Food for microbes. The interplay between indigestible carbohydrates, gut microbiota, and cardiometabolic disease

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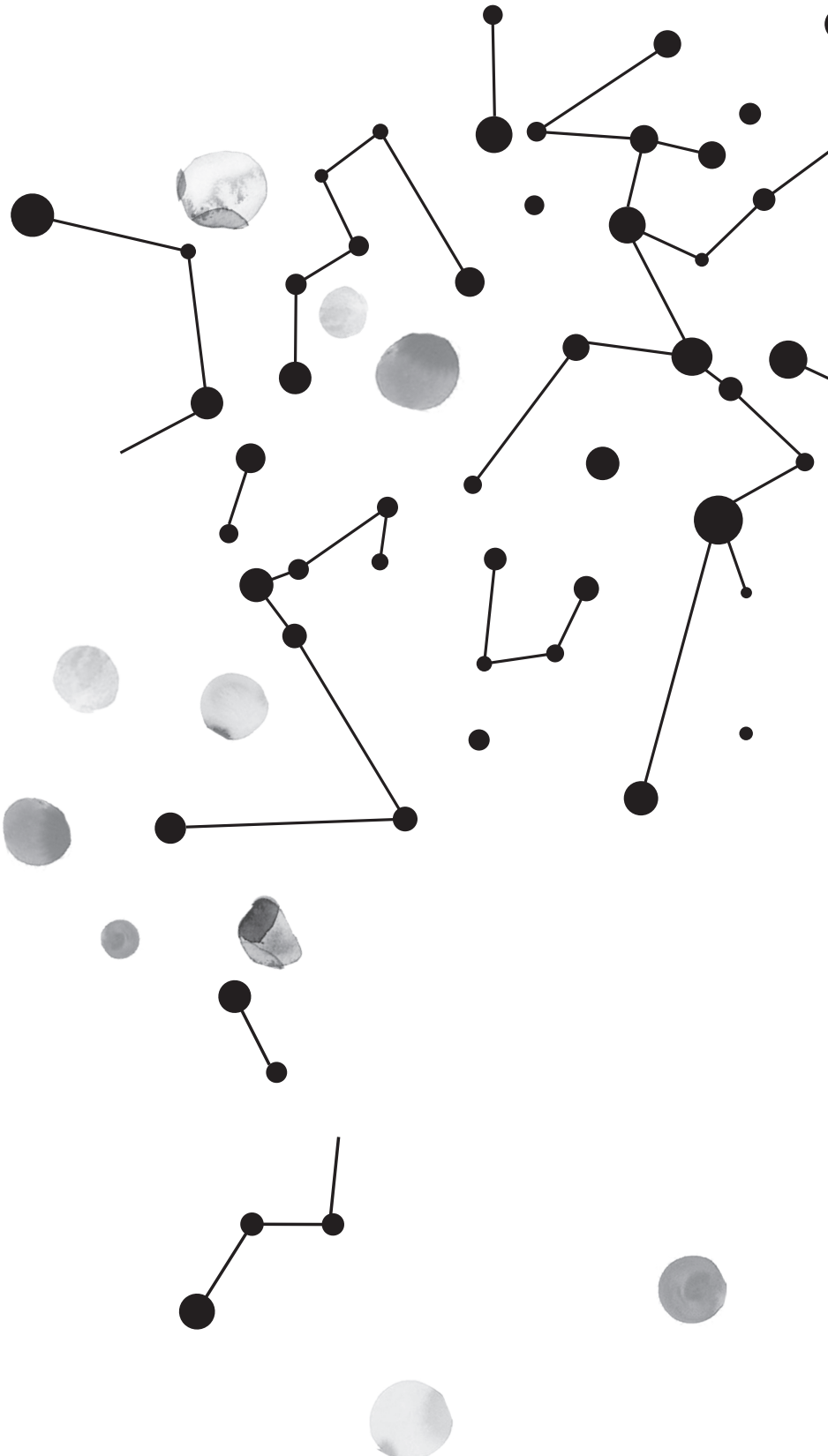

GC-MS ANALYSIS OF MEDIUM- AND LONG-CHAIN FATTY ACIDS IN BLOOD SAMPLES

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ABSTRACT

Our body contains a wide variety of fatty acids that differ in chain length, the degree of unsaturation, and location of the double bonds. As the various fatty acids play distinct roles in health and disease, methods that can specifically determine the fatty acid profile are needed for fundamental and clinical studies. Here we describe a method for the separation and quantification of fatty acids ranging from 8 to 24 carbon chain lengths in blood samples using gas chromatography-mass spectrometry following derivatization using pentafluorobenzyl bromide. This method quantitatively monitors fatty acid composition in a manner that satisfies the requirements for comprehensiveness, sensitivity, and accuracy.

INTRODUCTION

Lipids and fatty acids (FAs) are present in all organisms and constitute essential structural elements of biological membranes, regulate and control cellular function, and are involved in the onset and progression of various diseases [1]. The majority of FAs are present as esters in lipids, such as triacylglycerols, sterol esters, and phospholipids. Only a small fraction is nonesterified, generally termed free FAs (FFAs) [2]. The role of FAs in health and disease has gained extensive interest. FAs differ in chain length, the degree of unsaturation, and location of the double bond(s). As various types of FAs have different associations with disease outcomes, the assessment of the FA composition in biological samples may provide suitable information. Therefore, great effort has been put in the development of comprehensive, sensitive, and reliable methodologies to quantify the FA composition. Here we describe step-by-step the quantitative analysis of the FA profile in plasma using GC-MS, measuring FAs with a chain length ranging from 8 to 24 carbons.

GC-MS is an analytical technique that is well-suited for the analysis of the total amount of FAs as well as the FA composition within a sample [3]. As blood contains both esterified FAs and FFAs, a separate hydrolysis step is required during sample preparation to determine the total FA composition. For the analysis of FAs by GC-MS, it is of importance to convert FAs into suitable volatile derivatives by derivatization (e.g., alkylation or silylation) [4]. Traditionally in GC-MS analysis, FAs were being transformed into their methyl ester or trimethylsilyl ester derivatives [5]. Alternatively, pentafluorobenzyl bromide (PFBBr) can be used to derivatize FAs. It has been successfully applied for the analysis of FAs with different chain lengths [6–9]. The benzyl bromide group reacts with the carboxylic acid group to form a pentafluorobenzyl ester. In addition, this pentafluorobenzyl ester contains ideal properties for electron capture negative ionisation (ECNI), a highly selective and sensitive ionisation technique. The combination of PFBBr derivatization and ECNI ionisation allows for the analysis of negatively charged molecular ions. These ions are usually detected in the single ion monitoring (SIM) mode on quadrupole-based mass spectrometers. Isotopically labelled internal standards (IS) have to be used for quantitative analysis of FAs by GC-MS. The addition of IS

in GC-MS analysis enables quantitative analysis of biological samples and greatly improves detection specificity [10].

MATERIALS

Use only high-purity solvents (preferably LC-MS grade) in order to prevent increased background signals (*see* **Note 1**). If vendors from different materials are mentioned in this method section, the use of these chemicals is recommended based on our previous experiences. The use of 10 M NaOH forms an exception. In order for the method to succeed, it is urged to use the specific items mentioned in the Materials section. An overview of the amount of materials and chemicals is provided in Table 1.

MATERIALS FOR SAMPLE PREPARATION

1. Glass autosampler vials, inserts, and caps. It is recommended to use Agilent certified 2 mL vials with screw top; Agilent certified 250 μL inserts with polymer feet; and Agilent screw caps with PTFE/red silicone septum.
2. 1 μg/mL IS solution in ethanol (EtOH) (*see* **Note 2**): accurately weigh decanoic acid-d19, palmitic acid-d31, and arachidonic acid-d8, and dissolve in EtOH to a final concentration of 1 μg/mL. Store at −80°C.
3. Concentration series of FA standards in EtOH: use GLC reference standard 85 mix (Nu-Chek Prep), eicosapentaenoic acid (Cayman), docosapentaenoic acid (Cayman), and docosahexaenoic acid (Cayman), and serially dilute using EtOH. Prepare concentrations ranging from the lower limit of quantification (LLOQ) (*see* Table 2) to 50 μg/mL. Store at −80°C.
4. 172 mM PFBBr in acetone: add 26.8 μL PFBBr to 1 mL acetone. Prepare fresh daily.
5. 10 M NaOH in water. In order for the method to succeed, it is urged to use a prepared solution from Sigma-Aldrich (Art. No. 72068) (*see* **Note 3**).

Table 1. Chemicals and materials needed per sample for the quantification of FAs

Chemical / material	Calibration series per sample	Plasma / serum sample
Plasma / serum	-	10 µL
Acetone	250 µL	250 µL
10 M NaOH	-	10 µL
1 µg/mL IS solution	10 µL	10 µL
Standards in EtOH	10 µL[a]	-
EtOH	-	10 µL
172 mM PFBBr	100 µL	100 µL
n-hexane	500 µL	500 µL
Water	250 µL	250 µL
Glass autosampler vials	2	2
Glass autosampler inserts	1	1
Glass autosampler caps	2	2

For every batch of samples, take along three blank samples. Blank samples should be processed in exactly the same way as biological samples

[a] For every individual sample of the calibration series, a specific concentration of standards in EtOH is used

MATERIALS FOR GC-MS

1. GC with split/splitless injector, coupled to a quadrupole mass spectrometer with chemical ionisation source.
2. Injection: autosampler (recommended).
3. GC column: use an Agilent VF-5 ms column (5% phenylmethyl; 25 m × 0.25 mm internal diameter; 0.25 µm film thickness).
4. Pure helium (99.9990%) and methane (99.9995%) are used as carrier and chemical ionisation gas, respectively.

METHODS

Palmitic acid and stearic acid are ubiquitous. Hence, extra care has to be taken to prevent sample contamination. Sources of contamination include low-quality plastics and (low-purity) solvents (*see* **Note 1**).

SAMPLE PREPARATION

1. Facilitate rapid sampling. Store samples at −80°C upon collection if the samples are not prepared immediately (*see* **Note 4**).
2. Prepare a glass autosampler vial for every sample: for calibration samples, add 250 µL acetone and 10 µL of your calibration series FA standards at the desired concentration. An indication of the expected LLOQ for every FA is provided in Table 2. For biological samples, add 250 µL acetone (*see* **Note 5**), 10 µL EtOH (*see* **Note 6**), and 10 µL plasma or serum into a glass autosampler vial. For blank samples, add 250 µL acetone and 10 µL EtOH into a glass autosampler vial. For every experiment, three blank samples should be included.
3. Hydrolyse the biological and blank samples (*see* **Note 7**): add 10 µL 10 M NaOH to the biological and blank samples. Vortex all samples. Heat the biological and blank samples at 60°C for 30 min in a laboratory stove. Let the samples cool down to room temperature (approximately 15 min).
4. Add 10 µL 1 µg/mL IS solution (*see* **Notes 2** and **8**) to every sample and vortex all samples.
5. Add 100 µL 172mM PFBBr in acetone (*see* **Note 9**). Vortex the samples.
6. Heat the samples at 60°C for 30 min in a laboratory stove. Let the samples cool down to room temperature (approximately 15 min) (*see* **Note 10**).
7. Add 500 µL *n*-hexane and 250 µL water to the samples. Shake vigorously in vertical direction of the vial for 10 sec. Let the samples rest for 1 min at room temperature.
8. Prepare a new empty glass autosampler vial with a glass insert for every sample. Transfer 250 µL of the *n*-hexane (upper layer) into the glass insert.

Table 2. Overview of FAs

FA	Name	RT (min)	Monitored *m/z* (M^-)	LLOQ (ng/mL)[a]	IS
FA 08:0	Octanoic acid	10.14	143.1	200	FA 10:0-d19
FA 10:0	Decanoic acid	11.01	171.1	50	FA 10:0-d19
FA 10:0-d19	Decanoic acid-d19	10.92	190.3	N/A	N/A
FA 11:0	Undecanoic acid	11.42	185.2	50	FA 10:0-d19
FA 12:0	Lauric acid	11.87	199.2	100	FA 10:0-d19
FA 13:0	Tridecanoic acid	12.40	213.2	50	FA 10:0-d19
FA 14:0	Myristic acid	13.03	227.2	50	FA 16:0-d31
FA 14:1 (n-5)	Myristoleic acid	12.97	225.2	20	FA 16:0-d31
FA 15:0	Pentadecanoic acid	13.78	241.2	50	FA 16:0-d31
FA 15:1 (n-5)	10-Pentadecenoic acid	13.72	239.2	10	FA 16:0-d31
FA 16:0	Palmitic acid	14.68	255.2	500	FA 16:0-d31
FA 16:0-d31	Palmitic acid-d31	14.43	286.4	N/A	N/A
FA 16:1 (n-7)	Palmitoleic acid	14.50	253.2	50	FA 16:0-d31
FA 17:0	Heptadecanoic acid	15.72	269.3	20	FA 16:0-d31
FA 17:1 (n-7)	10-Heptadecenoic acid	15.53	267.2	10	FA 16:0-d31
FA 18:0	Stearic acid	16.54	283.3	500	FA 16:0-d31
FA 18:1 (n-9) cis	Oleic acid	16.37	281.3	100	FA 16:0-d31
FA 18:1 (n-9) trans	Elaidic acid	16.41	281.3	50	FA 16:0-d31
FA 18:2 (n-6)	Linoleic acid	16.34	279.2	50	FA 16:0-d31
FA 18:3 (n-6)	Gamma linolenic acid (GLA)	16.15	277.2	50	FA 16:0-d31
FA 18:3 (n-3)	Alpha linolenic acid (ALA)	16.40	277.2	50	FA 16:0-d31
FA 20:0	Arachidic acid	17.66	311.3	50	FA 20:4-d8
FA 20:1 (n-9)	11-Eicosenoic acid	17.55	309.3	20	FA 20:4-d8
FA 20:2 (n-6)	11,14-Eicosadienoic acid	17.54	307.3	10	FA 20:4-d8
FA 20:3 (n-6)	Homo-Gamma linolenic acid (DGLA)	17.43	305.3	10	FA 20:4-d8
FA 20:3 (n-3)	11,14,17-Eicosatrienoaic acid	17.58	305.3	10	FA 20:4-d8
FA 20:4 (n-6)	Arachidonic acid (AA)	17.28	303.2	10	FA 20:4-d8

(Continued)

Table 2. *Continued*

FA	Name	RT (min)	Monitored *m/z* (M^-)	LLOQ (ng/mL)[a]	IS
FA 20:4-d8	Arachidonic acid-d8 (AA-d8)	17.26	311.3	N/A	N/A
FA 20:5 (n-3)	Eicosapentaenoic acid (EPA)	17.33	301.2	10	FA 20:4-d8
FA 22:0	Behenic acid	18.48	339.3	50	FA 20:4-d8
FA 22:1 (n-9)	Erucic acid	18.40	337.3	20	FA 20:4-d8
FA 22:2 (n-6)	13,16-Docosadienoic acid	18.39	335.3	10	FA 20:4-d8
FA 22:4 (n-6)	Adrenic acid (AdA)	18.21	331.3	10	FA 20:4-d8
FA 22:5 (n-3)	Docosapentaenoic acid (DPA)	18.25	329.3	10	FA 20:4-d8
FA 22:6 (n-3)	Docosahexaenoic acid (DHA)	18.15	327.2	20	FA 20:4-d8
FA 24:1 (n-9)	Nervonic acid	19.28	365.4	20	FA 20:4-d8

For each FA, an indication of the retention time (RT), the *m/z* value, an indication of the LLOQ, and the IS to be used are shown. *N/A* not applicable

[a] An indication of the lowest concentration to be included in the calibration series. This LLOQ is determined for every individual experiment. The calibration series samples are measured twice. A specific concentration is included if signal/noise >10 and if the accuracy based on the calibration obtained ≥80 and ≤120% for both measurements

GC-MS ANALYSIS

1. Inject 1 μL in the GC-MS, splitless at 280°C.
2. Use helium as carrier gas at a constant flow rate of 1.20 mL/min.
3. Use the following temperature gradient: 1 min at 50°C, linear increase at 40°C/min to 60°C, held for 3 min at 60°C, linear increase at 25°C/min to 237°C, linear increase at 3°C/min to 250°C, linear increase at 25°C/min to 315°C, held for 1.55 min at 315°C.
4. Set the transfer line temperature at 280°C.
5. Keep the ionisation source temperature at 280°C.
6. Use methane as chemical ionisation gas at approximately 15 psi.
7. Detect ions obtained in the negative mode using SIM analysis (*see* **Notes 11** and **12**). Table 2 provides the *m/z* values to be monitored and an indication of retention times (RT).

As a consequence of small chromatographic differences (e.g., GC column length), the exact RT varies between various GC systems. Hence, calibration using external standards is mandatory.

DATA ANALYSIS

1. Integrate the obtained signal (*see* **Note 13**).
2. Calculate the relative retention times (RRT) and area ratios using the respective IS (*see* Table 2) (*see* **Notes 14** and **15**).
3. Determine the slope and LLOQ for every FA by performing linear regression. It is recommended to use a weighing factor of $1/x^2$ [11].
4. Calculate the FA concentrations by using the area ratios obtained from the biological samples, average signal of the blank samples as intercept (*see* **Note 16**), and the slopes obtained from the analysis of the calibration series samples.

NOTES

1. Palmitic acid and stearic acid usually give high background signals, resulting in a relatively high LLOQ (Fig. 1). Sources of these FAs include solvents and plastic containers of inferior quality. Background signals of these FAs can be diminished by using high-purity solvents (preferably LC-MS grade). Additionally, use glass vials for organic solvents.
2. The IS signal should be present in every sample. The IS are used to correct for differences in sample preparation between the samples. Use exactly the same batch of 1 μg/mL IS solution in EtOH for the entire experiment, as minor differences in IS composition might translate into systematic under or overestimation of FAs in samples.
3. Sodium hydroxide pellets can be heavily contaminated by FAs.
4. Collect the biological samples as quickly as possible, and store the samples at −80°C. Levels of FAs can change upon sample collection if the collection is performed slowly or when samples are stored improperly by auto-oxidation of polyunsaturated FAs and enzymatic hydroxylation.

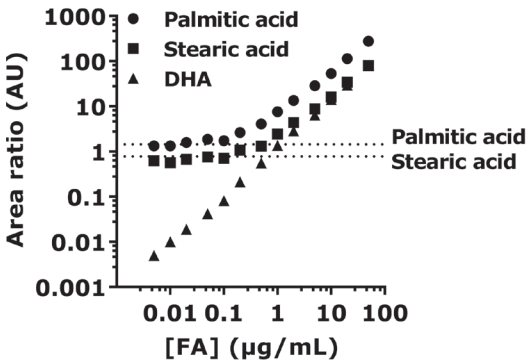

Fig. 1 Background signal of palmitic acid and stearic acid. Palmitic acid and stearic acid usually show a high background signal. As a consequence, the LLOQ for these FAs is higher than for FAs which do not show a high background signal like docosahexaenoic acid (DHA). The dashed lines in the graph show the size of the background signals.

5. Acetone facilitates the precipitation of proteins.
6. The addition of 10 µL EtOH to the samples ensures that the solvents of the biological samples are matched to the solvents in the calibration samples.
7. No esterified FAs are present in the calibration series samples. Therefore, no hydrolysation step is needed.
8. Under highly alkaline conditions, risk of hydrogen–deuterium exchange exists [12]. Therefore, the IS need to be added after hydrolysis.
9. Within this protocol no base is added to catalyse the derivatization reaction, since the addition of base can severely increase FA background [7].
10. *n*-Hexane is added after the samples have been cooled down in order to prevent evaporation and spilling.
11. Sensitivity is higher when the mass spectrometer is operated in SIM mode as compared to the full scan mode. However, the full scan mode can be very useful to detect FAs that are not incorporated in the SIM method or to determine the RT of a specific FA. If one

decides to operate in full scan mode, an *m/z* range of 100-400 can be used.

12. For isotopologue analysis, either *m/z* values corresponding to isotopologues can be added to the SIM method (e.g., M0, M1, M2, etc. for every FA) [7] or the MS can be operated in scheduled scan mode (e.g., scan window including *m/z* values corresponding to M0, M1, M2, etc., for every FA).
13. Oleic and elaidic acids have equal masses and are not baseline separated. In some cases, it is therefore not possible to accurately and precisely quantify one or both of these two FAs within the same sample. FA 20:1 (n-9) and FA 22:1 (n-9), co-elute with, respectively, FA 20:2 (n-6) and FA 22:2 (n-6). As a consequence, the M2 isotopes (containing $2 \times {}^{13}C$ instead of ${}^{12}C$) of FA 20:2 (n-6) and FA 22:2 (n-6) might contaminate the FA 20:1 (n-9) and FA 22:1 (n-9) signal.

14. $$\text{RRT} = \frac{\text{retention time analyte}}{\text{retention time IS}}$$

15. $$\text{Area ratio} = \frac{\text{area analyte}}{\text{area IS}}$$

16. The blank samples reflect the background signal of the biological samples more accurately than the intercept obtained from the linear regression of the calibration series samples. Therefore, use the average area ratio of the blank samples as background signal/intercept to calculate the concentrations of the biological samples. Use the following formula:

$$\text{concentration} = \frac{\text{area ratio - average area ratio blank samples}}{\text{slope}}$$

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