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Evaluation of lipid extraction and fatty acid composition of human plasma

Avaliação da extração lipídica e composição em ácidos graxos do plasma humano

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Aim: The objective of this study was to compare the efficiency of lipid extraction and to evaluate the fatty-acid composition in total lipids from human plasma, using a new technique and the established Folch, Lees and Stanley (FLS), Bligh and Dyer (BD), Rose-Gottlieb (RG), and Gerber (GM) methods. Method: A new technique, the alternative method, to extract total lipids using a microwave was proposed and evaluated. Results: The total lipids extracted from human plasma varied between 0.19% and 0.41%; the highest total lipid extracted were obtained by the Folch, Lees and Stanley (0.41%), alternative method (0.37%) and Rose-Gottlieb (0.36%) methods. The Gerber method was ineffective to extract total lipids from human plasma. A total of 24 fatty-acid species were quantified by gas chromatography. Of the methods studied, the highest concentrations were found using the Folch, Lees and Stanley method ($p \leq 0.05$). Conclusion: The alternative method is a fast lipid-extraction technique that can be used for the identification of human plasma fatty acids, but it is not suitable for their measurement.

Keywords: *Fatty acids/analysis; Lipids/analysis; Lipids/chemistry; Plasma; Comparative study*

Introdução

Blood is a highly specialized tissue composed of many different components. Four of the most important ones are red cells, white cells, platelets, and plasma. This essential fluid carries out the critical functions of oxygen and nutrient transportation to our cells and removes carbon dioxide, ammonia and other waste products.

Plasma is the relatively clear, yellow tinted solution made up of water (92%), sugar, fat, protein and salt that carries the red cells, white cells, platelets, and some other chemicals, such as lipid proteins, which are lipid carriers.[1]

Triacylglycerol (TG) is one of the most important classes of lipids in human plasma. It is composed of fatty acids (FA) that, as a constituent of the cell membrane [2] and a precursor of metabolites that control physiological and pathologic processes, play important roles in the human organism.[3] However, lipids can also be associated with the development of cardiovascular disease and cancer. [4-6] As a result, the study of the lipid constituents of human plasma is important

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to evaluate circulating fatty acids. Concentrations reflect ingestion in the diet and production by the organism. They also enable some understanding of the relationship between an individual's diet and the development of diseases.

This has motivated studies about techniques for the analysis of plasma lipids. Some authors analyzed non-esterified fatty acids and compared two methods to determine fatty acid concentrations; one using solvent extraction followed by esterification and the other by direct esterification.[7,8] However, none of these studies compared the different methods of lipid extraction. This is the most critical step in the analysis of lipid content; it can present problems such as low recovery and fatty acid oxidation, which affect the results and consequently, the analysis of fatty acid composition, especially of polyunsaturated fatty acids (PUFA), such as the omega-3 (n-3) and omega-6 (n-6) series.[9]

The number of studies that employ microwaves in chemistry has increased due to the need of faster, more efficient, and cleaner processes.

Hence, this study investigated the efficiency of the Folch, Lees and Stanley, also known as the Folch method (FLS),[10] Bligh and Dyer (BD),[11] Rose-Gottlieb (RG),[12] and the Gerber (GM)[13] extract methods to evaluate the fatty acid composition in human plasma. Additionally, an alternative method of extraction of lipids using microwaves applied to fatty acids in human plasma is described and evaluated.

Methods

This project was performed according to the guidelines of the Permanent Committee for Ethics in Research with Human Beings of the Universidade Estadual de Maringá (UEM), Protocol No. 256/2007.

Sampling

Sampling was carried out at the Hemocentro Regional de Maringá – UEM, in Maringá city, Paraná State. Blood samples (300 mL) were collected from volunteers in three satellite bags (450 mL) containing CPDA-l anticoagulant (sodium citrate, monohydrated citric acid, monophase monohydrated sodium phosphate, adenine, monohydrate dextrose and water) from April to December 2007. The bags were homogenized by inversion during collection. For the complete separation of the liquid part of the blood from the cells, the samples were centrifuged at 3,000 rpm for 15 min.

The homogenized plasma was pooled (450 mL) and stored in a 20 mL amber colored vial under N_2 atmosphere and frozen at -18°C until analysis.

Solvents, reagents and standards

Analytical grade solvents and reagents purchased from Merck® were used. Standard solutions were prepared of 10.03 mg/mL tripalmitin in chloroform, 1.0 mg/mL methyl tricosanoate in isooctane and a mixture of 37 fatty acid methyl ester standards (FAME) (Standard 189) in isooctane, all purchased from Sigma®.

Glassware and equipment

Manufacturer (Pyrex®) certified lot glassware was used. The certified equipment and instruments required for the study were: an analytical balance (Bel Engineering A42456GC), oven (Fanem® 315SE), microwave oven (Brastemp BMK27ABHNA – Jet Defrost Crisp), gas chromatograph (Varian® CP-3380) fit with split/splitless flame ionization detection and a fused silica column (CP-7420 - 100% bonded cyanopropyl, 100 m, 0.25mm i.d. and 0.25 μm stationary phase). The Workstation 5.0 software (Varian®) was used for peak area integration.

Analysis Methods

Moisture determination

Moisture was determined according to Association of Official Analytical Chemists (AOAC).[12] The samples were homogenized and analyzed in quadruplicate and the results were expressed in plasma mass percentage (%).

Total lipid extraction methods

The following methods were used for lipid extraction: FLS [10] in chloroform, methanol and water (2:1:1); BD [11] in chloroform, methanol and water (1:2:0.8); RG [12] in 25% NH_3 solution; GM [13] in sulfuric acid and an alternative method.

In the Alternative method, 2.0 g of plasma was weighed in a 50-mL screw-cap extraction tube, to which 2.5 mL distilled water was added. The mixture was agitated in a tube agitator for 2 min and heated for 10 s in the microwave oven at maximum power (1.7 kW). The mixture was added to 3.75 mL ethyl alcohol and agitated for another 2 min. Next, 6.5 mL of petroleum ether was added and the tube was vigorously agitated for 1 min. After phase separation, the top phase was transferred to a 50 mL flat bottom flask with known weight and the solvent was evaporated in a rotavapor, after which the total lipid content was determined by gravimetry.

The samples were homogenized and analyzed in quadruplicate for each method.

Validation of the alternative method

Precision

Intra-assay precision was determined by the coefficient of variation [14] of four extractions of total lipids (TL) from human plasma.

Spike and recovery test

Accuracy was estimated through spike and recovery tests.[14] Known amounts of tripalmitin standard of approximately 50% more than the mass of total lipids in human plasma samples were added. Additions were performed in quadruplicate. Recovery was carried out by the Alternative and FLS methods.

Analysis of fatty acid methyl esters

The Hartman and Lago method[15,16] was used to prepare FAME by fatty acid esterification and transesterification of triacylglicerol. Methyl esters were separated in a gas chromatograph (Varian CP-3380) equipped with a fused silica capillary column CP-7420 (100 m x 0.25 mm i.d. x 0.25 µm film) and a flame ionization detector. Injector and detector temperatures were 220ºC and 240ºC, respectively. The gas flow rates used were 1.4 mL/min carrier gas (H_2), 30 mL/min make-up gas (N_2), and 30 mL/min H_2 and 300 mL/min as flame gases (H_2 and synthetic air, respectively). The sample splitting rate was 1/80.

The operating parameters used were: column temperature 165°C for 12 min, increased to 180°C at 40°C/min, held for 15 min and then increased to 240°C at 15°C/min and held at this temperature until the finish. FAME were identified by comparing the retention times of the sample constituents with those of Sigma standards. The samples were analyzed in two replicates.

Quantitative analysis of fatty acids

FA extracted from human plasma was quantified in relation to the internal standard methyl tricosanoate (23:0).[17]

Statistical analysis

Mean values were statistically compared using the Tukey test at 5% standard error with one-way ANOVA. Data were processed by the Statistica 7.0 software (StatSoft, Tucksa, 2005).

Results and Discussion

The composition of human plasma obtained was: 92.23 ± 0.08% moisture with a total lipid range of 0.19 - 0.41%. The FLS (0.41 ± 0.04%), Alternative (0.37 ± 0.02%) and RG (0.36 ± 0.01%) methods gave the best results without any significant difference between them ($p > 0.05$) but a significant difference ($p \leq 0.05$) was found for BD (0.19 ± 0.02%).

GM did not extract TL very effectively possibly due to the low amounts of lipids in plasma and the high concentration of sulfuric acid used which may have degraded organic compounds. Therefore, it was not possible to analyze fatty acids by this method.

The coefficient of variation of replicate analysis of total lipids by the Alternative method was 5.41. This value is below the accepted threshold for analytes with percent concentrations in the order of 10^{-3}, such as in the case of TL in human plasma, which shows that the Alternative method is precise.[18]

To evaluate the accuracy of this Alternative method, approximately 50% more mass of TG (standard tripalmitin) was added than the lipid content of the sample as has been recommended by other authors.[14]

Table 1. Recovery of tripalmitin (TG 16:0) from human plasma using the Folch et al. (1957) and Alternative methods

Methods	Added Amount (mg)	Recovered amount* (mg)	Percent recovery (%)
Alternative method	4.01	3.90	97.22
Folch et al. (1957)	4.01	4.10	102.21

*Results given as means of four replicate extractions of total lipids.
TG = triacylglycerol

This validation parameter was applied to the FLS and Alternative methods to compare the results, as FLS is the most used method to extract TL. The results are given in table 1.

The percent recovery values for the Alternative and FLS methods ranged from 80.0 to 110.0%, which is an acceptable range in method validation studies.[19] This result suggests that the methods were appropriately accurate, thus demonstrating their efficiency. Therefore, the Alternative method was shown to be adequate for the analysis of TL in human plasma.

This study investigated the most important of the 24 fatty acids (FA) in human plasma which are 18:2n-6, 16:0, 18:1n-9, 18:0, and 20:4n-6. Their concentrations (mg/g total lipids) are given in table 2.

The saturated fatty acids with the highest concentrations were the palmitic (16:0) and stearic (18:0) acids. Palmitic acid predominated with concentrations ranging from 80.19 to 135.45 mg/g TL. The highest concentration was identified by the FLS method and the lowest by the RG method. Nevertheless, the BD and Alternative methods gave very close values without any significant differences ($p > 0.05$).

The concentration of stearic acid ranged from 28.19 to 48.20 mg/g TL, with a significant different ($p \leq 0.05$) between the methods. The FLS method was the most efficient, followed by the Alternative, BD and RG methods.

For the monounsaturated fatty acids (MUFA), the FLS method detected the highest ($p \leq 0.05$) and the RG method detected the lowest concentrations of oleic acid (18:1n-9). The results of the BD and Alternative methods were not significantly different ($p > 0.05$) with values ranging from 62.7 to 94.21 mg/g TL. Oleic acid reduces the oxidation of LDL-cholesterol, which is the most important to trigger the formation of atherosclerosis.[20]

Linoleic acid was the PUFA with the highest concentration in human plasma ranging from 150.94 to 223.87 mg/g TL. The FLS method gave the highest value, which was significantly different ($p \leq 0.05$) from the other methods. The Alternative method gave the second highest result, followed by the BD and RG methods. Other important PUFA in the Omega-6 series were arachidonic acid and dihomo-gamma-linolenic acid. The high concentrations of linoleic and arachidonic acids are a result of a diet rich in these FA.

Other PUFA found included omega-3 series acids; the highest concentration of alpha-linolenic acid, the precursor

Table 2. Concentrations of FA (mg/g TL) in human plasma using the Folch et al., Bligh and Dyer, Rose-Gottlieb and Alternative methods

Component	Folch et al. (1957)	Bligh and Dyer (1959)	Rose-Gottlieb (1998)	Alternative method
Fatty acid	Mean±SD (mg/g)	Mean-±SD (mg/g)	Mean±SD (mg/g)	Mean±SD (mg/g)
12:0	1.48±0.20 ^{a}	1.22±0.02 ^{ab}	1.13±0.03^{b}	1.08±0.05^{b}
14:0	6.72±0.32^{a}	5.12±0.03^{b}	4.34±0.34^{c}	5.17±0.27^{b}
15:0	1.52±0.08^{a}	1.19±0.01^{b}	0.95±0.01^{c}	1.21±0.06^{b}
16:0	135.45±3.25^{a}	100.00±0.21^{b}	80.19±6.76^{c}	101.62±2.07^{b}
16:1n-9	1.65±0.20^{a}	1.40±0.07^{ab}	1.28±0.09^{b}	1.29±0.02^{b}
16:1n-7	7.05±0.48^{a}	4.18±0.50^{c}	4.84±0.39^{c}	5.83±0.09^{b}
17:0	1.70±0.05^{a}	1.26±0.02^{b}	1.07±0.05^{c}	1.15±0.05^{bc}
18:0	48.20±0.52^{a}	33.81±0.26^{c}	28.19±1.60^{d}	37.19±0.87^{b}
18:1n-9t	5.84±0.17^{a}	2.96±0.27^{c}	3.58±0.37^{c}	4.88 ±0.20^{b}
18:1n-9c	94.21±1.86^{a}	73.27±1.98^{b}	62.70±4.49^{c}	78.72±1.94^{b}
18:1n-7	7.46±0.24^{a}	5.02±0.96^{b}	4.86±0.30^{b}	7.43±0.22^{a}
18:2n-6t,t	0.98±0.02^{a}	0.46±0.04^{b}	0.36±0.03^{c}	0.38±0.01^{c}
18:2n-6c	223.87±4.43^{a}	173.71±3.65^{c}	150.94±10.02^{d}	196.29±2.66^{b}
18:3n-6	1.81±0.12^{a}	1.91±0.01^{a}	1.46±0.09^{b}	1.35±0.08^{b}
18:3n-3	8.52±0.36^{a}	5.77±0.30^{b}	5.65±0.21^{b}	6.04±0.12^{b}
20:0	1.41±0.05^{a}	1.29±0.06^{b}	0.99±0.05^{c}	1.19±0.04^{b}
20:1n-9	0.84±0.06^{a}	0.62±0.02^{c}	0.55±0.04^{c}	0.72±0.02^{b}
20:2n-6	0.78± 0.02^{b}	0.69±0.02 ^{bc}	0.66±0.02 ^{c}	1.09±0.05^{a}
20:3n-6	5.11±0.21^{a}	3.84±0.03^{c}	3.23±0.14^{d}	4.37±0.24^{b}
20:4n-6	43.31±1.22^{a}	30.05±2.39^{c}	27.21±1.45^{c}	37.50±1.36^{b}
20:5n-3	16.48±0.80^{a}	12.69±0.15^{b}	10.72±0.52^{c}	13.61±0.36^{b}
24:1n-9	1.05±0.21^{a}	0.72±0.06^{b}	0.71±0.02^{b}	0.70±0.03^{b}
22:5n-3	3.37±0.21^{a}	2.59±0.13^{b}	2.10±0.08^{c}	2.74±0.04^{b}
22:6n-3	9.88±0.39^{a}	7.94±0.23^{c}	6.22±0.26^{d}	8.66±0.25^{b}
Total	Mean±SD (mg/g)	Mean±SD (mg/g)	Mean±SD (mg/g)	Mean±SD (mg/g)
SFA	196.48±4.28^{a}	143.89±0.57^{b}	116.86±8.61^{c}	148.62±2.75^{b}
MUFA	118.11±2.62^{a}	88.16±0.79^{c}	78.52±5.63^{d}	99.58±1.89^{b}
PUFA	314.12±6.95^{a}	239.66±6.59^{c}	208.55±12.27^{d}	272.02±3.95^{b}
n-6	273.13±6.35^{a}	209.35±7.93^{c}	182.18±10.81^{d}	238.30±4.09^{b}
n-3	38.26±1.31^{a}	29.00±0.51^{b}	24.69±0.98^{c}	31.05±0.47^{b}

Means and respective standard deviations (SD) of four extractions of TL, which resulted in two injections each. Means followed by different letters in the same line are significantly different by Tukey test at 5% probability

Abbreviations: TL = Total lipids; PUFA = Polyunsaturated fatty acids; MUFA = Monounsaturated fatty acids; SFA = Saturated fatty acids; n-6 = Omega-6 series FA; n-3 = Omega-3 series FA

of the series, was identified by the FLS method and was significantly higher ($p \leq 0.05$) than the results of the BD, RG, and Alternative methods, which were statistically similar ($p>0.05$). The FLS method identified the highest concentrations of 20:5n-3, 22:5n-3; the results of the BD and Alternative methods were statistically similar while the values identified by the RG method were the smallest. For 22:6n-3, all methods were significantly different ($p \leq 0.05$) with the most efficient being the FLS and Alternative methods. These omega-3 series PUFA play an important role in the treatment of cardiovascular diseases.[21]

The total of saturated fatty acids (Table 2) ranged from 116.86 to 196.48 mg/g TL depending on the method, with the highest being by the FLS method and the lowest by the RG method. The BD and Alternative methods gave statistically similar intermediate concentration values ($p > 0.05$).

The total MUFA and PUFA concentrations ranged from 78.52 to 118.11 mg/g TL, and from 208.55 to 314.12 mg/g TL, respectively (Table 2). These two totals were significantly different ($p \leq 0.05$) for all methods; the FLS method gave the highest value, followed by the Alternative, BD and RG methods.

Conclusion

The analysis of the results obtained shows that the Gerber method is not indicated for the extraction of lipids from human plasma, while the Rose-Gottlieb method did not

perform well for fatty acids, despite the good total lipid analysis results.

The Bligh and Dyer method was not very efficient in the extraction of total lipids, but was efficient for fatty acids in general. The Alternative method was efficient in the extraction of lipids, although it affects the composition of fatty acids quantitatively; it can be used with human plasma.

The Folch method stood out as the most efficient in the extraction of total lipids and fatty acids.

Resumo

Objetivo: O objetivo desse estudo foi comparar a eficiência da extração lipídica e avaliar a composição em ácidos graxos nos lipídios totais (LT) no plasma humano usando uma nova técnica e os métodos já conhecidos: Folch, Lees e Stanley (FLS), Bligh e Dyer (BD), Rose-Gottlieb (RG) e Gerber (MG). Método: A nova técnica para extração de lipídios totais utiza o micro-ondas, método Alternativo (MA). Resultados: Os lipídios totais (LT) extraídos do plasma variaram de 0,19% a 0,41%, os maiores teores foram obtidos por FLS (0,41%), MA (0,37%) e RG (0,36%). O MG foi ineficaz na extração dos lipídios totais do plasma. Um total de 24 espécies de ácidos graxos foi quantificado no plasma por cromatografia em fase gasosa. Entre os métodos estudados, as maiores concentrações de ácidos graxos foram obtidas usando-se o método de FLS (p≤ 0,05). Conclusão: O método Alternativo foi considerado uma técnica rápida de extração lipídica, a qual poderá ser utilizada somente na identificação de ácidos graxos em plasma humano, mas não adequado para a quantificação dos mesmos.

Descritores*: Ácidos graxos/analise; Lipídeos/análise; Lipídeos/química; Plasma; Estudo comparativo*

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