> (0.3)	<0.001	0.219	0.002
22:6n-3	0.7 <sup>ab</sup> (0.4)	0.4 <sup>a</sup> (0.4)	1.1 <sup>b</sup> (0.3)	1.9 <sup>*</sup> (0.4)	1.8 <sup>*</sup> (0.4)	2.2 <sup>*</sup> (0.4)	0.009	<0.001	0.487
18:2n-6	13.4 <sup>a</sup> (1.6)	17.6 <sup>b</sup> (2.9)	17.3 <sup>ab</sup> (2.3)	15.0 <sup>a</sup> (1.6)	21.3 <sup>b</sup> (1.5)	15.7 <sup>a</sup> (1.6)	<0.001	0.075	0.010
18:3n-6	0.4 (0.3)	ND	0.2 (0.03)	0.4 (0.4)	0.5 <sup>*</sup> (0.2)	0.4 (0.3)	0.621	0.013	0.098
20:3n-6	0.2 <sup>ab</sup> (0.2)	ND <sup>a</sup>	0.4 <sup>b</sup> (0.1)	0.1 (0.2)	0.3 (0.3)	0.3 (0.3)	0.132	0.823	0.044
20:4n-6	7.8 (1.2)	7.8 (1.7)	6.7 (0.8)	9.6 <sup>a</sup> (1.7)	7.5 <sup>b</sup> (1.2)	4.8 <sup>*c</sup> (0.7)	<0.001	0.782	0.006
16:1n-7	2.0 <sup>a</sup> (0.7)	0.5 <sup>b</sup> (0.1)	0.4 <sup>b</sup> (0.1)	2.0 <sup>a</sup> (0.7)	0.9 <sup>b</sup> (0.3)	0.7 <sup>b</sup> (0.4)	<0.001	0.187	0.439
18:1n-9	7.5 <sup>a</sup> (2.0)	10.9 <sup>b</sup> (2.0)	9.9 <sup>ab</sup> (1.4)	9.1 (2.8)	10.5 (2.1)	10.1 (1.9)	0.025	0.522	0.513
20:1n-9	0.3 (0.3)	ND	0.1 (0.02)	0.4 (0.3)	0.3 (0.4)	0.2 (0.3)	0.112	0.140	0.659
22:1n-9	0.3 (0.6)	ND	0.3 (0.04)	0.1 (0.2)	ND	ND	0.326	0.157	0.385

Table of materials/ equipment

Name of material/Equipment	Company	Catalogue number
Methanol:	Fisher Scientific	M/4056/17
Chloroform:	Fisher Scientific	C/4966/17
BHT:	Sigma- Aldrich	W218405
NaCl	Sigma- Aldrich	S9888
Hexane	Fisher Scientific	H/0406/17
Glacial acetic acid	Sigma- Aldrich	695084
Sulfuric acid	Sigma- Aldrich	339741
Potassium carbonate	Sigma- Aldrich	209619
Potassium bicarbonate	Sigma- Aldrich	237205
Ethyl acetate	Fisher Scientific	10204340
Toluene	Fisher Scientific	T/2300/15
Diethyl ether	Sigma- Aldrich	309966
Nitrogen (oxygen free) cylinder	BOC	44-w
Aminopropyl silica SPE cartridges	Agilent	12102014
Silica gel SPE cartridges	Agilent	14102010
Molecular sieves	Sigma- Aldrich	334324
Glass pasteur pipettes	Fisher Scientific	FB50251
Borosilicate glass test tube	Fisher Scientific	FB59527
Screw thread glass test tubes	Fisher Scientific	FB59555
Caps for screw thread test tubes	Fisher Scientific	FB51353
Solida phase extraction (SPE) tank	Agilent	
Luer stopcocks for SPE tank	Agilent	12131005
Vacuum pump	Sigma- Aldrich	2656-194GB-1EA
GC vials	Kinesis	STV 12-03TS
GC vial lids	Kinesis	SCC09-0.2B
GC inlet liners	SGE Analytical science	092002
GC septa	SGE Analytical science	041856
GC column	SGE Analytical science	054612
Gas chromatograph		

Comments/Description	MSDS (Caution notes)
'CAUTION' Fumes - HPLC Grade	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Fumes - HPLC Grade	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Dust fumes - Anhydrous	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
Anhydrous	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Fumes - HPLC Grade	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Burns - 99.85%	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Burns - 99.999%	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
99% ACS Reagent grade	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
99.7% ACS Reagent grade	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Fumes - 99+% GLC Specified	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Fumes	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Fumes	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
'CAUTION' Compressed gas - explosion risk	<a href="http://www.boconline.co.uk/en/sh">http://www.boconline.co.uk/en/sh</a>
Cartridge - Bead mass 100mg	
Cartridge - Bead mass 100mg	
Pellets, AW-300, 1.6 mm	<a href="http://www.sigmaaldrich.com/MSI">http://www.sigmaaldrich.com/MSI</a>
Non-screw cap	
13mm	
To fit 13mm tube	
VacElut 20 Manifold	
Short thread 9mm, TPX 0.2ml fused glass insert	
Short thread 9mm blue	
Split/splitless Focus liner ID 4mm, OD 6.3mm length 78.5mm	
11mm, MN material	
Length 30m, ID 0.22mm, Film thickness 0.25µm	
HP 6890 series	

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### AUTHOR:

Name: Fisk, Helena L  
Department: Human Developmental and health academic unit  
Institution: University of Southampton  
Article Title: The use of gas chromatography to analyse compositional changes of fatty acids in rat liver tissue during pregnancy  
Signature: H Fisk Date: 23.07.2013

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MS # (internal use):

**Editorial comments:**

1. Please revise step 1.3 to avoid the use of the word "kill." Please use a less graphic term such as "euthanize."

**This has been revised as suggested (see step 1.3).**

2. Please describe how to remove the liver in step 1.4. If these details have been reported elsewhere, please provide the appropriate citation.

**This has been described as suggested (see step 1.4).**

3. Please indicate how long one can store the samples in all steps where samples are stored (e.g., steps 2.5, 2.7, etc.)

**Storage time recommendations have now been included as suggested (see step 2.5, 2.7, 3.6, 3.9, 3.11, 3.13, 3.19, 3.21, 4.9, 5.9, 6.2)**

4. Please re-write step 7.5 in imperative tense.

**This has been revised as suggested (see step 7.5)**

5. You have only highlighted ~ 0.75 pages of your protocol to be filmed. Please note that highlighting is used by our script writers to translate your protocol into a script and plan the video portion of your article. Any information that is not highlighted will therefore not be included in your video. Your video should contain enough detail such that an entire, coherent story is presented to the viewer.

Additionally, the highlighting should include complete statements and complete actions and not portions of sentences or actions. For example, steps 2.5 and 2.7 are highlighted while step 2.6 is not highlighted. In order for the protocol to make sense in the video, please also highlight step 2.6.

Please re-evaluate your protocol considering the highlighting guidelines above and adjust your highlighting as you see fit to tell a complete, coherent story to be included in your video. You may highlight as much as 2.75 pages of text to be filmed.

**The protocol has been re-evaluated, and further sections highlighted as suggested for a more coherent story.**

**Reviewer #2:**

*Minor Concerns:*

I have just few comments (listed below), which I would appreciate if they will be addressed by the authors:

- p. 5 line 220: it is step 3.19, not 2.19.

**This has now been corrected (see step 4.1)**

- p. 7 line 273: step 6.2 is ambiguous. What do you mean by repeating step 5.1 and then combine the two hexane fractions? Maybe you mean repeat all the step 5 (from 5.1 to 5.9) or maybe repeat just step 6.1? Please, shine more light on this particular step of the protocol.

**This has now been revised to make the instructions more clear (see step 6.2).**

- p. 7 line 280: the temperature method of the GC instrument starts at 15 °C, I think it is 150 °C.

**This has now been amended to 115 °C (see step 7.1).**

- p. 8 line 345: the authors affirm that 16:0 and 18:2n6 are the most identifiable peaks within the samples expressed in Figure 2. If I am correct, I see other two peaks that present a higher intensity at ~ 9 and 12 minutes, which fatty acid methyl esters are they?

**We can confirm that the two main peaks are 16:0 and 18:2n-6, and the 'box' on the figure has been adjusted to fall into the correct position. We have also included peak identities for the other major fatty acids within this sample (see Figure 2).**

- p. 9 line 364: 20:4n6 content (close to 8% of the total) is not present in figure 5 so I would add in the caption also this fatty acid. The five (not four) major fatty acids within CE that are not shown.

**Figure 5's title has been corrected to state that the five major fatty acids within CE are not shown.**

- By examination of the GC run, I have noticed that retention time in Figure 1 is shortened with respect to the others present in Figure 2-4. It is matter of fact that RT changes its value run by run, for this reason I suggest the authors to label with the fatty acid name at least the six main peaks in each figure in order to help the reader to analyze the chromatograms.-

**All figures have now been revised to ensure that the main peaks on each chromatogram are identified (see figure 2, 3 and 4).**

Moreover, the peak intensity in the chromatograms is different in all the figures, suggesting a different fatty acid composition of the analyzed sample. Maybe it could be of reader's interest adding to each caption which kind of sample it is shown (FAME of CE coming from virgin or pregnant rat? type of diet?).

**Details of the sample used to generate each chromatogram are now included in the title of figures 1, 2 and 4 as suggested.**



*Additional Comments to Authors:*

The present paper is focused on n6-n3 PUFA content and these values are reported in Table 1. As said by the authors, there are other fatty acids that are the major ones, which give also the higher contribution to the total fatty acid composition (methyl palmitate, stearate and oleate). Which is their contribution in terms of relative percentage? Perhaps it might be interesting to add their value to Table 1.

**Table 1 now also includes data for saturated and monounsaturated fatty acids as suggested.**

**Reviewer #3:**

1. The authors should include more details of the GC instrument conditions all of which can markedly impact separation of peaks. These include: the type of gas used (nitrogen, hydrogen or helium) and the volume of gas flow through the column; the injector and detector temperatures; the makeup gas and flow rate.

**Further details of GC instrument conditions have now been incorporated into step 7.1 as suggested.**

2. It would be helpful to the reader to provide a representative trace of either a standard mixture of fatty acids or an actual sample, in which all relevant peaks are identified. This could well replace Figure 2.

**Figure 2 has now been amended to include peak identities for all relevant peaks, and includes both chromatograms from a standard mixture of fatty acids, and an actual sample.**