Polyunsaturated Fatty Acids in Dried Milk Samples: Validation of a Lipid Separation-Free Method

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A Rapid, Lipid Separation-Free Method to Determine Polyunsaturated Fatty Acids in Milk Samples

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ABSTRACT

A rapid method to determine polyunsaturated fatty acids (linoleic and α-linolenic acids and its long chain metabolite, DHA) in milk samples, avoiding the fat separation step, was developed and validated. The transesterification reaction was carried out directly on freeze-dried milk samples to produce fatty acid methyl esters. Separation, identification and quantification of analytes were performed by GC-MS. In these experimental conditions the matrix effect was negligible. Values for repeatability and intermediate precision demonstrate excellent method precision.

To test the method applicability, goat's, cow's and human's milk with different fat contents and essential fatty acids concentrations were examined.

(**Key words:** Fatty acids, milk fat, gaschromatographic method)

INTRODUCTION

Essential fatty acid are nutrients of primary importance for human health, and research over the last 15 years has demonstrated the potential role of an adequate intake of n–3 polyunsaturated fatty acid (**PUFA**) in the prevention of several diseases, cardiovascular diseases in particular [1]. As linoleic and α-linolenic acids compete for

the same enzymes in the synthesis of other biologically-important polyunsaturated fatty acids, e.g. DHA, their optimal intakes are mutually dependent [2, 3].

In recent years, recommended dietary allowances have been published in several countries, not only for linoleic and α -linolenic acids but also for long-chain n–3 PUFAs, especially docosahexaenoic acid (**DHA;** $C_{22:6,\ n-3}$) and eicosapentaenoic acid [4]**.**

Breast milk is an important source of DHA and essential fatty acids in early human life. Formula-fed infants, who do not receive exogenously preformed long chain PUFA, have lower levels of DHA in the membranes of the central nervous system [5].

Milk and dairy products are recognized as important sources of nutrients in the human diet, providing energy, high-quality protein, and essential minerals and vitamins. However, milk fat is relatively more saturated than most plant oils, and this has led to negative perception among consumers and public-health concerns related to excessive intake of saturated fats. Milk fat content and fatty acids (**FA**) composition significantly depend on the diet fed to dairy cows, offering the opportunity to respond to market forces and human-health recommendations [6].

Although several methods have been developed for the determination of FA in biological specimens, most studies either do not include any assay validation or report incomplete assay validation. Furthermore, such methods require previous extraction of FA from the milk sample[7]; a step that increases analysis time and may be a source of inaccuracy .

In this study, a gas chromatographic method to determine linoleic and α-linolenic acids and DHA in lyophilized milk samples, avoiding the extraction step, was developed and validated.

Sample milk from cows fed with n–3 rich fodder was examined and compared with other cow's, goat's and human milks, using the proposed method.

MATERIALS AND METHODS

Chemicals and Reagents

The chemical reagents used were n-hexane (HPLC grade, Fluka), sodium chloride (analytical grade, BDH), boron trifluoride-methanol solution (ref. 15716, Fluka). Linoleic acid (cis 9,12-octadecadienoic acid, ref. 62230), α -linolenic acid (cis 9,12,15-octadecatrienoic acid, ref. 62160) and DHA (cis 4,7,10,13,16,19-docosahexaenoic acid, ref. 43938) were from Fluka, while linoleneaidic acid (trans 9,12,15-octadecatrienoic acid, ref. L 2406) was from Sigma. Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Samples

The samples of both goat's and cow's milk were collected in a farm near Turin (Italy), whereas the samples of human milk were kindly provided by a colleague of

ours; the other milk samples, included one sample of n–3-enriched bovine milk, were purchased in different shops in Turin. All these milk samples were immediately frozen after collection, lyophilized and stored at -20°C.

The validation procedure was performed using lyophilized n–3 enriched milk. This sample was also used as QC sample. Since the analyte content was unknown, the standard addition method was used to determine sample concentrations and to evaluate bias.

All procedures to evaluate the method were performed on a sample of n–3 enriched lyophilized bovine milk, which was then taken as reference sample.

Methylation Procedure.

The methylation procedure proposed by Morrison [8] was followed, with minor changes.

A volume of 60 µl of a 500 µg/ml standard solution of linoleneaidic acid (internal standard) was evaporated to dryness under nitrogen in a 20 ml centrifuge tube provided with a Teflon-lined screw cap. Carefully weighed amounts of sample were added to the residue. 3 ml of boron trifluoride-methanol reagent were added to the mixture under nitrogen. The tube was closed, heated at 80 °C for 45 minute, cooled and opened. For fatty acids methyl esters (**FAME**) extraction, 1 ml of a NaCl saturated aqueous solution and 3 ml of n-hexane were added. The mixture was vigorously shaken

and phase separation was achieved by centrifugation. 1.5 ml of clear supernatant was transferred in an autosampler vial.

Instrumentation and Chromatographic Conditions

GC-MS determination was carried out on a VARIAN 3800 gaschromatograph equipped with VARIAN 8400 autosampler and SATURN 2000 ion-trap mass selective detector. A VF-23 MS (Varian) (50% cyanopropylsilicone) fused silica capillary column (30 m long, 0.25 mm i.d. and 0.25 mm film) was used. Both injector and detector temperatures were set to 250 °C. The samples (1 μl) were injected in splitless mode for 90 sec after which the split ratio was 25:1. The oven was programmed as follows: 70°C for 1.0 min, increased at 5°C/min to 230°C, and held constant for 10 min. Helium was used as carrier gas at a flow rate of 1.0 ml/min. Quantification was carried out measuring the area produced by the mass fragments at m/z 67-81, 79-91 and 67-79 for linoleic acid, DHA and linoleneaidic and α -linolenic acids, respectively.

Compounds were identified by both retention times and mass spectra.

Detection Limit.

In order to determine the method's detection limit, measures were carried out at decreasing concentrations of standards, until the analyte-related signal disappeared. The analysis at the minimum concentration which produced an appreciable signal was

repeated eleven times, with independent measures. The limit of detection (**LOD**), defined as the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty, was calculated by extrapolation applying the formula:

LOD = 3 x SD x C/ M

where: SD is the standard deviation, C is the analyte concentration and M the mean area. The limit of quantification (**LOQ**) was calculated from the formula:

LOQ = 10 x SD x C/M.

Both LOD and LOQ values were confirmed by the injection of the suitable standards.

Precision

The first type of precision study is instrument precision or injection repeatability [9]. In this study, 10 injections of the same solution of standards were carried out to test the performance of the chromatographic instrument. The repeatability or intra-assay precision is obtained by repeatedly analyzing, on the same day, aliquots of a homogeneous sample, each of which has been independently prepared following the method procedure. The intermediate precision was evaluated by measuring 6 replications over 3 days; the ANOVA statistical test was used to interpret the results.

Calibration Curves

Three calibration experiments were performed. The complete analytical procedure described above was applied to all calibration samples.

Linearity was checked by an *a posteriori* F-statistical test, comparing the residual standard deviation of the linear model with that of the quadratic model [10].

External calibration: a set of six calibration standards was prepared from the pure commercial standards for each of the analytes and for linoleneaidic acid (internal standard). All calibration solutions were prepared using n-hexane as solvent. Suitable volumes of the four calibration solutions were introduced into a centrifuge tube and evaporated to dryness under nitrogen. The residue, after the addition of 3 ml of boron trifluoride-methanol reagent, was treated as described above. Four replications were performed for each calibration point.

Standard-added calibration: standard-added calibration (**AC**) was achieved by addition of different amounts of standard fatty acid solutions to a constant weight of sample; this curve includes the value of "zero" addition. The lowest concentration of the standard addition calibration curve was chosen in order to double the response of the analyte in the sample.

Youden calibration: the Youden calibration (**YC**) curve was established with increasing amounts of sample. In this curve, the value that corresponds "zero" to sample volume is not included. Sample amounts ranged between 0.01 g and 0.06 g of lyophilized milk. As in the above calibrations, linoleneaidic acid was added as internal standard.

RESULTS AND DISCUSSION

The analyte concentrations were generally too low compared to the major components to produce appreciable peaks in the TIC chromatograms, but they could easily be recognized by their characteristic mass fragments.

The LOD and the LOQ of the three analytes, expressed in terms of injected amount, were respectively 43 and 140 pg for linoleic acid, 30 and 99 pg for α -linolenic acid, and 240 and 780 pg for DHA.

Table 1 gives the results for injection repeatability. As mentioned above, the data were obtained by measuring the three methylated fatty acids standards 10 times. The instrumental precision was good, ranging from 3.12% for DHA to 1.41% for α -linolenic acid (Tab.1).

Values for repeatability and intermediate precision were obtained using the reference sample and are reported in Table 2. The percent relative standard deviations at each day for each analyte were below 10, demonstrating excellent method precision. The ANOVA statistical test did not detect any significant differences among the results.

Calibration

Table 3 shows the regression parameters of the external calibration curves, standard addition curves and Youden calibration curves.

External calibration

The external calibration curves (**EC**) were obtained over the concentration range 0.5 to 10 μg/ml for DHA, 2 to 25 μg/ml for linoleic acid and 1 to 10 μg/ml for α -linolenic acid. The linearity was good for all analytes in the range tested, as shown by the determination coefficients (table 3). In order to evaluate the upper limit of linearity, higher concentrations (up to 100 μg/ml) were assayed. A deviation from the linear pathway occurred at concentrations above 40 μg/ml for α -linolenic acid and DHA, and above 50 μg/ml for linoleic acid.

Standard-added curves

Analyte content in the samples was assessed by the standard-added curve, as described in the literature [11]. Comparison of the values obtained by this method and those calculated by external calibration curves allowed bias to be estimated [12]. The standard-added curve also produces an estimate of the matrix effect: proportional bias can be estimated by comparing the slopes of the straight lines of external calibration and addition calibration. If the slopes are similar, then no component of proportional bias is involved. Table 3 shows the regression parameters for the three compounds. As can be seen, the slopes of the external calibration curves and standard-added curves are generally very close. To check the similarity of the external calibration and standard-added curves slopes, a Student's t test was applied. For all analytes considered, the

significance level was below 5%, thus the hypothesis of the presence of a proportional bias was rejected.

Youden calibration

The Youden calibration was performed for several purposes. Firstly, the YC enables the suitability of the esterification conditions to be evaluated. Both reaction time and amounts of BF_3/CH_3OH are reported [8] to be critical for methylation yield: generally, samples containing different kinds (i.e. free fatty acids, mono- di- or triglycerides, etc) and quantities of reactive species require different reaction conditions. In the YC, the response linearity for increasing amounts of sample was checked; YC linearity indicates that conditions in the methylation reaction are maintained pseudo-first order; as the method had to be applied to the analysis of milk samples of different sources, it was very important to verify that the experimental conditions were suitable for a wide range of contents of different fats. In the tested range, the linearity of the YC calibration curves attests that the methylation reaction occurred in pseudo-first order conditions, which is the basis for accuracy of the analysis (Table 3). Moreover, a difference between the intercepts of the EC and YC curves indicates a constant bias due to sample matrix effect [13]. Because the two intercepts were obtained from different independent variables (sample amount and standard dilution, respectively, for EC and YC), a comparison test could not be established. However, a difference may be considered to exist between the two values if the value of the Youden calibration intercept is not included within the confidence interval value of the external calibration intercept. In this study, all YC intercept values were included in this range, and thus no constant bias was detected.

Finally, the concentration values of the analytes in the sample can be estimated from the ratio of the external and Youden calibration slopes. The comparison between these values and those obtained by the standard-addition method is of a great aid in evaluating accuracy.

Table 4 gives the concentration values obtained by the two independent methods and by external calibration. As may be seen, the data were in excellent agreement; the table also shows the high accuracy of concentration results estimated by the external calibration method.

The proposed method was applied to the analysis of milk samples from different sources. The results for the three fatty acids, with the related uncertainty (95% confidence level) are shown in Table 5. Linoleic acid and DHA are present in high concentrations in human milk. The relevance of the cow's nutrition to enrich the essential fatty acid content of milk is demonstrated by the comparison of the different cow milk samples. There was a significant increase in linoleic and α -linolenic acids, and especially in DHA (from below the LOD to 41.6 mg/L) in milk from cows fed with n–3 fatty acids supplementation, whereas the fatty acid contents of the other cow milk samples, unlike the goat's milk samples, was within a very small range.

Conclusions

Several protocols for milk fatty acid analysis have been described, and are now established. All methods required an initial separation of the lipids from the liquid milk

sample, either by extraction [14] or by centrifugation [15, 16], followed by transesterification to form FAME [7]. With the method described here, starting from a freeze-dried sample, the extraction step is not required and the sample can be immediately subjected to the transesterification procedure. This saves the time required for lipid separation and reduces chemical and environmental costs, because it does not require solvents. Furthermore the extraction step is very often a source of inaccuracy.

The accuracy of the proposed method is evident from the validation parameters: in particular, it seems not to be affected by any matrix effect, indeed the concentration values obtained by external calibration, standard-added calibration and YC are very close.

The method was validated on a sample "naturally" enriched with n–3 FA and was then applied to other freeze-dried samples of milk from different sources; the results are in agreement with data reported in the literature [16-18]. In this study we focussed on DHA and on linoleic and α-linolenic acids, because they are essential fatty acids, and could be used as the principal markers of dairy products naturally enriched in n-3 fatty acids.

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Table 1: Instrumental precision. Values of means and standard deviations are given in counts

	mg/L	Mean	St.Dev.	CV %
Linoleic acid	20	371918	8961.0	2.41
alfa-linolenic acid	20	284612	4013.7	1.41
DHA	6	40376	1259.9	3.12

Table 2: Intra-day and inter-day precision for linoleic acid, α-linolenic acid and DHA in the reference sample. Areas are expressed in counts

		Day 1	Day 2	Day 3	Interday precision
Linoleic acid	Mean Area	297771	314126	311586	
	SD	14976	4181	19571	
	RSD%	5.03	1.33	6.28	
					14431 (4.69%)
α-linolenic acid	Mean Area	45105	43374	45832	
	SD	3358	4018	2047	
	RSD%	7.45	9.26	4.47	
					3246 (7.25%)
DHA	Mean Area	4923	5125	5234	
	SD	290	347	180	
	RSD%	5.89	6.78	3.44	
					281 (5.52%)

Table 3: Estimated regression parameters for the three analytes in the three different calibration studies. R^2: coefficient of determination; SE: standard error for regression

	Intercept	Slope	R^2	SE
External calibration				
Linoleic acid	-7004	17430	0.999	4495.0
α- linolenic acid	-2483	14203	0.998	2350.2
DHA	-5206	7915	0.997	1722.9
Standard Addition calibration				
Linoleic acid	314578	17987	0.999	3370.9
α-linolenic acid	47082	14428	0.999	2896.1
DHA	5204	8850	0.995	2568.5
Youden calibration				
Linoleic acid	-3961	31508371	0.999	8253.3
α-linolenic acid	-1892	4850189	0.998	6030.5
DHA	-3861	933963	0.999	656.71

Table 4. Comparison of analyte concentrations in the reference sample obtained by different calibrations

	AC mg/g	YC mg/g	EC mg/g
Linoleic acid	5.36	5.43	5.41
α-linolenic acid	1.03	0.997	0.918
DHA	0.353	0.354	0.332

Table 5: Essential fatty acid concentrations in different milk samples

	Linoleic acid mg/L	α-linolenic acid mg/L	DHA mg/L
Untreated goat's milk	832 ± 88	79.8 ± 12	8.4 ± 1.2
Commercial goat's milk (UHT treatment)	1417 ± 150	96.6 ± 15	9.2 ± 1.4
Untreated cow's milk	853 ± 90	220 ± 35	N.D.
Commercial cow's milk (UHT treatment)	810 ± 86	194 ± 31	N.D.
Pasteurized commercial cow's milk	854 ± 90	224 ± 36	N.D.
Commercial milk from cows fed with n-3 rich food	1184 ± 126	346 ± 55	41.6 ± 6.1
Human milk	2394 ± 254	198 ± 32	79.8 ± 11.7