Revista do Instituto Adolfo Lutz
https://doi.org/10.53393/rial.2022.v.81.37903
ISSN: 1983-3814 (on-line)
Original Article/Artigo Original

Determination of lipids and fatty acids in infant formula: comparison of the conventional x direct method

Lipídios e ácidos graxos em fórmula infantil: comparação de metodologias de extração convencional x direta

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Received/Recebido: 06.08.2021 - Accepted/Aceito: 18.02.2022

ABSTRACT

Lipids in food are conventionally analyzed in two stages: extraction with organic solvent and fat esterification reaction, in this case, the type of fat of each food influences the choice of extraction and esterification reagents. In the direct method, such procedures are performed in one step. This work compared the conventional extraction method and quantification of lipids and fatty acids, with a direct method in infant formula. A reference sample of infant formula conteining certified lipids and fatty acids values from the National Institute of Standards and Technology was used. The conventional method for lipid analysis used the acid hydrolysis method; for the determination of fatty acids, the fats were extracted with a mixture of ethyl ether and petroleum ether. The direct method consisted of direct trans esterification with sodium methoxide. In the analysis of fatty acids, the majority of the results showed statistically equal values ($\alpha < 0.05$) for both methods. The direct method proved suitable, mainly because of reduction in analytical time and quantity of solvents.

Keywords. Direct Transesterification, Fat, Fatty Acid Methyl Ester, Gas Chromatography.

RESUMO

Os lipídios em alimentos são analisados, de forma convencional, em duas etapas: extração com solvente orgânico e reação de esterificação da gordura, neste caso o tipo de gordura de cada alimento influencia na escolha dos reagentes da extração e esterificação. No método direto, estes procedimentos são realizados em uma etapa única. Este trabalho comparou a metodologia convencional de extração e quantificação de lipídios e ácidos graxos, com um método direto em fórmula infantil. Foi utilizada uma amostra de referência de fórmula infantil com valores certificados para lipídios e ácidos graxos da *Nacional Institute of Standards and Technology*. A metodologia convencional para a análise de lipídios empregou método com hidrólise ácida; para a determinação dos ácidos graxos, a gordura foi extraída com uma mistura de éter etílico e éter de petróleo. O método direto fundamentou-se na transesterificação direta com metóxido de sódio. Na análise dos ácidos graxos, a maioria dos resultados demonstrou valores estatisticamente iguais ($\alpha < 0,05$) para os dois métodos. O método direto demonstrou ser apropriado, principalmente pela diminuição do tempo de análise e quantidade de solventes.

Palavras-chave. Transesterificação Direta, Gordura, Ésteres Metílicos de Ácidos Graxos, Cromatografia Gasosa.

INTRODUCTION

Since the 1950s, the idea of feeding children using infant formulas since birth has been emerged. In recent years, there has been a noticeable improvement in infant formulas, and currently, there are several options that are increasingly being supplemented to become similar to the composition of human breast milk[1].

During the commercial preparation of infant formulas, a part of the milk fat is replaced by a mixture of vegetable oils, among them: corn oil, canola oil, coconut oil, sunflower oil, palm oil and palm kernel oil. The addition of these oils provides a change in the composition of fatty acids from milk, with the addition of essential fatty acids (FAs), mainly linoleic acid and α-linolenic, thus making the composition closer to that of human milk[1,2]. The constituents of infant formula raw materials are blended, pasteurized, homogenized, concentrated, and dried or sterilized by manufacturers to ensure that these products have good quality and long shelf life[3].

Lipid extraction from foodstuffs is traditionally carried out with organic solvents (ethyl ether, petroleum ether, chloroform or methanol) and gravimetric determination. Specific factors may influence the extraction, such as the polarity of the extraction solvent and pretreatment of the sample (e.g., hydrolysis, washes etc.). The choice of the most appropriate solvent for lipid extraction in food matrices is one of the most critical steps in this determination, due to the diversity of dietary matrices and their lipid composition. The extraction process can take time, requiring multiple steps and different solvents or a combination of these for adequate and complete solubilization of lipid components in food matrices[4].

Food matrices can be treated before extraction, since lipids can be linked to other food compounds, such as proteins and carbohydrates, and thus only the action of the solvent is not sufficient for the release of lipid components. In the case of dairy products, including infant formulas, an alkaline pretreatment is used, usually with ammonium hydroxide, which breaks down the lipid emulsion, neutralizes some acid, and solubilizes the protein for later extraction with ether. The Roese-Gottlieb (AOAC 905.02) and Monjonnier (AOAC 989.05) methods use a blend of ethyl ether and petroleum to extract fat from residues treated with ammonia hydroxide in ethanol[5]. In this case, mono-, di- and triacylglycerols and traces amounts of other lipids are effectively extracted from dairy products, including milk, cheese, and milk-based infant formula. These lipid extraction methods, with a prior alkaline hydrolysis, are recommended for the determination of FAs[5,6].

Analysis of FAs by gas chromatography involves the preliminary conversion of triacylglycerols into more volatile derivatives and typically fatty acid methyl esters (FAME) are prepared. Several procedures are described and each with its advantages and limitations, according to characteristics of the lipid matrices to be analysed[4,7].

The conventional procedure to obtain FAME is esterification reactions, usually catalysed by acids or bases and they involve two processes: hydrolysis and esterification or transesterification[8].

Direct transesterification ("in situ") method without prior extraction of fats are currently being used. They consist of mixing the sample with esterification reagents such as methanol hydrogen chloride; methanol acetyl chloride; methanol sodium methoxide; methanol sodium hydroxide (NaOH), hydrochloric acid and methanol solution; sulfuric methanol acid and boron trifluoride solution (BF_3). There is an obvious saving of analytical time and solvents as the lipid extraction and derivatization steps of the later occur the same time. However, the water content of

the food, the sampling, the choice of reagents (catalyst), and the conditions of the analysis are key factors in the effectiveness of direct reactions[9-12].

Several studies are carried out to compare the traditional method, namely the extraction of lipid and the subsequent derivation of FA in FAME with the direct method (DM) or "in situ". O'Fallon et al[13] obtained similar results between traditional and "in situ" methods for samples of fish oil, meat product, and conjugated linoleic acid (CLA) capsules. Cruz-Hernandez et al[14] observed that the direct method can be used for the fatty acids quantification in human milk. Other authors also demonstrated similarity in terms of the results of the methods: Abdulkadir and Tsuchiya[15] for samples of marine animals; Wang et al[16] when analyzing egg yolks; Golay et al[10] for dairy products; and Castro-Gómez et al[11] for milk powder and conjugated linoleic acid supplementation. However, Mazalli and Bragagnolo[17] verified that the direct method was not adequate to determine polyunsaturated fatty acids (PUFA) in powdered eggs. The direct method is also currently used to produce biodiesel from algae and vegetable oils in order to save[18,19]. In 2007, the American Oil Chemists' Society (AOCS)[20] published the Ce 2b-11 method of direct methylation of lipids using alkaline hydrolysis with methanolic NaOH, organic solvent extraction (n-hexane), and methylation with BF_3. Total fat is also determined directly by calculation, based on fatty acids obtained by gas chromatography with flame ionization detection (GC/FID) in accordance with the AOCS Ce 1h-05 method. This method, however, is not applicable for milk fats and marine oils or oils with long-chain polyunsaturated fatty acids (LC-PUFAs) or micro-encapsulated oils, in these cases AOCS recommends the use of the Ce 2c-11 method[20].

The comparison of analytical methods is due two separate reasons. The first is when you wish to check a new method with an existing one, to evaluate whether the results of the two methods are indistinguishable or whether they differ in a major way. The second reason is to calibrate one method to another so that the measurements made by one method can be converted to those others[21].

ABNT ISO/IEC 17025[22] norm defines reference material (RM) as a sufficiently homogeneous and stable material concerning one or more specified properties, which has been established as being suitable for its intended use in a measurement process, and certified reference material (CRM) as a reference material (RM) characterized by a metrologically valid procedure for one or more specified properties, added by a certificate providing the specified property value, its associated uncertainty and a metrological traceability declaration. The CRM has multiple utilities within a laboratory, and among the various possibilities, the ones that most stand out are the calibration and metrological control of equipment, the verification of the accuracy and precision of analytical methods, through the validation of methods, and the training of analysts.

One of the techniques that can be used for the validation of a methodology is the comparison with results obtained by other validated methods, and in this sense, the CRM has outstanding relevance because it is a tool that guarantees the comparability between the method already standardized and the method in the study. The guarantee of comparability is of important because it is through it that the reliability of measurements is attested, possible commercial technical barriers are eliminated and, consequently, a guarantee of fair exchange relationships, besides allowing substantial increases in terms of quality, innovation, and competitiveness[22,23].

The objective of this study was to compare the conventional method for analysis of lipids and fatty acids in infant formula with the direct method in a sample of certified reference material.

MATERIAL AND METHODS

Material

A National Institute of Standards and Technology (NIST) 1849/2006 infant formula reference sample was used, with both certified and reference values for lipids and FAs.

Reagents, solvents and standards

The solvents and reagents of analytical grade used were: petroleum ether, ethyl ether, 95% ethanol, hydrochloric acid, ammonium hydroxide, potassium hydroxide, sodium sulfate, NaOH, sodium methoxide, ammonium chloride, and sodium citrate; and chromatographic grade solvent: n-hexane

The following chromatographic standards were used: C11:0 and C13:0 in triacylglyceride (TAG) form, with an approximate purity of 98% and 99%, respectively; C11:0, C13:0, C21:0, and C23:0, in the form of PUFAs, with an approximate purity of 99%; a mixture with certified quantities of 37 PUFAs (Supelco); a FAME mixture of the *cis-trans* isomers of linoleic acid (C18:2) (Sigma) and a FAME mixture of the *cis-trans* isomers of α-linolenic acid (C18:3) (Sigma), and individual methyl ester standards (Sigma), being: eladic (C18:1 9t); vacenic (C18:1 11c); *trans* vacenic C18:1 7c; C18:1 12c; CLA (C18:2 9c,11t and C18:2 10t,12c); palmitoelaid (C16:1 9t), palmitic (C16:0); linolenic (C18:2 9t,12t); eicosapentaenoic (C20:5 5c,8c,11c,14c,17c); araquidonic (C20:4 5c,8c,11c,14c); and docosahexaenoic (C20:6 4c,7c,10c,13c,16c,19c).

Methods

Figure 1 shows the flow chart for the methodologies used in the study, the conventional (CM) and direct methods. In the CM, two methods of fat extraction were used, the AOAC 963.155 method for the gravimetric determination of total fat, and the Roese-Gottlieb method AOAC[5] for fat extraction and subsequent analysis of FAs.

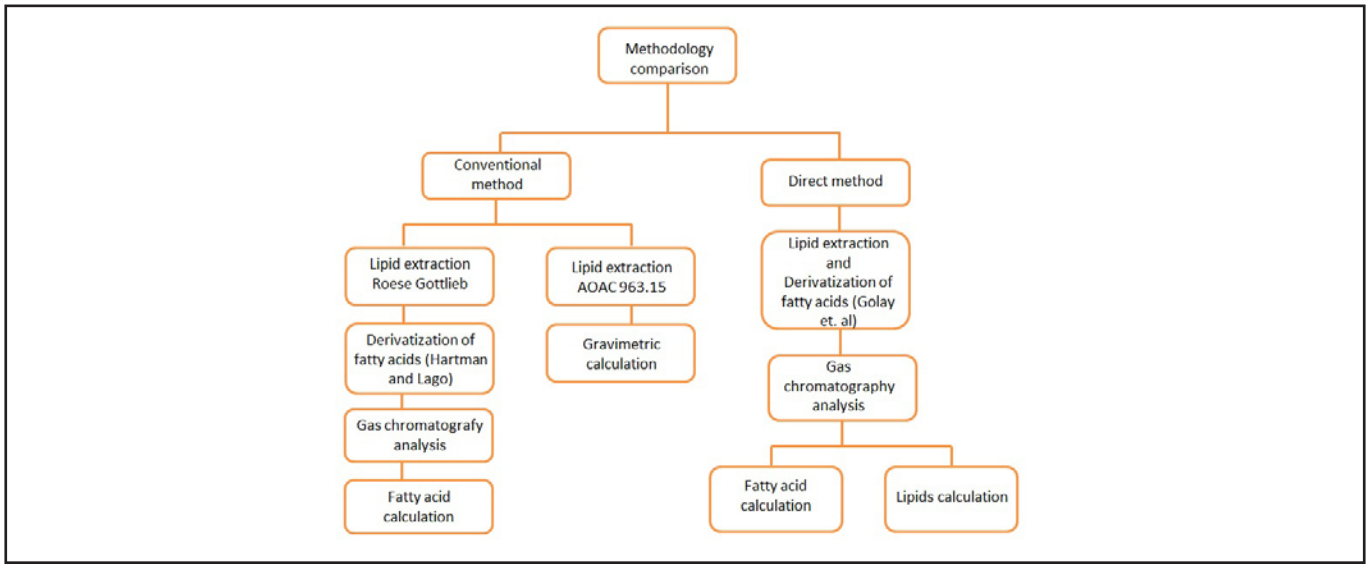

Figure 1. Flow chart for the conventional method (CM) and the direct method (DM) used in the study

Conventional method – CM (lipid extraction and transesterification of fatty acids)

Method AOAC 963.155 is acid hydrolysis and subsequent extraction with petroleum ether. For the analysis by the Roese-Gottlieb analysis (AOAC, 2005)[5], about 2 g of sample was weighed in a centrifugal tube, and 10 mL of distilled water was added and homogenized. So 10 mL of 95% ethyl alcohol were added, 25 mL of ethyl ether, and 25 mL of petroleum ether, and the tube was centrifuged (New Technique – NT 812) for 5 min at 3000 rpm. Following this step, the ether phase was separated and collected in a flask suitable for evaporation. The aqueous extract was twice extracted with additional 15 mL of ethyl ether and 15 mL of petroleum ether, and the ether phase was collected in the appropriate flask. The solvent was evaporated under nitrogen gas flow, and lipids were determined by gravimetry up to constant weight.

For the preparation of PUFAs, the mixed catalysis method was used, according to Hartman and Lago[24] modified by Maia and Rodrigues-Amaya[25]. In approximately 100 mg of the fat extracted by the Roese-Gottlieb method, weighed in a 50 mL tube with a lid, 1 mL of each internal standard (IS) was added, which were PUFAs of C11, C13, C21, and C23, with a concentration of 2.5 mg/mL, and 4 mL of solution 2 M potassium chloride in methanol. Following agitation in the vortex, for 1 min, this solution was maintained for 5 min in a heating bath at 70 ºC, and the tube was removed and cooled under running water. 5 mL of esterifying solution (prepared with sulfuric acid and ammonium chloride in methanol) was added, the tube was stirred in the vortex and the heating bath (5 min, 70 ºC) was maintained. Following the cooling, 6 mL of a saturated solution of sodium chloride and 3 mL of n-hexane were added, and the tube was agitated (1 min in the vortex) and left at rest for separation of the phases. The 1 µL volume of the organic phase was injected by a gas chromatography (GC).

Direct method ("in situ")

The direct method (DM) used was based on Golay et al[10] study, with changes implemented in this study. The analytical steps were as follows: about 100 mg of the sample was weighed in a centrifuge vial of 50 mL; 2 mL of n-hexane was added, the vial was stirred for 30 s and then 300 µL of the following internal standards (PUFAs C21:0 and C23:0, and triacylglycerides of fatty acids C11:0 and C13:0) were added, all with an approximate concentration of 2.5 mg/ mL and 500 µL of 2 M sodium methoxide solution in methanol. The flask was shaken in a vortex for 2 min and heated in a heating bath 50 ºC for 20 min. Then, the vial was cooled in running water until room temperature, and 2 mL of n-hexane and 10 mL of an aqueous solution of 1.5% sodium citrate and 1.0% of sodium chloride were added. The flask was shaken for a minute in a vortex. After the separation phase, 1 µL from the top phase was injected in a GC.

Analysis of fatty acids by gas chromatography with flame ionization detector

Fatty acids analysis by gas chromatography using a flame ionization detector (GC/FID) (Shimadzu, model 17A), coupled with a 100% phase fused silica capillary column with a phase of 100% bis-cyanopropyl polysiloxane (SP 2560, Supelco) of 100 m, 0.25 mm in diameter and 0.20 µm film thickness, according to the conditions described by Kramer et al[26]: injector and detector temperature: 250 °C; flow: 1.90 mL/min; temperature ramp: 45 °C (1 min); 13 °C/min to 175 °C (27 min); 4 °C/min to 215 °C for 35 min; drag gas: hydrogen (flow: 30 mL/min); makeup gas (nitrogen): 30 mL/min; synthetic airflow: 300 mL/min; column pressure: 175 kPa; dividing ratio of the 1:15 sample. The inject mode was manual.

The separate components were identified by co-injection of standards and comparisons with

absolute and relative retention times to the internal pattern, which was calculated by dividing the retention time of each component by the retention time of the peak of the internal standard.

Quantification of polyunsaturated fatty acids

The theoretical response factor for FID was calculated based on equations 1 and 2, and in relation to IS[27-29].

$$K_{FA_i} = \frac{M_{FA_i}}{(n_{FA_i} - 1).A_c} (1)$$

Where:

K_{FA_i} = response correction factor for fatty acid "i";

M_{FA_i} = molecular mass of the fatty acid methyl ester "i";

N_{FA_i} = number of carbon atoms of the fatty acid methyl ester "i";

Atomic mass as used for the calculation: carbon = 12.01 (A_c), hydrogen = 1.0079, oxygen =15.994.

$$K'_{FA_i} = \frac{K_{FA_i}}{K_{IS}} (2)$$

Where:

K'_{FA_i} = relative correction factor for fatty acid "i";

K_{IS} = FID response factor for IS;

K_{FA_i} = FID response factor for fatty acid "i".

Two procedures were used to quantify fatty acids, including the addition of an internal standard. In a previous lipid extraction, the calculations were performed according to equation 3[29]. The internal standard used for this determination was the methyl ester C23:0 fatty acid.

$$conc_{FA_i} = \frac{M_{IS}.A_{FA_i}.K'_{FA_i}.f_{FA_i}.L}{m.A_{IS}} (3)$$

Where:

M_{IS} = mass of the internal standard added to the sample;

A_{FA_i} = PUFAs area in the sample chromatogram;

K'_{FA_i} = response correction factor of each PUFA in the FID to the IS;

f_{FA_i} = conversion factor from PUFAs to FA[29];

L = lipid content in grams percent grams of sample;

m = mass of sample, g;

AIS = area of the FAME internal standard in the sample chromatogram.

In the direct method, the total fat mass was calculated. Triacylglycerol C13:0, C21:0 and C23:0 FAME were added at initial extraction. Fatty acids were calculated using equations 2 and 3, and lipids were calculated from the sum of the triacylglycerol-like components according to AOAC method 996.06 using equation 4[5].

$$M_{FAME_i} = \frac{A_{FA_i}.m_{IS}.1.0059.K'_{FA_i}}{A_{FA_{IS}}} (4)$$

Where:
M_{FAME_i} = mass of each FAME component;
A_{FA_i} = peak area of each component;
m_{IS} = mass of the internal standard;
1.0059 = conversion factor of the IS TAG from corresponding FAME;
K'_{FA_i} = experimental response factor of the FID, about the IS;
$A_{FA_{IS}}$ = peak area of the IS.

$$M_{FA_i} = M_{FAME_i} . f_{FA_i} \quad (5)$$

$$M_{TAG_i} = M_{FAME_i} . f_{TAG_i} \quad (6)$$

Where :
M_{FA_i} = mass of each component expressed as corresponding FA;
f_{FA_i} = Conversion factor of FAME in FA[5];
M_{FAME_i} = mass of each component expressed as the corresponding TAG;
f_{TAG_i} = conversion factor from FAME to TAG[5].

The calculation of recovery of ISs added in the infant formula sample was based on the Validation Guide of INMETRO[30].

Statistical analysis

All analyses were performed in triplicate and the values were expressed on average ± standard deviation. The comparison of the procedures was performed by the analysis of variance (ANOVA) and subsequent *Tukey* test, with a significance level of 95%, where the means followed by the same letter do not differ in the group. Statistical calculations were performed in Statistica® software, version 10. The correlation between the analyzed methods and values of the NIST certificate and Pearson coefficient (r) calculation were performed and evaluated according to the methodology described by Ludbrook[21].

RESULTS

Table 1 shows the lipid content determined by CM and DM, given the use of 3 different IS. The value of lipids by CM was 31.50 ± 0.30 g/100 g, with a coefficient of variation of 0.95%. The lipid values obtained by calculation for the DM, according to Equation 4, were 29.07 ± 1.66 g/100 g for IS C13:0 (TAG), from 29.40 ± 1.10 g/100 g for IS C21:0 (FAME) and 31.41 ± 0.45 g/100 g for IS C23:0 (FAME), which are statistically equal (ANOVA, $\alpha < 0.05$). Lipids values were also calculated using two ISs: C13:0 (TAG) and C23:0 (FAME), with a value of 30.9 ± 0.4 g/100 g. Lipid values per DM and CM showed statistically equal values (ANOVA, $\alpha < 0.05$) on the NIST certificate (31.0 ± 0.6 g/100 g).

Table 1. Lipid content obtained for the conventional and direct methods

Method	Mean ± Standard deviation (g/100g)
Direct – TAG 13:0	29.07 ± 1.66[a]
Direct – FAME 21:0	29.40 ± 1.10[a]
Direct – FAME 23:0	31.41 ± 0.45[a]
Conventional	31.50 ± 0.30[a]

Analysis in triplicate; values in g/100 g of sample; direct method: methodology adapted from Golay et al. (2007)[7]; means followed by the same letter do not differ within the group, according to ANOVA and *Tukey* test (α = 0.05). CV: coefficient of variation. TAG: triacylglycerol; FAME: fatty acid methyl esters

Table 2 shows the levels of FAs obtained for the NIST sample 1849 by CM and DM, in addition to the certified and reference values provided by NIST. It is possible to verify that only the values for FAs C20:4, C22:6, and C20:3, determined by DM, were statistically different (ANOVA and *Tukey* test, α = 0.05) about NIST and CM certified values. In addition to statistical comparison by the ANOVA method and *Tukey* test, the relation between the FAs calculated by CM and DM was performed, and the relation by the DM and the values of the NIST certificate and the Pearson coefficient (r), or correlation coefficient was calculated; these data can be observed in **Figure 2**. For the relationship between CM and DM, the value of r was 0.9956 and for the DM and the values present in the NIST certificate was 0.9955, demonstrating a strong correlation between the methods and between the DM and the NIST certificate values. **Table 2** presents the calculated values for saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs, obtained by the CM and DM methods, and values certified by NIST.

Table 2. Comparison of certified fatty acid contents obtained by the conventional and direct method in the NIST 1849 sample

Fatty acids	NIST	Conventional method	Direct method
6:0[R]	0.061 ± 0.011[a]	0.061 ± 0.019[a]	0.061 ± 0.011[a]
8:0[C]	0.638 ± 0.067[a]	0.640 ± 0.025[a]	0.653 ± 0.050[a]
10:0[C]	0.473 ± 0.019[a]	0.475 ± 0.026[a]	0.494 ± 0.029[a]
12:0[C]	3.712 ± 0.075[a]	3.709 ± 0.299[a]	3.717 ± 0.024[a]
14:0[C]	1.521 ± 0.021[a]	1.511 ± 0.050[a]	1.519 ± 0.038[a]
15:0[C]	0.007 ± 0.0003[a]	0.010 ± 0.001[a]	0.010 ± 0.001[a]
16:0[C]	2.500 ± 0.1600[a]	2.550 ± 0.310[a]	2.510 ± 0.170[a]
16:1 n-7[C]	0.0262 ± 0.0016[a]	0.027 ± 0.001[a]	0.028 ± 0.001[a]
17:0[R]	0.015 ± 0.001[a]	0.015 ± 0.002[a]	0.015 ± 0.001[a]
18:0[C]	0.905 ± 0.056[a]	0.935 ± 0.138[a]	0.939 ± 0.087[a]
18:1 n-9 cis[C]	10.63 ± 0.88[a]	10.400 ± 1.050[a]	11.430 ± 0.810[a]
18:1 n-7 cis[C]	0.203 ± 0.021[a]	0.199 ± 0.008[a]	0.200 ± 0.025[a]
18:2 n-6 cis[C]	6.02 ± 0.1[a]	6.190 ± 0.120[a]	5.820 ± 0.300[a]
18:3 n-3 cis[C]	0.561 ± 0.043[a]	0.558 ± 0.058[a]	0.506 ± 0.012[a]

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Continuation

Fatty acids	NIST	Conventional method	Direct method
20:0[C]	0.095 ± 0.003[a]	0.092 ± 0.001[a]	0.094 ± 0.011[a]
20:1[R]	0.062 ± 0.007[a]	0.065 ± 0.005[a]	0.062 ± 0.007[a]
20:3[R]	0.02 ± 0.004[a]	0.0203 ± 0.001[a]	0.010 ± 0.004[b]
20:4[C]	0.206 ± 0.022[a]	0.200 ± 0.009[a]	0.095 ± 0.008[b]
22:0[R]	0.08 ± 0.007[a]	0.080 ± 0.010[a]	0.080 ± 0.007[a]
22:6[C]	0.067 ± 0.006[a]	0.061 ± 0.003[a]	0.021 ± 0.002[b]
24:0[C]	0.039 ± 0.003[a]	0.040 ± 0.002[a]	0.040 ± 0.002[a]
24:1[R]	0.024 ± 0.004[a]	0.024 ± 0.002[a]	0.024 ± 0.004[a]
SFA[CA]	10.046 ± 0.202[a]	9.539 ± 0.467[a]	10.129 ± 0.205[a]
MUFA[CA]	10.945 ± 0880[a]	10.715 ± 1.050[a]	11.744 ± 0.810[a]
PUFA[CA]	6.874 ± 0.111[a]	7.029 ± 0.134[a]	6.462 ± 0.300[a]

Analyses in triplicate; values expressed as mean ± standard deviation in g/100 g sample; [C]: certified fatty acid values; [R]: reference fatty acid values; [CA]: sum of individual fatty acid values provided by NIST; SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; PUFA: sum of polyunsaturated fatty acids; means followed by the same letter do not differ within the group according to ANOVA and *Tukey* test (α = 0.05). Limit of quantification of the method = 0.01 g/100 g

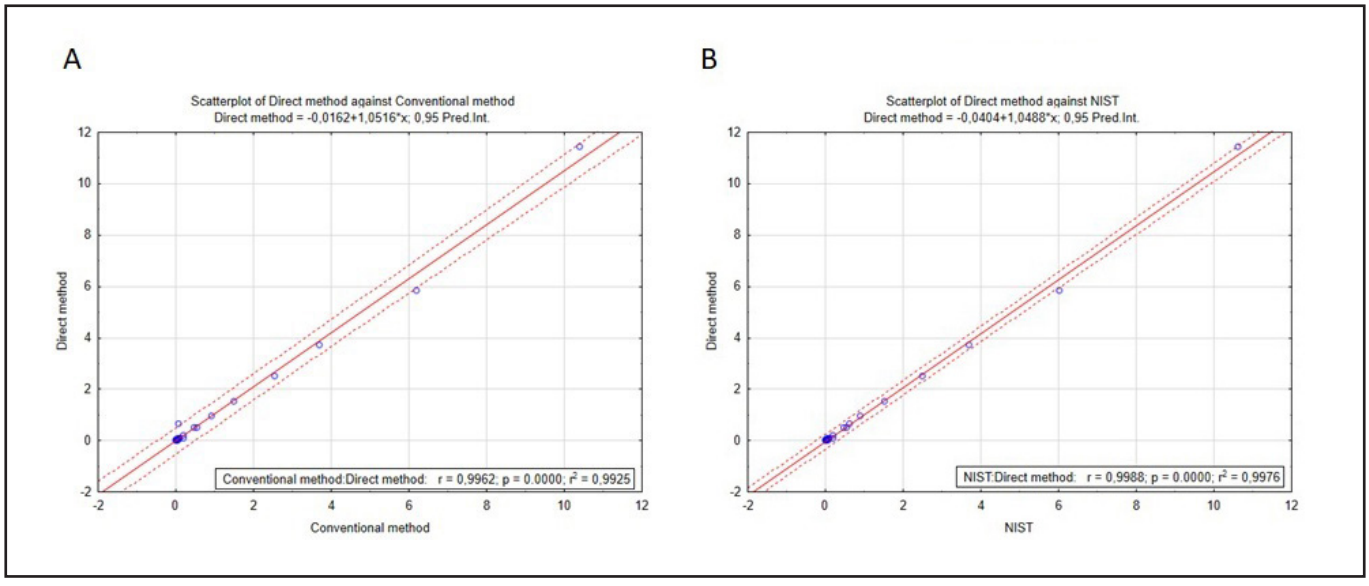

Figure 2. Correlation graph. A) Relation between the values of fatty acid for conventional method and direct method. B) Relation of the fatty acid values for the direct method to the NIST certificate values. r = correlational coefficient

DISCUSSION

The conventional method routinely used in the laboratory consists of two lipid extractions, one being performed for the quantification of total fat and the other for acid analysis; after this step, the transesterification reaction is performed, and then the analysis by gas chromatography. These reactions may take 3 to 5 days, with a high use consumption of organic solvents. Thus, a comparison was made

with a direct method, where lipid extraction and fatty acids esterification occur together, reducing analysis time and solvent expenditure. For methodological comparison, a sample of NIST infant formula was used, linked to the USA. Department of Commerce, and is considered to be a CRM, i.e., CRMs characterized using state-of-the-art methods to determine chemical composition, thus ensuring that it is an excellent material for comparing the methodologies, besides having been manufactured by a company in the sector, following the step s of production and using the similar ingredients of infant formulas available in the market[31]. The NIST sample used for comparison of methods can therefore be considered representative of end-use infant formula.

When available, CRMs should be used in the validation process of a test method because they have a concentration value for each parameter and the associated uncertainty. A use of CRM is its analysis to assess the performance of the analytical method[30].

Lipid Determination

In CM, prior acid hydrolysis was conducted with subsequent extraction with petroleum ether for gravimetric determination of lipids (**Figure 1**). An infant formula typically consists of a variety lipid sources such as milk and vegetable fats and encapsulated fatty acids. Thus, the use of previous acid hydrolysis can help to break down microcapsules, releasing fatty acids more efficiently[32,33].

The study used for DM did not provide for the determination of lipids by calculation, and this modification was added in this study to simplify quantification; the calculations were performed according to Equations 4 and 6 and expressed as equivalent triacylglycerols.

In methods of quantification of lipids by calculation, the choice of the internal standard is extremely importance. Lipids are calculated as the sum of fatty acids and condense in the triacylglycerol molecule in accordance with AOAC5 and AOCS Ce 1h-0520. In the experimental work, the EMAG 21:0 and C 23:0 IS have been used for the calculations, in addition to the IS of TAG C13:0.

According to the values in **Table 1**, there was no statistical difference between the ISs for the calculation of lipids. However, if we consider the elution in which PUFA is eluated in a chromatogram, the higher the PUFAs calculated from the IS, the higher the difference in its concentration[34]. The lipid values obtained with the C21:0 and C23:0 IS reflect the contribution of short-chain fatty acids present in dairy products, since these FAs elude far from the IS, thus having relative detector response factors greater than those calculated with a closer IS (**Figure 1**). In the case of PI TAG 13:0, it is the AGPI-CL that influences the calculation, because the values of the FID response factors are lower when calculated to this IS, because these FAs are distant from the IS in the chromatogram, as can be observed in **Figure 3**. Thus, when there is a variety of FAs in the food matrix, the ideal is quantification with two IS, that is, to use as IS the 13:0 TAG from the beginning of the elution to the stearic acid region (18:0), and the IS of the FAME 23:0 for the fatty acids between C18:0 and C22:6.

To ensure the reliability of the IS lipid results, recoveries were calculated for IS C13:0 and C23:0, with results ranging from 98 to 99% (coefficient of variation of 0.58%) and from 97 to 99% (coefficient of variation of 1.00%), considered adequate as recommended by the Validation Guide of INMETRO[30] for this concentration range, i.e., recoveries between 95 and 105%[30] for 0.1% of IS added to the sample, demonstrating the accuracy of the data presented.

Therefore, it can be verified that lipid analysis in infant formula can be performed by the DM when adding two PI for the calculation since it showed a statistically equal value (ANOVA and *Tukey* test, $\alpha = 0.05$) compared with both the CM and the CRM of the NIST.

Figure 3 shows a NIST 1849 infant formula chromatogram that checks the elution order of the PUFAs and the ISs additions.

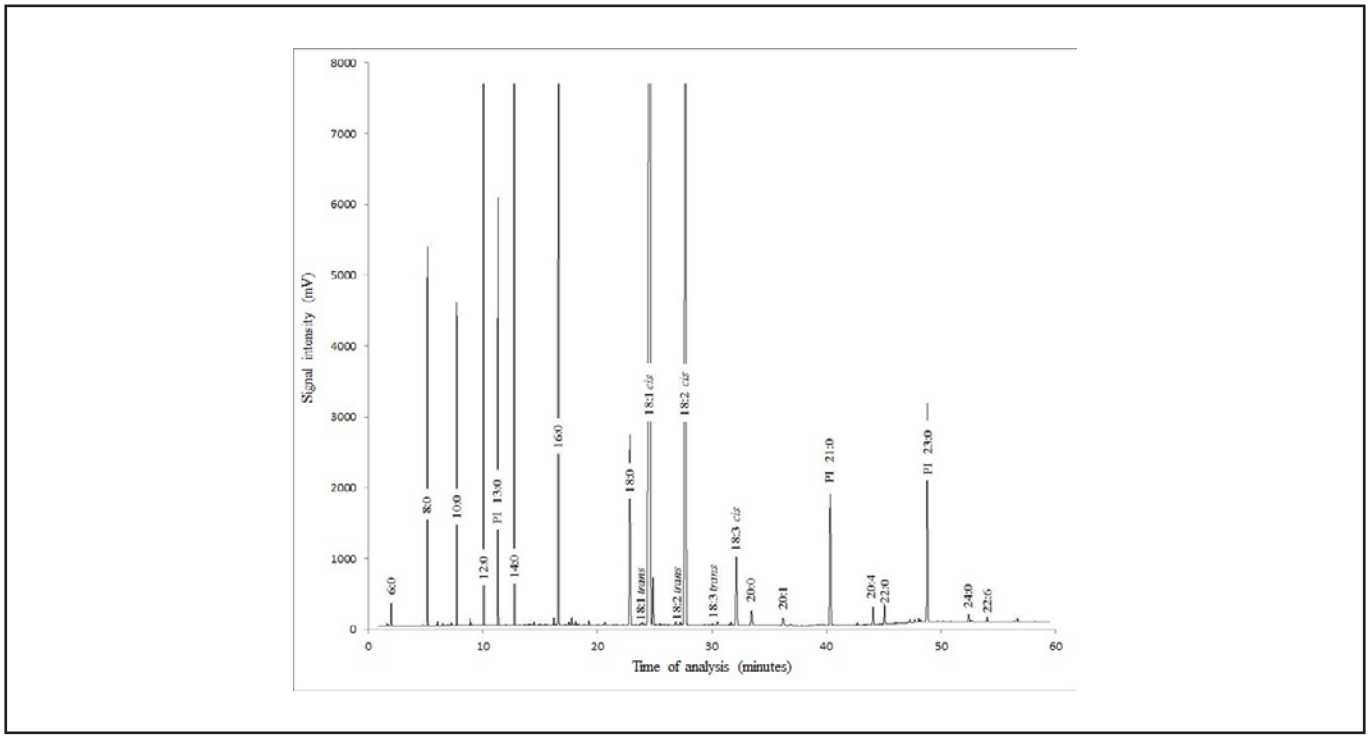

Figure 3. Chromatogram of the major fatty acids methyl esters from the gas chromatography analysis of sample NIST 1849. IS: internal standard

Quantification of fatty acids in the sampled NIST 1849

The fatty acid calculations were carried out using the TAG C13:0 IS, for the eluted FA before C18:0, and FAME C23:0, for those after C18:0 (**Figure 3**). The theoretical response factor for correction of the FID was that of the IS itself. For CM, equation 3 and for DM equations 4 and 5 have been used. In the infant formula CRM certificate, there are two classes of values reported to the FAs, being certified values value for FAs C8, C10, C12, C14, C15, C16, C16:1, C18, C18:1, C18:2, C18:3, C20, C20:4, C22:6, and C24 and reference values for FAs C6, C17, C20:1, C20:3, C22, and C24:1.

NIST define as a certified value the data for which there is greater reliability and accuracy, in which all known or suspected sources of bias have been fully investigated or accounted for. The uncertainty associated with a certified value usually specifies a range within which the true value is expected to be at a confidence level of approximately 95% and a study of homogeneity was carried. The reference value in the certificate is defined as the best estimate of the true value provided, where all known or suspected sources of bias outside of the certificate have not been fully investigated by NIST. The uncertainty associated with a reference value may not include all sources of uncertainty and may be only a measure of the precision of the measurement method[35].

For the FAs declared with certified values, the majority had very close results between the CM and the DM, being statistically equal (ANOVA and *Tukey* test, α = 0.05), and also to the CRM, except for the polyunsaturated FAs C20:4 and C22:6, a fact observed in **Table 2**. In the case of FAs declared as a reference, only C20:3 had a statistically different value for the CM and DM methods and also for the infant formula sample (**Table 2**). Regardless of the rigidity with which the value was determined by the CRM manufacturer, those FAs with longer chains and with double unsaturations had lower values for the DM, thus demonstrating a limitation of the analytical methodology.

The DM uses a higher temperature, which may contribute to the degradation of polyunsaturated fatty acids; the steps of lipid extraction and derivatization of fatty acids in the sample may not have been efficient, especially in the case of micro-encapsulated fatty acids. In addition, the direct method was applied three weeks after the conventional method. Considering the justifications presented those polyunsaturated fatty acids which are more susceptible to degradation and/or oxidation, since they present more reactive sites, may have been degraded, which may explain the lowest levels obtained[36]. On the other hand, methods under softer conditions, such as those used in CM, do not affect the composition of polyunsaturated fatty acids[3].

The correlation observed for the values of FAs between DM and MC was 0.9955 for the FAs calculated by the DM and those of the NIST certificate were 0.9988, higher than 0.99, demonstrating a strong correlation between the methods and between the MD and the values of the NIST certificate[37]. Thus, we can make sure that these methods are equivalent, and can be used to analyze FAs in infant formula.

FAs are typically indicated on food labels, including infant formula, such as the sum of FAs, *trans* fatty acids (TFA), MUFA, and PUFA. The NIST certificate does not contain the AGT and therefore they were not calculated. Thus, by performing this on the basis of the data in **Table 2**, it is possible to verify that these values are statistically equal (ANOVA and *Tukey* test, α = 0.05). The difference between the values obtained by the DM and those reported in the NIST certificate was 0.82% for SFA, 7.31% for MUFA, and 6.00% for PUFA, showing that the DM can be used to calculate the sum of these FAs.

The results obtained in the present study, considering the matrix studied and using the direct methodology, were satisfactory and the DM can be used in the laboratory for quantification of FA, since when comparing its results with those of the MC and the values of the NIST certificate, of the 22 FAs analyzed, only three (13.6%) showed values for the DM different from the CM and from that provided for in the certificate of infant formula NIST. Difference percentages have been observed for SFA, MUFA, and PUFA, but they correspond to the 20% tolerance of nutritional information provided in the legislation. For the sum of the PUFAs, there were no differences between the values of DM, CM, and NIST values, and these results were statistically equal (ANOVA and *Tukey* test, α = 0.05). However, when analyzing specific FAs like C20:3, C20:4 and C22:6, large variations were found; therefore, if the objective of the analysis is to quantify these FAs individually, further studies and adaptations of the DM are necessary.

It is noteworthy that, for infant formula, there is no official direct method, and the AOCS Ce 2b-11 and 2c-11 methodologies[20] can be used, which are indicated for food matrices in general.

CONCLUSION

The direct method for quantification of lipids and fatty acids in infant formula proved to be appropriate and similar to the conventional method, with the advantages of being faster and using fewer organic solvents. The calculation with the addition of two internal standards proved suitable for lipid quantification, as well as for fatty acids. Therefore, the direct method may be considered an alternative to the laboratory and used in the calculation of the concentration of most individual fatty acids, as well as for the sum of saturated, monounsaturated, and polyunsaturated fatty acids in infant formula.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

FINANCING

Not declared.

ACKNOWLEDGEMENTS

Not declared.

AUTHORS' CONTRIBUTION

Analytical and statistics analysis and writing of the article: Mahyara Markievicz Mancio Kus Yamashita; Guidance in the analysis, data analysis and writing, correction and final review of the article: Sabria Aued Pimentel; Work guidance and article correction: Jorge Mancini Filho.

PRESENTATION NOTE: This article is part of Mahyara Markievicz Mancio Kus master's thesis entitled "Determination of polyunsaturated fatty acids in infant formulas: comparison of methodologies in analysis by gas chromatography" that was developed in the Graduate Program in Food Sciences – Area of Bromatology, at the Pharmaceutical Sciences School of the University of São Paulo, under the guidance of Prof. Dr. Jorge Mancini Filho and defended in August 2009.

REFERENCES

1. Kus MMM, Aued-Pimentel S, Mancini-Filho J. Comparação de métodos analíticos para determinação de lipídios e ácidos graxos polinsaturados por cromatografia gasosa em fórmula infantil. Rev Inst Adolfo Lutz. 2009;68(1):12-20. Available from: https://periodicos.saude.sp.gov.br/index.php/RIAL/article/view/32737

2. Kus MMM, Silva SA, Aued-Pimentel S, Mancini-Filho J. Informação nutricional de fórmulas infantis comercializadas no Estado de São Paulo: avaliação dos teores de lipídeos e ácidos graxos. Rev Nutr. 2011;24(2):209-18. https://doi.org/10.1590/S1415-52732011000200002

3. Kus MMM, Aued-Pimentel S, Mancini-Filho J. Estabilidade dos ácidos graxos poli-insaturados presentes em fórmulas infantis comerciais. Braz J Food Tech. 2011;14(2):145-53. Available from: http://bjft.ital.sp.gov.br/arquivos/artigos/v14n2457a.pdf

4. Aued-Pimentel S. Avaliação de procedimentos analíticos para a determinação de lipídios e ácidos graxos em produtos alimentícios [tese de doutorado]. São Paulo (SP): Coordenadoria de Controle de Doenças da Secretaria de Estado da Saúde de São Paulo; 2007.

5. Official Methods of Analysis of AOAC. 21. ed., Gaithersburg: AOAC International, 2019. [Accessed on 2019 Jun 18]. Available from: http://www.eoma.aoac.org/

6. Adrian J, Potus J, Poiffait A, Dauvillier P. Análisis nutricional de los alimentos. Métodos fisico químicos generales. Zaragosa: Editorial Acribia; 2000.

7. Christie WW. Preparation of esters derivatives of fatty acids for chromatographic analysis. In: Advances in lipid methodology – two, 1993; 2: 69-111. [Accessed on 2006 Out 05]. Available from: https://lipidlibrary.aocs.org/lipid-analysis/selected-topics-in-the-analysis-of-lipids/preparation-of-ester-derivatives-of-fatty-acids-for-chromatographic-analysis

8. Carpenter DM, Ngeh-Ngwainbi J, Lee S. Lipid Analysis. In: Carpenter DE, Sullivan DM, organizer. Methods of analysis for nutritional labeling. Arlington: AOAC International; 1993. p.85-104.

9. Carrapiso AI, Garcia C. Development in lipid analysis: some new extraction techniques and in situ transesterification. Lipids. 2000;35(11):1167-77 https://doi.org/10.1007/s11745-000-0633-8

10. Golay PA, Dionisi F, Hug B, Giuffrida F, Destaillats F. Direct quantification of fatty acids in dairy powders with special emphasis on trans fatty acids content. Food Chem. 2006;101(3): 1115-20. https://doi.org/10.1016/j.foodchem.2006.03.011

11. Castro-Gómez P, Fontecha J, Rodríguez-Alcalá LM. A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples. Talanta. 2014;128:518-23. https://doi.org/10.1016/j.talanta.2014.05.051

12. Liu Z, Wang J, Li C, Rochfort S. Development of one-step sample preparation methods for fatty acid profiling of milk fat. Food Chem. 2020;315:126281. http://dx.doi.org/10.1016/j.foodchem.2020.126281

13. O'Fallon JV, Busboom JR, Nelson ML, Gaskins, CT. A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. J Anim Sci. 2007; 85(6):1511-21. https://doi.org/10.2527/jas.2006-491

14. Cruz-Hernandez C, Goeuriot S, Giuffrida F, Thakkar SK, Destaillats F. Direct quantification of fatty acids in human milk by gas chromatography. J Chromatogr A. 2013;1284:174-9. https://doi.org/10.1016/j.chroma.2013.01.094

15. Abdulkadir S, Tsuchiya M. One-step method for quantification and qualitative analysis of fatty acids in marine animal samples. J Exp Mar Biol Ecol. 2008;354:1-8. https://doi.org/10.1016/j.jembe.2007.08.024

16. Wang Y, Sunwoo H, Cherian G, Sim JS. Fatty acid determination in chicken egg yolk: a comparison of different methods. Poult Sci. 2000; 79(8):1168-71. https://doi.org/10.1093/ps/79.8.1168

17. Mazalli MR, Bragagnolo N. Validation of two methods for fatty acids analysis in eggs. Lipids. 2007;42(5):483-90. https://doi.org/10.1007/s11745-007-3046-4

18. Ehimen EA, Sun ZF, Carrington CG. Variables affecting the in situ transesterification of microalgae lipids. Fuel. 2010;89(3):677-84. https://doi.org/10.1016/j.fuel.2009.10.011

19. Velasquez-Orta SB, Lee JGM, Harvey A. Alkaline in situ transesterification of Chlorella vulgaris. Fuel. 2012;94:544-50. https://doi.org/10.1016/j.fuel.2011.11.045

20. Official Methods and Recommended Practices of the AOCS. Revisions 2017. 7. ed., Champaign: American Oil Chemists' Society, 2017. [Accessed on 2018 Nov 14]. Available from: https://www.aocs.org/attain-lab-services/methods/methods

21. Ludbrook J. Confidence in Altman-Bland plots: a critical review of the method of differences. Clin Exp Pharmacol Physiol. 2010;37(2):143-9. https://doi.org/10.1111/j.1440-1681.2009.05288.x

22. ABNT NBR ISO/IEC 17025. Requisitos gerais para a competência de laboratórios de ensaio e calibração. 2017.

23. Melo EDA. O uso dos MRC (Materiais de Referência Certificados) na avaliação dos métodos analíticos. Rev Analytica. 2020. [Accessed on 2021 Jun 10]. Available from: https://revistaanalytica.com.br/o-uso-dos-mrc-materiais-de-referencia-certificados-na-validacao-dos-metodos-analiticos/#_edn1

24. Hartman L, Lago RAC. Rapid preparation of fatty acid methyl esters from lipids. Lab Prac. 1973;22(6):475-6. Available from: https://www.scienceopen.com/document?vid=5334de68-6e03-446b-85e7-244d0ac1ce1b

25. Maia EL, Rodrigues-Amaya DBR. Avaliação de um método simples e econômico para a metilação de ácidos graxos com lipídios de diversas espécies de peixes. Rev Inst Adolfo Lutz. 1993;53(1-2):27-35. Available from: https://periodicos.saude.sp.gov.br/index.php/RIAL/article/view/35982

26. Kramer JKG, Blackadar CB, Zhou J. Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP sil 88) for analysis of milkfat with emphasis on CLA, 18:1, 18:2 and 18:3 isomers, and short- and long-chain FA. Lipids. 2002;37(8):823-35. https://doi.org/10.1007/s11745-002-0967-2

27. Ackman RG, Sipos JC. Application of specific response factors in the gas chromatographic analysis of methyl esters of fatty acids with flame ionization detector. J Am Oil Chem Soc. 1964;41(5):377-8. https://doi.org/10.1007/BF02654818

28. Bannon CD, Graske JD, Hillker AE. Analysis of fatty acid methyl esters with high accuracy and reliability: Validation of theoretical relative response factors of unsaturated esters in the flame ionization detector. J Am Oil Chem Soc. 1986;63(1):105-10. https://doi.org/10.1007/BF02676134

29. Instituto Adolfo Lutz (São Paulo – Brasil). Métodos físico-químicos para análise de alimentos: normas analíticas do Instituto Adolfo Lutz. 4. ed. [1. ed. digital]. São Paulo (SP): Instituto Adolfo Lutz; 2008. Available from: http://www.ial.sp.gov.br/resources/editorinplace/ial/2016_3_19/analisedealimentosial_2008.pdf

30. Instituto Nacional de Metrologia, Qualidade e Tecnologia. Guia de Orientação sobre validação de métodos analíticos. DOQ-CGCRE-0008 – revisão 08. 2020. [Accessed on 2021 Jun10]. Available from: http://www.inmetro.gov.br/credenciamento/organismos/doc_organismos.asp?torganismo=calibensaios

31. National Institute of Standards e Technology (NIST). Department of Commerce. United States of America. Certificate of Analysis – Standard Reference Material 1849 Infant/Adult Nutritional Formula. 2010. [Accessed on 2021 Jun 10]. Available from: https://www-s.nist.gov/srmors/certificates/archives/1849.pdf

32. Hirayama KB, Speridião PGL, Fagundes-Neto U. Ácidos graxos poli-insaturados de cadeia longa. Elect J Ped Gastroent Nutr Liver Dis. 2006;10(3):1-10.

33. Currtis JM, Berrigan N, Dauphinee P. The determination of n-3 fatty acid levels in food products containing microencapsulated fish oil using the one-step extraction method. Part 1: measurement in the raw ingredient and dry powdered foods. J Am Oil Chem Soc. 2008;85(4):297-305. https://doi.org/10.1007/s11746-008-1194-1

34. Schreiner M. Quantification of long chain polyunsaturated fatty acids by gas chromatography: evaluation of factors affecting accuracy. J Chromatogr A. 2005;1095(1-2): 126-30. https://doi.org/10.1016/j.chroma.2005.07.104

35. National Institute of Standards e Technology (NIST). Department of Commerce. United States of America. NIST Special Publication 260-136 – Standard Reference Materials Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements. 2000. [Available from: 2021 Jun 10]. Available from: https://www.nist.gov/system/files/documents/srm/SP260-136.PDF

36. Maduko CO, Park YW, Akoh CC. Characterization and oxidative stability of structured lipids: infant milk fat analog. J Am Oil Chem Soc. 2008;85(3):197-204. https://doi.org/10.1007/s11746-007-1192-8

37. Twomey PJ, Kroll MH. How to use linear regression and correlation in quantitative method comparison studies. Int J Clin Pract. 2008;62(4):529-38. https://doi.org/10.1111/j.1742-1241.2008.01709.x