

Automated Sample Preparation for Profiling Fatty Acids in Blood and Plasma using the Agilent 7693 ALS

Application Note

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Abstract

A miniaturized automated sample preparation method is described for the determination of fatty acid methyl esters in blood or plasma samples using the Agilent 7693 Series Automatic Liquid Sampler. Fifty-microliter samples are derivatized using acetyl chloride in methanol and the fatty acid methyl esters are extracted in iso-octane. Extracts are analyzed by GC-FID. Excellent repeatability is obtained and the method can be applied for fully automated fatty acid profiling in blood and plasma samples during dietary, biomedical or metabolomic studies.



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Introduction

The determination of the fatty acid profile in plasma and blood samples is used as a diagnostic tool in biomedical analysis. The relative composition of the lipidic fraction can be applied to nutritional and epidemiological studies [1-3]. These include the study of the relationship between dietary fat intake and human health, the relationship between fatty acid profiles and some pathologies such as cardiovascular diseases, and, more recently, fatty acid profiling, which is also used in metabolomic (lipidomic) studies.

In general, fatty acid profiling is performed using GC-FID or GC-MS. The lipidic fraction in blood or plasma samples is hydrolyzed and methylated into its fatty acid methyl esters (FAMES). A 1 μ L sample of the iso-octane extract containing the FAMES is separated using a polar column, providing separation according to carbon number and number of double bonds. Separation of cis-trans isomers is also obtained using a cyanopropyl silicone column with high cyano content (e.g. HP-88).

Separate steps are used for hydrolysis and methylation. Typically, sample preparation requires several hundreds of microliters (up to a few milliliters) of plasma or blood and includes sequential lipid extraction, hydrolysis, and methylation steps. Some of these steps are relatively slow and require considerable handling and manipulation (fraction transfer, evaporation, etc). Recently, miniaturized methods were developed that allow the determination of fatty acids in a single drop of blood [2,3]. Best results were obtained using a single derivatization step using a methanolic solution of acetyl chloride [3].

In this application note, this miniaturized method was translated into a fully automated method using the Agilent 7693 Series Automatic Liquid Sampler (ALS). The method was tested on plasma and blood samples and the reproducibility was evaluated.

Experimental

The analyses were performed on an Agilent 7890 GC System equipped with a split/splitless (S/SL) inlet with a splitless liner (p/n 5062-3587) and flame ionization detector (FID). Automated sample preparation and injection were performed using a 7693A ALS (G4520A). The autosampler consisted of two injection towers and a tray. The front injection tower was

equipped with a 10- μ L syringe with a PTFE-tipped plunger (5181-3354) and used for sample injection. The back injection tower was equipped with a 100- μ L syringe with a PTFE-tipped plunger (5183-2042) for reagents and extraction solvent addition. The sample tray was equipped with a heater and bar code reader/mixer. The solvent vials in the rear and front turrets were filled as follows:

Rear turret

- Wash A1: methanol
- Wash A2: acetone
- Wash A3: iso-octane
- Wash B1: 5% acetylchloride in methanol (freshly prepared for each sequence)
- Wash B2: iso-octane (dilution)

Front turret

- Wash A1 – A2: iso-octane
- Wash B1 – B2: iso-octane

The sample preparation method was based on the method developed for the determination of fatty acids in a single drop of blood [3]. The method starts with a 50- μ L blood or plasma sample placed into a 250- μ L insert (5181-3377) in a 2-mL vial (5182-0714) with a screw cap (5185-5829). The autosampler adds the derivatization reagent (5% acetyl chloride, Sigma-Aldrich, in methanol) and the sample is heated and mixed at 75°C for 30 min. In this step, the lipidic fraction in blood or plasma consisting of free fatty acids, sterol esters, glycerol esters (mainly triglycerides), and phospholipids is hydrolyzed and transmethylated into fatty acid methyl esters (FAMES). Three sequential heating and mixing steps are done 10 min apart during the 30-min period. After this, 100 μ L of iso-octane is added as an extraction solvent. The fatty acid methyl esters are extracted and analyzed by GC-FID. The sample preparation sequence programmed in the GC ChemStation is given in Table 1.

Table 1. Sample Preparation Sequence

1. Return all vials
2. Move vial from front sequence vial to back turret position #1
3. Wash syringe in Back tower, drawing 50 μ L from Wash A1 dispensing into Current waste vial 1 times
> wash with MeOH
4. Dispense 150 μ L from vial Wash B1 to vial Sample 1 on Back tower
> Add 5% AcCl to sample
5. Wash syringe in Back tower, drawing 50 μ L from Wash A1 dispensing into Current waste vial 1 times
> Wash syringe with MeOH
6. Wash syringe in Back tower, drawing 50 μ L from Wash A2 dispensing into Current waste vial 1 times
> Wash syringe with Acetone
7. Wash syringe in Back tower, drawing 50 μ L from Wash A3 dispensing into Current waste vial 2 times
> Wash syringe with iso-octane
8. Move vial from back turret position #1 to mixer
9. Mix at 4000 rpm 1 times for 10 seconds
10. Move vial from mixer to heater
11. Heat vial at 75 $^{\circ}$ C for 600 seconds
12. Move vial from heater to mixer
13. Mix at 4000 rpm 1 times for 10 seconds
14. Move vial from mixer to heater
15. Heat vial at 75 $^{\circ}$ C for 600 seconds
16. Move vial from heater to mixer
17. Mix at 4000 rpm 1 times for 10 seconds
18. Move vial from mixer to heater
19. Heat vial at 75 $^{\circ}$ C for 600 seconds
20. Move vial from heater to back turret position #1
21. Wash syringe in Back tower, drawing 50 μ L from Wash A3 dispensing into Current waste vial 1 times
> Wash syringe with iso-octane
22. Dispense 100 μ L from vial Wash B2 to vial Sample 1 on Back tower
> Extract with 100 μ L iso-octane
23. Wash syringe in Back tower, drawing 50 μ L from Wash A2 dispensing into Current waste vial 1 times
> Wash syringe with Acetone
24. Wash syringe in Back tower, drawing 50 μ L from Wash A1 dispensing into Current waste vial 1 times
> Wash syringe with MeOH
25. Move vial from back turret position #1 to mixer
26. Mix at 3000 rpm 4 times for 30 seconds
27. Wait for 2 minutes
> wait for phase separation
28. Move vial from mixer to front sequence vial

The analytical conditions are based on the GC-MS method described by Bicalho et al [3]. A long polar Agilent HP-88 GC column is used for optimal resolution of cis-trans fatty acids and determination of important omega-3 (n-3) and omega-6 (n-6) fatty acids. The analytical conditions are summarized in Table 2.

Table 2. Analytical Conditions

Injection	1.0 μ L sampling depth: 15 mm (*)
Inlet	S/SL in splitless mode, 250 $^{\circ}$ C
Column	100 m \times 0.25 mm, 0.2 μ m HP-88, p/n 112-88A7
Carrier gas	Helium Inlet pressure: 322 kPa, constant pressure (Flow = 2.14 mL/min @ 50 $^{\circ}$ C)
GC oven temp	50 $^{\circ}$ C (1 min) @ 15 $^{\circ}$ C/min to 175 $^{\circ}$ C, @ 1 $^{\circ}$ C/min to 250 $^{\circ}$ C Total run time = 84.33 min (**)
FID	250 $^{\circ}$ C, 30 mL/min H2, 400 mL/min air

(*): This sampling depth was visually checked on a prepared sample. The needle depth was adjusted to penetrate the upper iso-octane layer and avoid the lower aqueous/methanol layer.

(**): The last FAME elutes before 60 min. The additional 25-min hold time was needed to elute sterols (cholesterol). The maximum operating temperature of the HP-88 column is 250 $^{\circ}$ C. Total analysis time could eventually be reduced by backflushing using a pressure-controlled capillary flow technology device after the column (for example, purged Ultimate Union).

Nomenclature:

FAMEs listed in tables and figures are abbreviated using the shorthand annotation according to the formula

$$Ca = b * nx * z$$

where Ca is the number of carbon atoms in the fatty acid chain (not including the methyl alcohol part), b is the number of double bonds, nx is the location of the double bond on the xth carbon-carbon bond, counting from the terminal methyl carbon towards the carbonyl carbon, and z is the geometrical configuration expressed as c for cis and t for trans.

Results and discussion

A typical chromatogram showing the fatty acid methyl ester profile from a plasma sample is shown in Figure 1.

Peak identification is given in Table 3. The main peaks are identified as the methyl-esters from palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 n9 c), linoleic acid

(18:2 n6 cc) and arachidonic acid (C20:4 n6). Also, the important omega-3 fatty acids, C18:3 n3 (alpha-linolenic acid, ALA), C20:5 n3 (eicosapentadienoic acid, EPA) and C22:6 (docosahexaenoic acid, DHA) are determined. The separation on the long HP-88 column also allows differentiation of cis and trans fatty acids, which is important since the trans fatty acids are a direct expression of the diet.

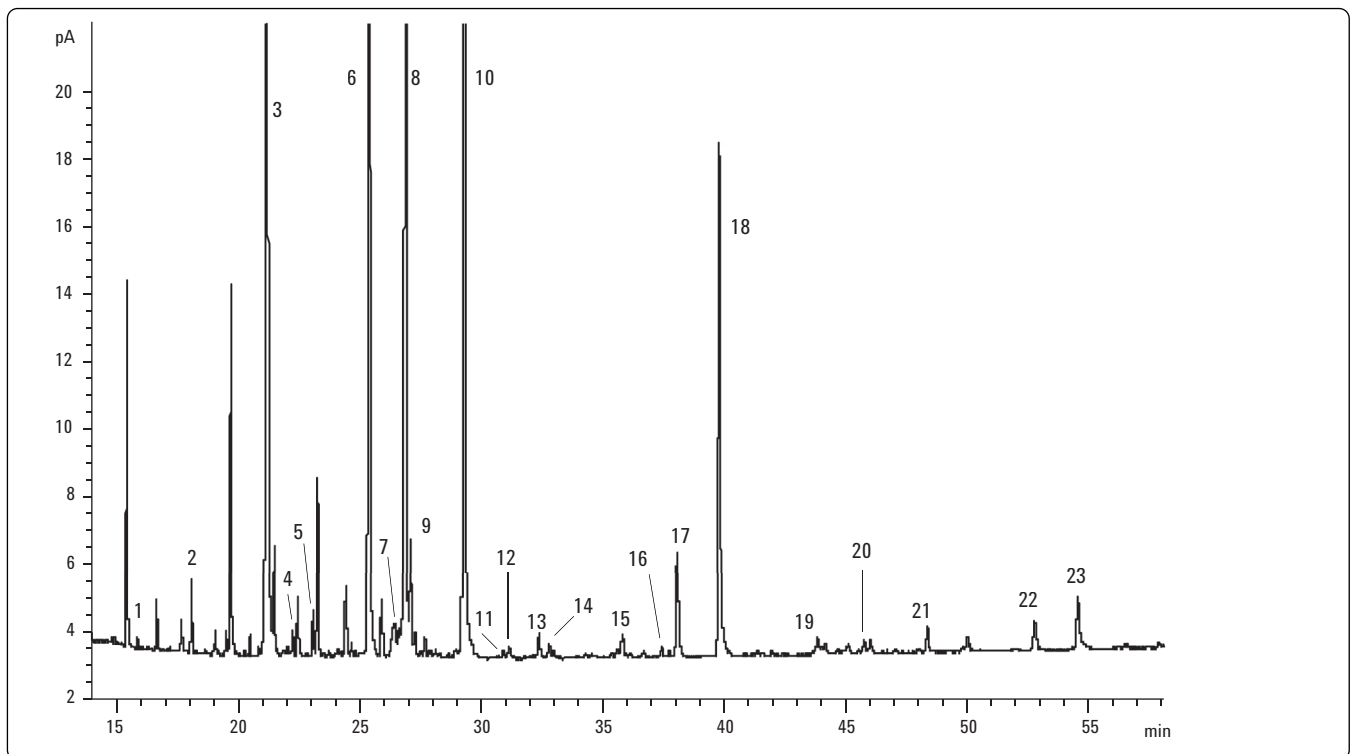


Figure 1. Fatty acid methyl ester profile from a plasma sample.

The relative composition of the fatty acids was calculated using peak area normalization, as is done with the standard approach. The analysis was repeated six times with aliquots of the same plasma sample. Sample preparations and analyses were performed in a fully automated sequence. The results are summarized in Table 3, showing the mean area % (n=6), the standard deviation and the %RSD. The data show that excellent reproducibility was obtained. The average RSD was 2% and for the most abundant fatty acids (relative area > 0.5 %) mostly below 1%. These precision values represent a composite of both sample preparation and analysis steps.

The reproducibility is graphically illustrated in Figure 2 showing an overlay of the FID chromatograms of the six analyses.

Table 3. Peak Identification and Reproducibility of Relative Peak Areas of Fatty Acids in Plasma.

Peak	tR (min)	Name	Mean Area %	s	RSD (%)
1	15.877	C12:0	0.059	0.003	4.43
2	18.143	C14:0	0.557	0.010	1.81
3	21.216	C16:0	33.239	0.185	0.56
4	22.465	C16:1 n7	0.579	0.008	1.37
5	23.118	C17:0	0.384	0.005	1.39
6	25.408	C18:0	17.819	0.113	0.64
7	26.454	C18:1 trans	0.909	0.007	0.76
8	26.897	C18:1 n9	12.153	0.067	0.55
9	27.114	C18:1 n7	1.009	0.007	0.67
10	29.324	C18:2 n6 cc	20.047	0.169	0.84
11	30.906	C20:0	0.071	0.004	5.31
12	31.186	C18:3 n6	0.126	0.005	3.60
13	32.381	C18:3 n3 (ALA)	0.335	0.005	1.38
14	32.789	C20:1 n9	0.111	0.006	5.12
15	35.789	C20:2 n6	0.294	0.004	1.50
16	37.417	C22:0	0.128	0.002	1.73
17	38.051	C20:3 n6	1.556	0.011	0.71
18	39.779	C20:4 n6	9.050	0.063	0.70
19	43.826	C20:5 n3 (EPA)	0.177	0.008	4.76
20	45.730	C24:0	0.175	0.009	5.19
21	48.295	C22:4 n6	0.429	0.005	1.08
22	52.707	C22:5 n3	0.349	0.006	1.81
23	54.466	C22:6 n3 (DHA)	1.028	0.013	1.28

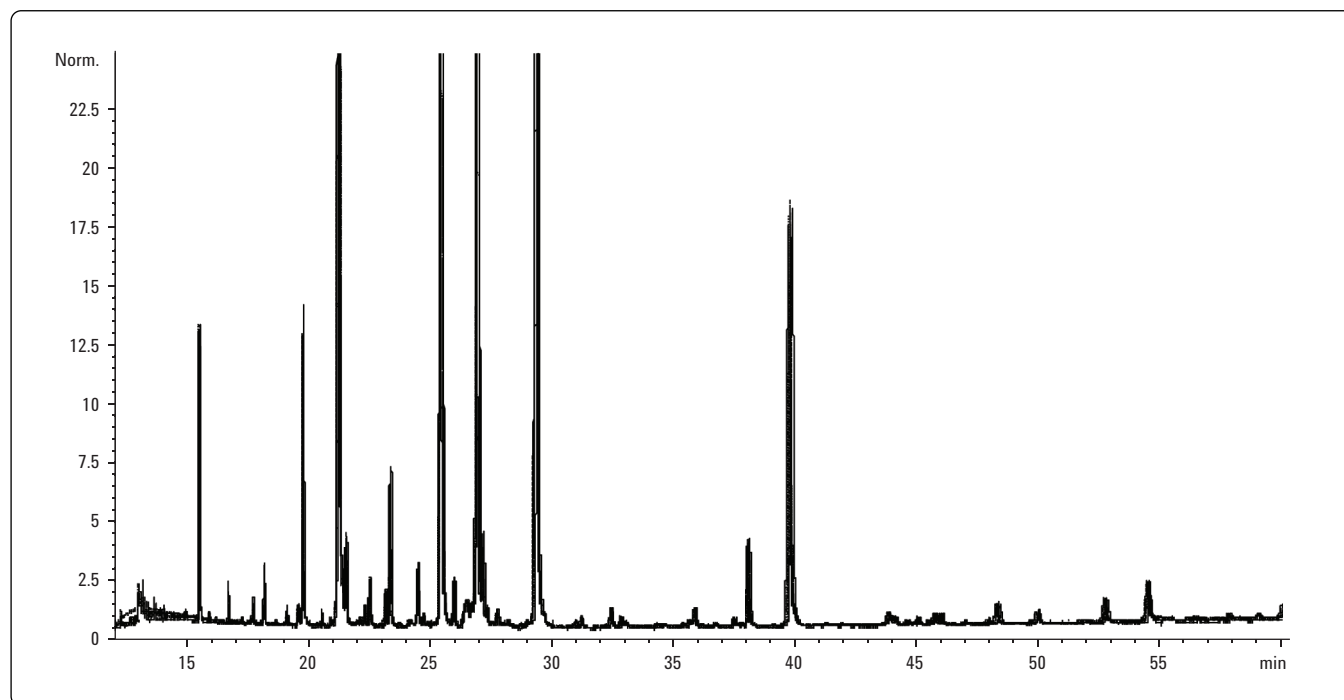


Figure 2. Overlay of the FID chromatograms of the six analyses.

The method is also applicable to blood samples. The fatty acid methyl ester profile obtained by the automated sample preparation GC-FID method for a 50- μ L blood sample (single drop of blood) is shown in Figure 3. In comparison to plasma, some interesting differences are noted. As already reported by Marangoni et al [2], the relative concentration of the highly unsaturated, long chain fatty acids, such as C20:5 n3, C22:4, C22:5 and C22:6 is higher in whole blood than in plasma.

Conclusion

The determination of fatty acids in blood and plasma samples can be fully automated using the 7693 ALS. A miniaturized

sample preparation method, only requiring 50 μ L of blood or plasma, was translated into a single vial reaction. Hydrolysis and transmethylation, followed by micro liquid-liquid extraction is performed using the back injection tower with the tray enabling vortex mixing and sample heating. Injection from the upper extract layer was performed using the front injector.

Excellent reproducibility was obtained, resulting in better than 2 % RSD for most fatty acid methyl esters. The method can be applied for fully automated fatty acid profiling in biomedical, dietary and metabolomic studies.

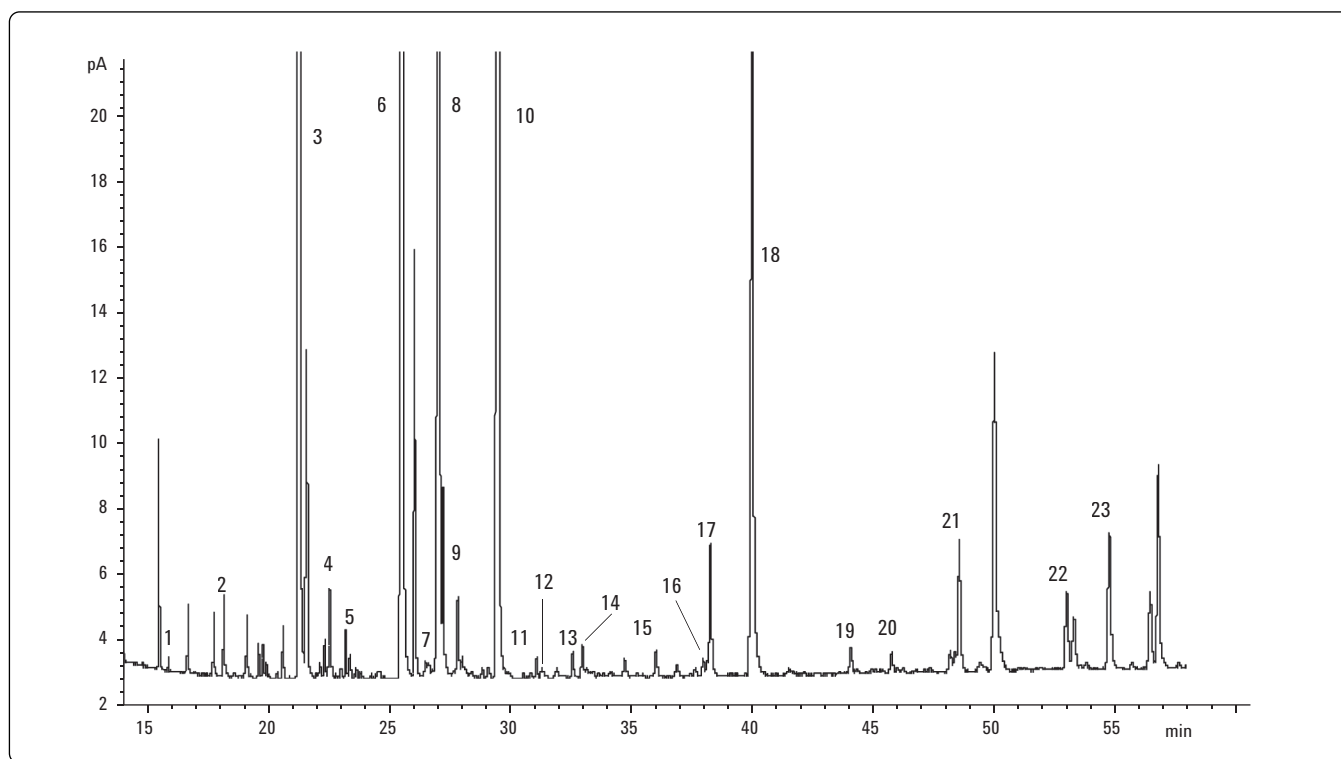


Figure 3. Fatty acid methyl ester profile obtained by the automated sample preparation GC-FID method for a 50 μ L blood sample.

References

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