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Technical Report

Evaluation of Fatty Acids Profiling in a Blood Drop Spotted on DBS Card by using a Robot-assisted GC Method

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Abstract:

The aim of present scientific research is to describe an analytical strategy to investigate the fatty acid content in a blood drop. Detailed knowledge of fatty acid status is a useful tool in disease prevention and health care. A rapid and reliable analytical procedure was developed in fully automatic manner. Sample collection was performed using the dried blood spot (DBS) approach. Fatty acids derivatization was directly performed using a robotic preparative station. Sodium methoxide and boron trifluoride were used as derivatizing agents. Fatty acid methyl esters (FAMEs) analysis was carried out by using a gas chromatograph equipped with an ionic liquid capillary column that allowed the chromatographic separation of the analytes of interest.

Keywords: fatty acid methyl esters, dried blood spot, direct derivatization, human blood

1. Introduction

Accurate fatty acid profiling of blood helps provided detailed information on current metabolic states. Lipidomic compounds are involved in numerous vital biological processes and enable to understand the human nutrition and health condition [Firl, 2013]. Normally, FA analysis by using a gas chromatography technique requires a derivatization step, following lipid extraction, to convert the FAs into more volatile and less polar compounds, often fatty acid methyl esters (FAMEs) [Micalizzi, 2020]. Morrison and Smith proposed a FA trans-esterification method based on the use of boron trifluoride (BF_3) in methanol [Morrison and Smith, 1964]. Metcalfe et al. [Metcalfe, 1966] developed a modified procedure of the Morrison and Smith's method providing firstly a rapid saponification with methanolic sodium hydroxide (NaOH) followed by esterification of FA adding BF_3 in methanol. Some procedures for the determination of FA in which lipids are extracted and transesterified at the same time have been also described. These protocols defined as direct derivatizations give better recovery values, time and solvent requirements are greatly reduced [Christie and Han, 1986]. Focusing on total FA analyses in biological fluids, Galli et al. [Galli, 2009] determined the FA content of circulating lipids in whole blood from fingertip puncture by using HCl in methanol as methylating agent. Recently, Rizzo et al. [Rizzo, 2010] described a direct derivatization procedure for total blood FAMEs assay by using CH_3ONa in methanol 3.33% (w/v) as basic catalyst. Other authors used a dual-stage derivatization procedure in which firstly CH_3ONa in anhydrous methanol (0.5% w/v) was added to lipid sample as basic catalyst for trans-esterification of FA linked by ester bonds to alcohols (e.g. cholesterol, glycerol) and after an acid catalyst, BF_3 in methanol, for esterification of all free fatty acids (FFAs) to methyl esters [Tranchida, 2008; Bondia-Pons, 2004].

The research herein describes an automated analytical strategy for accurate profiling of fatty acid content in a single blood drop from a dried blood spot card. The method has been optimized starting from the sample collection. DBS collection support has been introduced because requires lower sample volume and is more patient friendly compared to venipuncture. In addition, DBS paper cards avoids the often complicated and expensive processes due to the transport of biological fluids [Zakaria, 2016]. Direct derivatization procedure has been applied by using CH_3ONa and BF_3 as derivatizing agents. Developed analytical method was performed in fully automatic manner by using a robotic preparative station.

2. Experiment

2.1 Sample, Chemicals and Material

In this study, a blood sample from an informed donor was used. DBS cards (903 Whatman Protein saver cards) (Merck Life Science, Darmstadt, Germany) were used as sampling support for the collection of a blood drops drawn by lancet from the finger of the donors. All solvents, reagents, and standard materials were purchased from Merck Life Science. CH_3ONa and BF_3, both in methanol solution at a concentration of 0.5 % (w/v) and 14 % (w/v) respectively, were used for converting the lipid molecules in methyl esters form. FAME compounds were extracted from reaction mixture by using n-heptane (for HPLC, ≥ 99%). A C_4-C_{24} even carbon saturated FAMEs (1000 µg mL–1) standard mixture in *n*-hexane was utilized for FAMEs linear retention index (LRI) calculation. Supelco 37 Component FAME Mix, PUFA No.1 (Marine Source) neat, PUFA No.2 (Animal Source) neat, PUFA No.3 (from menhaden oil) neat, Bacterial Acid Methyl Ester (BAME) Mix were used to determine FAME reference LRIs.

To this purpose, a dedicated MS database of the standard FAMEs containing LRI values was constructed, helpful to identified the analytes of interest.

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2.2 Sample Preparation

For FAMEs evaluation, a blood drop was obtained by punching the fingertip of an donor and spotted on 903 Whatman protein saver card. The blood sample was dried for 60 min at room temperature. After, the blood spot was cut out and transferred directly into a 2.0 mL glass vial equipped with a metal cup and a polytetrafluoroethylene/silicone septum. FA fully automated derivatization was carried out by using a preparative station AOC-6000 (Shimadzu). 500 µL of CH_3ONa in methanol (0.5% w/v) was added to the vial as first derivatizing agent. After, the reaction mixture was automatically agitated for 100 seconds at 2000 rpm in the vortex mixer and heated for 900 seconds to the temperature of 95°C in the oven. As second derivatizing agent, 500 µL of BF_3 methanolic solution was added to the vial again agitated and heated at the same conditions previously described. For FAMEs extraction, 350 µL of n-heptane were added into the vial, while 300 µL of a NaCl saturated solution were used to facilitate the separation of the biphasic system. Finally, the mixture was incubated at room temperature for 180 seconds, waiting for the gravitational separation phase. The n-heptanic upper layer was collected and injected in the GC instrument.

2.3 GC-MS/FID Equipped with AOC-6000 (Shimadzu)

The separation and detection of the FAME compounds was carried out by using a Nexis GC-2030 (Shimadzu) instrument equipped with a GCMS-TQ8050 NX triple quadrupole mass spectrometer (Shimadzu) and flame ionization detector (FID). A split-splitless injector (280°C) and an AOC-6000 preparative station were installed on GC instrument. The capillary column was a SLB-IL60 30 m × 0.25 mm id, 0.20 µm df (Merck Life Science) connected to a SilFlow GC-3 port splitter (Trajan). The chromatographic column was connected to position 1 of the 3-port connector, while two uncoated tubings, 1 m × 0.10 mm I.D. and 0.35 m × 0.10 I.D., were connected to the positions 2 and 3. This configuration allowed the eluate to be simultaneously revealed in MS and FID detectors. The temperature program was as follows: 70°C to 180°C (10 min) at 3°C/min and after to 280°C at 3°C/min. Injection volume was 3.0 µL with a split ratio of 1:10. Helium was used as carrier gas at 30 cm s-1 of linear velocity and an initial inlet pressure of 147.1 KPa. Triple quad MS system operated in full scan mode for MS spectra acquisition. The MS parameters were as follows: the mass range was 40–550 amu, the ion source temperature was 220°C, and the interface temperature was 250°C. The GCMSsolution software (version 4.50 Shimadzu) was used for data collection and data processing. The peak assignment was carried out through the use of MS similarity spectra (over 85 %) and ±10 LRI tolerance window. For mass spectral identification, LIPIDS Mass Spectral Library (Shimadzu) was used and a lab-constructed LRI database were used.

The quantification of FAMEs was performed using the GC-FID data. The FID temperature was set at 280°C (sampling rate: 40 ms), while the gas flows were 40 mL min–1 for H2, 30 mL min–1 for the make-up gas (nitrogen) and 400 mL min–1 for air. Data were collected and processed using the LabSolutions software (version 5.92, Shimadzu).

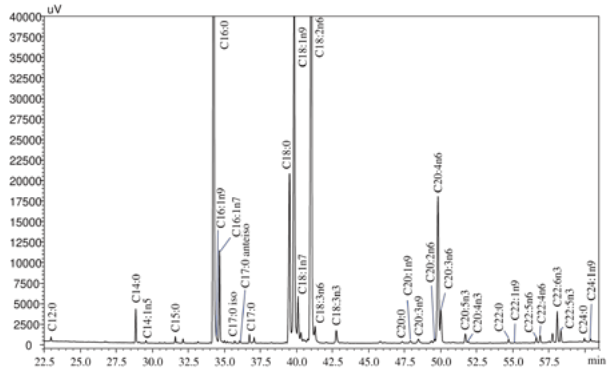

Fig. 1 GC-FID chromatogram of a blood drop spotted on DBS support by using robot-assisted direct derivatization protocol.

3. Results and Discussions

3.1 Robot-assisted GC Analysis

FAMEs analysis are usually carried out using GC capillary columns coated with polar stationary phases such as polyethylene glycol or cyanopropyl-based phases. In this application, it has been developed a chromatographic method by using a medium polarity ionic liquid (IL) capillary column. Compared to the polar stationary phases, the IL columns exhibit lower bleeding with the advantage of not compromising the quantification of the analytes with low concentration levels [Fanali, 2017]. Developed chromatographic method allowed the separation of 31 FAMEs as showed in Fig. 1. A linear temperature program was applied, except in C18 region, where a short 10-minutes isotherm allowed an improved resolution of FAME compounds.

The robot-assisted sample preparation allowed the conversion of lipid molecules in FAME compounds by the direct derivatization method. Derivatization protocol resulted absolutely compatible with the AOC-6000 providing consistent precision and eliminating errors associated with mundane preparation procedures such as solvent or derivatizing agents additions. Automated derivatization strategy was very simple and compatible with large-scale clinic studies requiring thousands of lipid biological samples.

In order to improve the reliability of peak assignment, a double check control was conducted. Both mass spectral similarity and LRI correspondence were evaluated. This identification procedure is applied especially when the molecular fragmentations are almost identical, such as in the case of FAMEs, especially in double-bond positional isomers. The combined use of MS similarity and LRI tolerance on a polar IL capillary column allowed a univocal identification of FAs (Table 1).

3.2 FAMEs Profiling

The developed analytical strategy was applied for evaluating the FA content of a blood drop. FA content, expressed in percentage terms, is summarized in Table 1. Percentage values in relation to saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and omega-3 (ω-3) and omega-6 (ω-6) classes, as well as specific ratios, are also reported in Table 2. FAMEs profile of the analyzed blood sample showed a high content of SFA family (37.1 %). Palmitic acid (C16:0), final product of the enzymatic system responsible for saturated fatty acid synthesis, was the most abundant SFA. Another important SFA is

stearic acid (C18:0) obtained from palmitic acid by the activity of the elongase enzyme. The MUFA class (27.45) is represented particularly by oleic acid (C18:1n9). Its abundance in blood is due to the fact that it has a biosynthetic origin, starting from C18:0 by the desaturase enzyme activity in position 9 of the carbon chain, and dietary origin such as olive oil in the form of triglyceride [Ferreri and Chatgilialoglu, 2015]. Palmitoleic acid (C16:1n7) is another relevant MUFA, which is obtained by enzymatic desaturation of C16:0. PUFA class is divided in ω-6 and ω-3 families. The most abundant PUFA in blood samples is linoleic fatty acid (C18:2n6), the precursor of ω-6 fatty acids that derives primary from the diet. Instead α-linolenic acid (C18:3n3) is the precursor of ω-3 family. The presence of erythrocyte membranes, mainly composed of arachidonic acid (C20:4n6) and docosahexanoic acid (C22:6n3) bonded in phospholipid structures, reflects the levels of C20:4n6 and C22:6n3 registered in blood [Micalizzi, 2020].

4. Conclusion

The rapid and robot-assisted developed method has proved its suitability for the qualitative and quantitative profiling of FAs in blood. The analytical strategy involves the easy collection of blood drops by using the DBS support and represents an ideal condition for large population studies. Future directions include the development of quantitative fatty acid databases for disease and non-disease samples. Such databases may be beneficial in the development of future therapies that improve fatty acid balance.

Table 1. Identity of the FAMEs contained in the blood sample spotted on DBS support. Abbreviations: LRI exp.: experimental LRI; LRI ref.: reference LRI; MS Sim%: database spectral similarity. The FAMEs contents are expressed in % values.

FAME	MS Sim (%)	LRI exp	LRI ref	Area (%)
Lauric acid ($C_{12:0}$)	93	1198	1200	0.06
Myristic acid ($C_{14:0}$)	97	1398	1400	0.72
Myristoleic acid ($C_{14:1n5}$)	91	1425	1425	0.02
Pentadecanoic acid ($C_{15:0}$)	96	1500	1500	0.24
Palmitic acid ($C_{16:0}$)	97	1599	1600	23.16
Palmitoleinic acid ($C_{16:1n9}$)	95	1603	1605	0.39
Palmitoleic acid ($C_{16:1n7}$)	95	1613	1615	1.32
iso-Heptadecanoic acid (*iso*-$C_{17:0}$)	88	1651	1652	0.14
anteiso-Heptadecanoic acid (*anteiso*-$C_{17:0}$)	92	1664	1666	0.1
Margaric acid ($C_{17:0}$)	96	1695	1700	0.35
Stearic acid ($C_{18:0}$)	98	1798	1800	11.79
Oleic acid ($C_{18:1n9}$)	96	1807	1809	23.56
cis-Vaccenic acid ($C_{18:1n7}$)	99	1814	1820	1.73
Linoleic acid ($C_{18:2n6}$)	99	1836	1845	18.22
γ -Linolenic acid ($C_{18:3n6}$)	96	1847	1856	0.36
α -Linolenic acid ($C_{18:3n3}$)	93	1886	1898	0.12
Arachidic acid ($C_{22:0}$)	97	1996	2000	0.12
Gondoic acid ($C_{20:1n9}$)	94	2010	2011	0.28
Eicosatrienoic acid ($C_{20:3n9}$)	93	2027	2031	0.25
homo- γ -Linolenic acid ($C_{20:2n6}$)	97	2055	2053	0.17
Arachidonic acid ($C_{20:4n6}$)	97	2063	2063	10.42
dihomo- γ -Linolenic acid ($C_{20:3n6}$)	98	2067	2066	1.5
Eicosapentaenoic acid ($C_{20:5n3}$)	98	2116	2119	0.33
Eicosatetraenoic acid ($C_{20:4n3}$)	89	2119	2123	0.01
Behenic acid ($C_{22:0}$)	93	2196	2200	0.19
Erucic acid ($C_{22:1n9}$)	89	2212	2216	0.03
Docosapentaenoic acid ($C_{22:5n6}$)	93	2271	2275	0.26
Adrenic acid ($C_{22:4n6}$)	98	2280	2285	1.35
Docosahexaenoic acid ($C_{22:6n3}$)	95	2328	2333	1.75
Docosapentaenoic acid ($C_{22:5n3}$)	93	2335	2341	0.67
Lignoceric acid ($C_{24:0}$)	98	2395	2400	0.19
Nervonic acid ($C_{24:1n9}$)	89	2412	2416	0.18

Table 2. FAME classes and as well as in specific ratios calculated in blood sample.

	Rate of Classification (%)
SFA	37.1
MUFA	27.5
PUFA	35.4
Omega-6	32.3
Omega-3	2.1
Omega-6/Omega-3	14.5
$C_{18:0}/C_{18:1n9}$ (Δ9 desaturase index)	0.5
$C_{16:0}/C_{16:1n7}$ (Δ9 desaturase index)	17.6
$C_{18:2n6}/C_{20:3n6}$ (Δ6 desaturase + elongase index)	12.2
$C_{20:4n6}/C_{20:3n6}$	7
$C_{20:4n6}/C_{20:5n3}$	31.7

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