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Comparison between third derivative spectrophotometric method and HPLC-DAD method in detection of malondialdehyde in infant formulae, human and cow milks

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ABSTRACT

In the present work, malondialdehyde (MDA) levels in infant formulae (IF), human and cow milks were monitored using the thiobarbituric acid method (TBA-test) with third derivative spectrophotometry and the derivatization with 2,4-dinitrophenylhydrazine followed by HPLC-DAD analysis. We compared these two detection methods applied to different clean-up procedures in order to evaluate their applicability and improve the recovery yields and the detection limit. Different commercial types of infant formulae, cow and human milks were analysed. The obtained results showed high reproducibility and precision for both analytical methods and evidenced the importance of the correct clean-up procedure. Some of the applied clean-up procedures allowed obtaining quantitative recovery yields and better results, with a good overlapping between the two tested analytical methods. Infant milk formulae showed MDA levels of 380-410 ng g^{-1}, while in human milk 230-340 ng $g^{-}1$ levels were found. The formula with hydrolyzed proteic fraction (HA), the long chain polyunsaturated fatty acids (LC-PUFA) added formula and two cow milk samples were also analysed, permitting to demonstrate the extensibility and the effectiveness of the adopted methods.

Keywords: Malondialdehyde, infant formulae, TBA-test, third derivative spectrophotometry, HPLC-DAD derivative method.

INTRODUCTION

Malondialdehyde (MDA) is one of the major secondary oxidation products formed from the breakdown of peroxides that are usually indicated as primary oxidation products. MDA is a low molecular weight, secondary product of the hydroperoxides fragmentation, a marker of the beginning of the lipid autoxidation process [1] and also a dangerous, mutagenic and genotoxic molecule [2-4]. Because of its electrophilic nature, it is very reactive towards amino groups of proteins and neurotransmitters, nucleic acids and thiolic groups [5-7] and it is able to play an important toxicological role. Besides its toxicity, MDA detection in food and tissues is relevant because is one of the most useful and simple approaches to estimate the extension of the oxidative process [8-10].

Autoxidation represents a limit for all fat/oil containing food. The lipid deterioration process leads to loss of nutrients, sometimes specifically added to improve the nutritional value of the food, as in the case of polyunsaturated fatty acids in infant formulae [11]. Furthermore the formation of off-flavours makes food very unpleasant, with the generation of very complex mixtures of radicalic, toxic and dangerous compounds involved in many human deseases [12].

For this reason it is of great importance to prevent food oxidation both using all the manufacturing procedures that limit the contact with oxygen and inhibit oxidation during storage with synthetic or natural antioxidants added during the food preparation [13].

In this context, it appears of great interest to have analytical methods that permit to evaluate the extension of the autoxidation process [8,14]. According to us, among the different available methods, the most important ones are based on MDA detection, because it is one of the first molecules to be formed, thus representing the initial step of autoxidation and for this reason MDA is often used as an index of the beginning of the whole process. In particular way we consider it of paramount importance in food devoted to early childhood.

Precedent studies of the oxidative processes in infant milk formulae are reported in the literature [15,16]. The interest on this topic is due to several reasons. Infant formulae have a high lipid component and represent the only food for non breastfed babies, without any possible alternative. Furthermore newborns are more exposed to toxicological risks than other people [17]. Finally, European law [18] completely lack of indications about the limits of accepted autoxidation in such delicate dietetic foods.

Infant formulae are prepared from cow milk adapting it to the requirements of newborns, so its composition is deeply modified. Many components, for example polyunsaturated fatty acids and long chain polyunsaturated fatty acids (LC-PUFA), are added to make it as similar as possible to human milk. The enrichment in unsaturated lipids, although considered essential for the neuronal development of babies, especially prematures [19,20], may increase significantly the possibility of autoxidation of these products. Human milk is produced and immediately and directly consumed, without any contact with air and heat. On the contrary infant milk formulae are produced with processes requiring several heating steps, packaged and stored till two years. Heating steps and storage conditions could begin the deterioration of the PUFA-rich lipid phase of the infant formulae, with consequent loss of food quality and formation of potentially toxic substances.

As previously said, MDA is a perfect marker for the beginning of the autoxidation process. Moreover MDA easily forms coloured and easy to detect products for reaction with other molecules. The well known TBA-test, based on the spectrophotometric quantification of the pink pigment formed from the condensation of MDA with two molecules of 2-thiobarbituric acid, is one of the most used methods to detect malondialdehyde in food and biological samples [21,22].

However this rapid and simple method has been often subjected to criticism by several authors that consider the results overestimated because not absolutely specific for MDA [23,24].

To evaluate the extent of this overestimation and the limit of its applicability, in the present work a comparative evaluation was made between the third derivative spectrophotometric procedure (thiobarbituric acid method, TBA-test) and the derivatization with 2,4-dinitrophenylhydrazine followed by HPLC-DAD analysis [23-26]. Different commercial types of infant milk formulae were analysed together with bovine and human milks. Different clean-up steps, extraction procedures and analytical methods were applied. This investigation allowed verifying the reproducibility and accuracy of the procedures. Furthermore the contents of malondialdehyde in cow milk, characterised by a very low unsaturated lipid fraction, and in human milk, the golden standard for feeding of newborns, were compared with the levels found in infant formulae.

EXPERIMENTAL SECTION

2.1. Chemicals and Samples

Trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxyl toluene (BHT), *1,1,3,3*-tetrametoxypropane (TMP), 2,4-dinitrophenylhydrazine (DNPH) and ammonium acetate were purchased from Sigma–Aldrich (Deisenhofen, Germany), bovine trypsin from Roche Holding LTD (Basel, Switzerland), bi-distilled water, acetonitrile and *n*-hexane for HPLC from Carlo Erba (Milan, Italy). All reagents and solvents were of analytical or HPLC grade.

The infant milk formulae and the bovine milk samples were commercially available on the market. The fresh human milk was kindly given as a gift. All cow and human milk samples were freeze-dried and stored frozen until the analysis.

2.2. Instrumentation

A UV spectrophotometer Lambda 40 (Perkin Elmer, Milan, Italy), with 1 cm absorption cell and a scanning speed of 120 nm min^{-1}, was used for all measurements.

A freeze-drier LIO5P (Cinquepascal, Milan, Italy) was used for cow and human milk samples.

HPLC apparatus (Perkin Elmer, Milan, Italy) was made up from a Series 200 pump and a 235C diode array detector, equipped with a Lichrospher 100 (Agilent Technologies, Geneva, Switzerland) C18 column (250 x 4,6 mm ID,

5µm) was employed for HPLC analyses; data acquisition and processing were carried out with a Perkin Elmer Totalchrom software.

2.3. Preparation of malondialdehyde standard solutions
Stock standard solutions of MDA were prepared by acid hydrolysis of TMP. Solutions of TMP (70 mg) in 0.1 N HCl (10 ml) were stirred for 5 min at 100°C and then quickly cool down to room temperature. Working solutions of MDA were prepared by diluting aliquots (1 ml) of the stock solutions with water. These working solutions were used for the calibration curves and the recovery experiments.

2.4. Spectrophotometric analysis
1 ml of the MDA working solution, prepared as previously described, was mixed with 1.5 ml of 5% aqueous TCA and 1.5 ml of 0.8% aqueous TBA. The solutions were incubated for 30 min at 70°C in a screw capped bottle and then analysed by third derivative spectrophotometry in the range 580-500 nm, at a scan speed of 120 nm min^{-1}. Reaction mixtures no containing MDA were used as blank.

The calibration curve, obtained by plotting the value of peak height at 526 nm *vs* MDA concentration in ng g^{-1}, had correlation coefficient $R^2 = 0.9997$; it was expressed by: $y = 9\ 10^{-6} x + 1.0\ 10^{-5}$ and was obtained in the range 100-2000 ng g^{-1}. The detection limit was 25 ng g^{-1} and quantisation limit was 78 ng g^{-1}.

Each recovery experiment was performed in triplicate, by two different operators, spiking with different amounts of MDA.

2.5. HPLC-DAD analysis
1 ml of the work solutions was mixed with 1 ml of bi-distilled water at 40°C, 0.7 ml of 5% aqueous TCA and 0.7 ml of 0.8 % BHT in *n*-hexane. The obtained solution was processed as for the spectrophotometric analysis.

Two calibration curves were prepared. Calibration curves were obtained by plotting the value of peak area at 307 nm *vs* MDA concentration in ng and were obtained in the range 100-2000 ng g^{-1}. The first, with *n*-hexane as extracting solvent, had a correlation coefficient $R^2 = 0.9827$ and was expressed by: y = 3,5156x - 1,696; the detection limit was 30 ng g^{-1}. The second, with diethyl ether as extracting solvent, had a correlation coefficient $R^2 = 0.9992$, was expressed by y = 4,24x + 10,193 and was obtained in the same range. In this case the detection limit was 5 ng g^{-1}.

Identification was based on the retention time of MDA-DNPH adduct. Each recovery experiment was performed in triplicate by two different operators, spiking with different amounts.

All the recovery yields were calculated as reported in the literature [22].

2.6. Sample preparation
Preliminary hydrolysis experiments, when necessary, and sample preparation were carried out as previously described [16].

In the centrifugation experiments, 2.00 g of sample, treated as described before, were centrifuged for 45' at 800 rpm. The top hexane layer was discarded, the cloudy mixture was heated for 20' at 70°C and then centrifuged again in the same conditions. The aqueous layer, containing insoluble particles, was filtered and the residue was washed with water; the filtered solution and the washing water were collected up to a final volume of 25 ml.

In the ultracentrifugation experiments 1.00 g of sample was weighed (in a ultracentrifuge screw capped Teflon tube) and 5 % aqueous TCA (4 ml) and 0.8 % BHT in n-hexane (2.5 ml) were added. The mixture was manually shaken and then ultracentrifuged for 15' at 8000 rpm (T = 10°C). The top hexane layer was discarded, the mixture was heated for 20 min at 70°C and then ultracentrifuged again in the same conditions. The aqueous layer was filtered and the residue was washed with water; the filtered solution and the washing water were collected up to a final volume of 10 ml.

1 ml of the solutions obtained after the centrifugation or ultracentrifugation steps, was mixed with 0.8% aqueous TBA (1.5 ml) and 5% aqueous TCA (1.5 ml) and incubated for 30 min at 70°C in a screw capped bottle. At the end the solutions were analysed by third derivative spectrophotometry against a blank reaction mixture [10].

The clean-up procedures were simplified for human milk samples. In this case the heating step was not required and only the centrifugation or ultracentrifugation step was performed.

For HPLC-DAD analysis, a 0.1 g sample was weighed in an ultracentrifuge screw capped Teflon tube and 1 ml of bi-distilled water at 40°C, 0.7 ml of 5% aqueous TCA and 0.7 ml of 0.8 % BHT in *n*-hexane were added. The mixture was manually shaken and then ultracentrifuged 15' at 8000 rpm. The top hexane layer and the proteic phase were discarded, the aqueous solution was added with 0.1 ml of 1 mM DNPH in HCl 2N, incubated for 60 min at room temperature and then extracted four times with 1 ml of hexane or diethyl ether. After evaporation, the dry residue was dissolved with 0.1 ml acetonitrile/water 50/50 v/v. Analyses were carried out injecting 20 µl of sample in isocratic mode, using as eluant 50 mM ammonium acetate/acetonitrile (55/45, v/v) at a flow-rate of 1ml min^{-1} and monitoring at 307 nm.

Each experiment was replicated four times by two different operators: rsd % were calculated for a single repeated experiment and for different procedures applied to the same sample.

RESULTS AND DISCUSSION

Four infant milk formulae (samples ***1,1a,2,3***), two "high quality" cow milks (***4,4a***) and two human milk samples (***5,6***), were submitted to different clean-up procedures and analysed for their MDA content by two analytical methods: the conventional spectrophotometric procedure (thiobarbituric acid method, TBA-test) applied with third derivative differentiation and the derivatization with 2,4-dinitrophenylhydrazine followed by HPLC-DAD analysis. The chosen formulae were representative of three different classes of products: sample ***1*** and ***1a*** were standard infant formulae, sample ***2*** was a HA-formula having the proteic fraction partially hydrolyzed; sample ***3*** was a formula enriched with LC-PUFA, besides vegetable oils that are always used in the formulations. The two cow milk samples (***4*** and ***4a***) were "high quality" products with a lipid content ≥3.5%, according to the Italian law. The last two samples were of human milk: sample ***5*** derived from the 2nd lactation week (transition milk), sample ***6*** derived from the 12th and the 13th lactation weeks (mature milk). The lipid content of all the analysed samples was comparable, at least in weight.

The samples were submitted to different clean-up procedures before the determination of MDA, with the aim of obtaining better recovery yields. Recovery experiments were performed at least in triplicate on each sample in all the different applied procedures. MDA content of all the samples was determined before and after the spiking with different levels of the analyte. Results obtained in the recovery experiments performed on sample ***1*** are reported in **Table 1**.

Table 1. Recovery experiments on sample *1*

WORK-UP	added MDA (ng g^{-1})	found MDA – initial MDA (ng g^{-1})	RECOVERY* %
CENTRIFUGATION	1719	1202 – 219 = 983	57
	1724	1201 – 219 = 982	57
	1243	928 – 219 = 709	57
	1233	959 – 219 = 740	60
	688	597 – 219 = 378	55
	683	588 – 219 = 369	54
	281	373 – 219 = 155	55
	279	373 – 219 = 156	56
ULTRACENTRIFUGATION	1713	1477 – 244 = 1233	72
	1718	1481 – 244 = 1237	72
	1456	1251 – 244 = 1007	69
	1460	1257 – 244 = 1013	69
	1328	1079 – 244 = 835	63
	1309	979 – 244 = 735	56
	675	662 – 244 = 418	62
	684	661 – 244 = 417	61

**Recovery yields % are calculated as: (found ng/g MDA) – (initial ng/g MDA) /added ng/g MDA*

As shown, different levels of spiking were tested and all the resulting data are consistent with a very high reproducibility. When MDA was spectrophotometrically detected after a simple centrifugation step, a recovery yield of about 57% was achieved. The recovery yield significantly increased from 57 to 66% when an ultracentrifugation step was introduced. However, the comparison of these results, taking into account the recovery, showed high reproducibility not only for the recovery yields inside the same set of experiments, but also for MDA level in the sample.

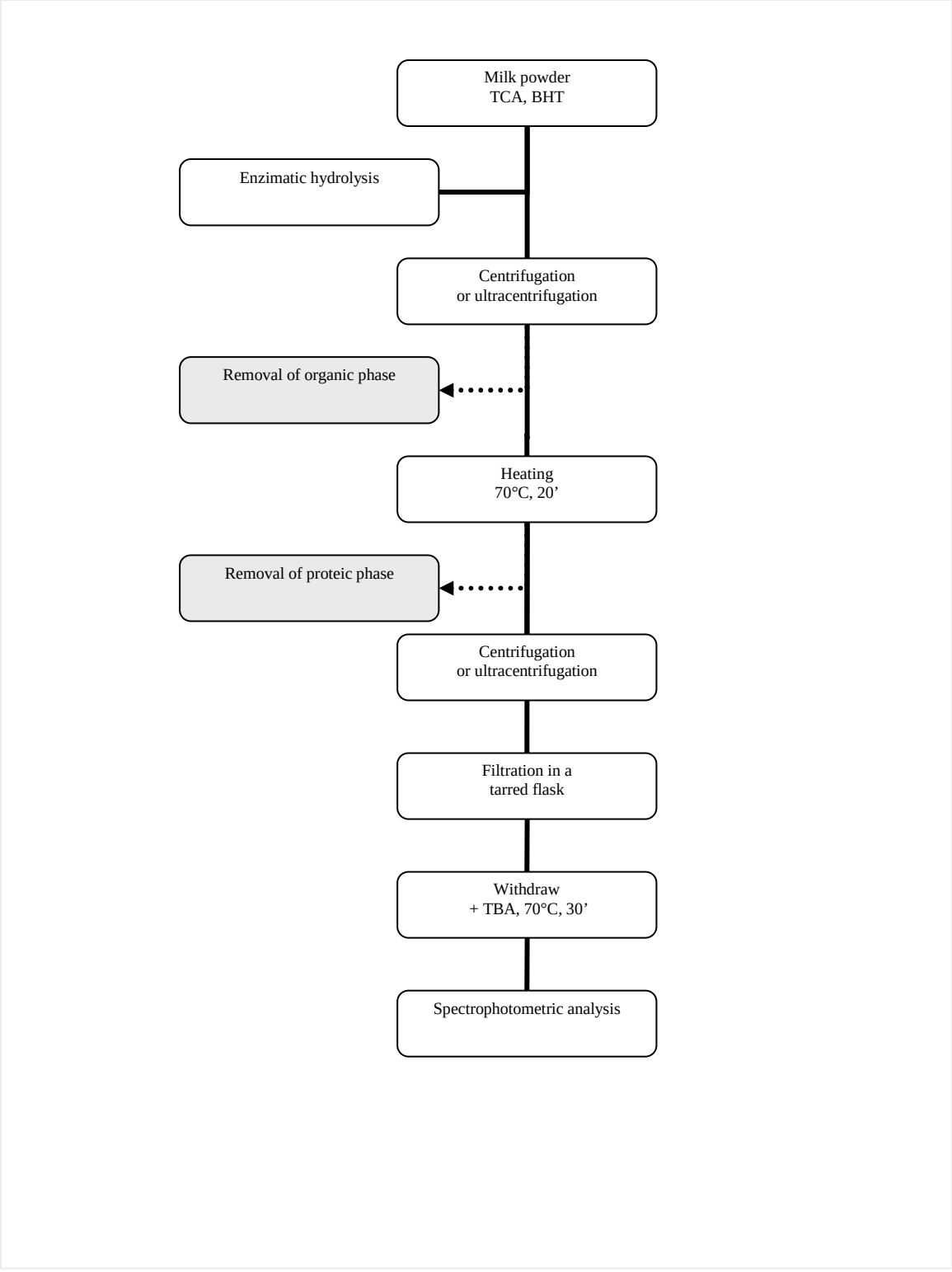

Figure 1. Infant milk formulas and cow milk samples clean-up for spectrophotometric analysis

Recovery experiments carried out on each sample showed highly reproducible results; the recovery yields (%) reported in **Table 2,3,4** take into account the whole set of done experiments that were used to estimate the real content of MDA in the analysed milks. MDA values, corrected on the basis of the previously calculated recovery yields, are also reported. As an example, in the case of sample ***1***, the base value of 219 ng g^{-1} (**Table 1**) becomes 384 ng g^{-1} taking into account the recovery of 57% (see **Table 2**, centrifugation), while the base value of 244 ng g^{-1} (**Table 1**) becomes 370 ng g^{-1} considering the mean recovery of 66% obtained with the ultracentrifugation of the sample (**Table 2**).

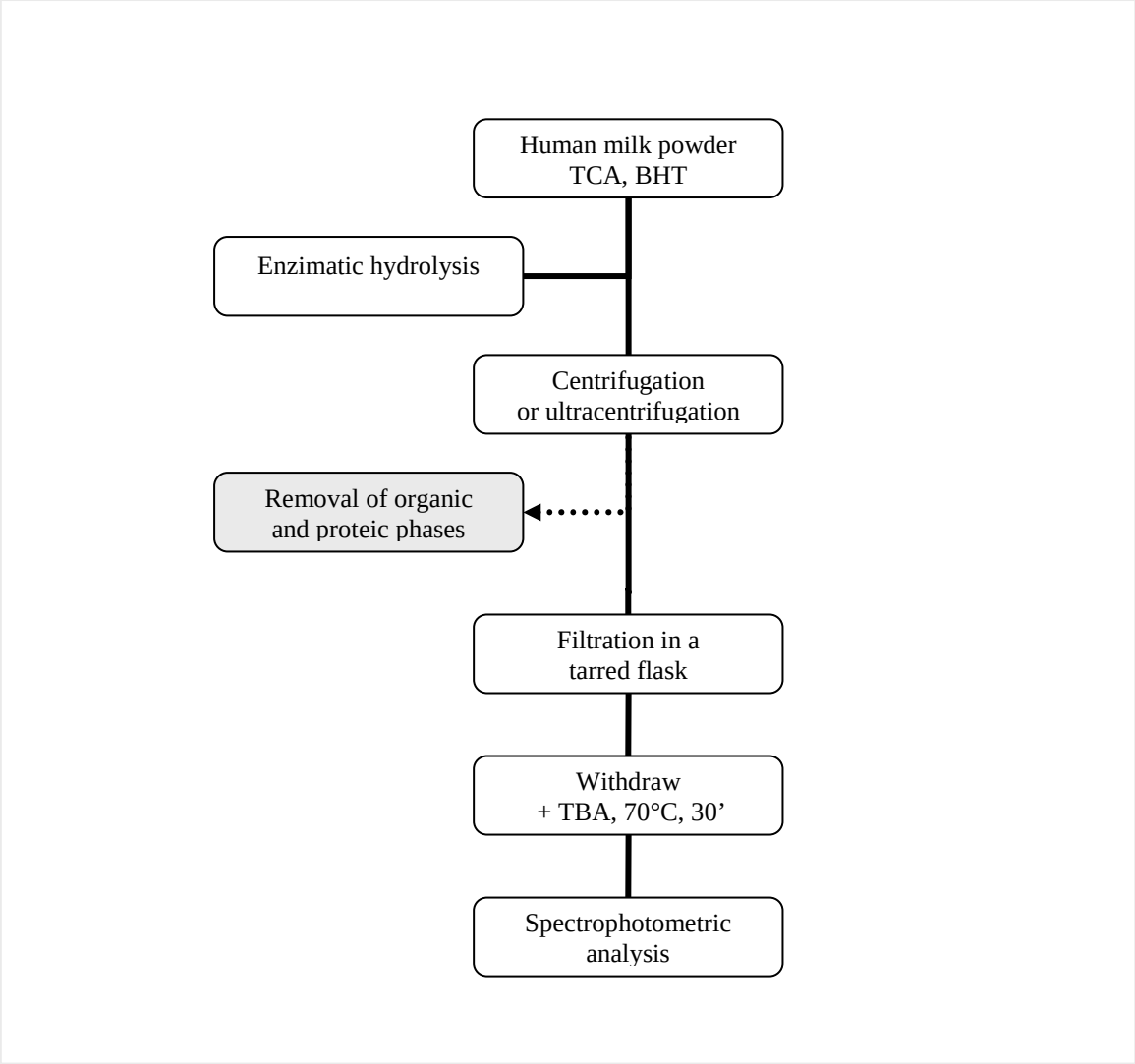

Figure 2. Human milk samples clean-up for spectrophotometric analysis

Table 2. MDA levels found with centrifugation or ultracentrifugation by third derivative spectrophotometric analysis

SAMPLE	MDA* (ng g^{-1})) ± SD CENTRIFUGATION rsd%		RECOVERY %	MDA* (ng g^{-1}) ± SD ULTRACENTRIFUGATION rsd%		RECOVERY %
1	384 ± 2.0	0.5	57	370 ± 2.6	0.7	66
1a				429 ± 12.0	2.8	83
2	1495 ± 10.5	0.7	60	1418 ± 25.5	1.8	63
3	878 ± 0.9	0.1	63	550 ± 2.8	0.5	82
4	198 ± 6.9	3.5	55	185 ± 8.7	4.7	93
4a				200 ± 3.8	1.9	72
5	227 ± 3.4	1.5	52	239 ± 3.3	1.4	62
6	323 ± 13.9	4.3	47	340 ± 1.7	0.5	63

**MDA levels are corrected taking into account the recovery %.*

As shown in **Figure 1** (infant formulae and cow milk) and **Figure 2** (human milk), in the first applied work-up, two centrifugation steps were combined with a third derivative spectrophotometric analysis, obtaining recovery yields quite low (47-60%, mean of 56%). When two ultracentrifugation steps were employed, the recovery yields rose of 10-20% (average recovery yields of 73%).

With the aim to further improve the recovery yields, hydrolysis experiments of the proteic fraction were performed (**Table 3**). In fact, proteic coagulation represents a constant problem in the clean-up of milk, both in the separation and in the filtration step. If samples were preliminarily submitted to an enzymatic hydrolysis with bovine trypsin [16], an overall simplification of the procedure was obtained. Sample ***2***, being a HA formula specially designed for babies allergic to the proteic fraction and already hydrolysed during manufacturing, was not submitted to hydrolysis.

Table 3. MDA levels found with centrifugation or ultracentrifugation after hydrolysis by third derivative spectrophotometric analysis

SAMPLE	MDA* (ng g^{-1}) ± SD HYDROLYSIS CENTRIFUGATION rsd%		RECOVERY %	MDA* (ng g^{-1}) ± SD HYDROLYSIS ULTRACENTRIFUGATION rsd%		RECOVERY %
1	386 ± 4.2	1.1	71			
1a				425 ± 4.2	1.0	87
3	791 ± 3.2	0.4	75			
4	170 ± 0.3	0.2	67			
4a				182 ± 1.6	0.9	80
5	250 ± 1.7	0.7	84	223 ± 3.1	1.4	88
6	355 ± 1.4	0.4	80	337 ± 5.0	1.5	86

**MDA levels are corrected taking into account the recovery %.*

When proteic hydrolysis is combined with centrifugation steps, a significant increase of the recovery yields took place. On the contrary when hydrolysis was combined to ultracentrifugation, smaller increments were obtained, probably because the ultracentrifugation assured the same phase separation as the hydrolysis and centrifugation combined together. Different results were obtained with human milk (samples ***5,6***). In this case, hydrolysis increased much more the recovery yields from 47-63% to 80-88%, both with the centrifugation and ultracentrifugation steps. These results could be explained taking into account the different proteic composition of human milk. The limited presence of caseins in human milk makes the centrifugation step sufficient for the separation, and limits the improvement obtained introducing the ultracentrifugation step.

The cleaned-up milk samples were submitted to a third derivative spectrophotometric evaluation of MDA levels at 527 nm [10]. Comparable levels of MDA were found in the samples ***1*** and ***1a*** (standard formulae, 370-430 ng MDA g^{-1}) as well as in the samples ***4*** and ***4a*** (cow milk, 170-200 ng MDA g^{-1}). The relative standard deviations were always good, with a maximum of 4.7% for sample ***4*** when cleaned-up with ultracentrifugation (**Table 2**).

Excellent rsd% (respectively of 5.2 and 3.8%) were obtained for samples ***5***,***6*** tested with all the four procedures. Overall the results showed a good agreement between the found levels of MDA for all the samples, except sample ***3*** probably for problems due to the clean-up procedure and not to the derivatization with the thiobarbituric acid or to interference in the spectrophotometric analysis.

Table 4. MDA levels found with HPLC-DAD analysis after extraction with *n*-hexane or diethyl ether

SAMPLE	MDA* (ng g^{-1}) ± SD *n*-HEXANE HPLC-DAD ANALYSIS λ=307nm rsd%		RECOVERY %	MDA* (ng g^{-1}) ± SD DIETHYL ETHER HPLC-DAD ANALYSIS λ=307nm rsd%		RECOVERY %
1	363 ± 18.9	5.2	81			
1a				371 ± 5.2	1.4	100
2	1417 ± 79.3	5.6	91			
3	563 ± 25.8	4.6	80			
4	175 ± 14.0	8.0	71			
4a				186 ± 10.5	5.6	96
5	239 ± 2.0	0.8	76	221 ± 1.4	0.6	95
6	340 ± 1.0	0.3	69	321 ± 2.0	0.6	98

**MDA levels are corrected taking into account the recovery %.*

The same milk samples were submitted to HPLC-DAD determination of MDA, after derivatization of this last with 2,4-dinitrophenylhydrazine (DNPH, **Figure 3**).

The derivatization step carried out at room temperature together with the chromatographic separation [27], allowed to by-pass the problem of potential interferents of TBA-test raised by some authors [23,28]. Samples were cleaned-up with only one step of ultracentrifugation. Even in these cases, many recovery experiments were performed and good reproducibility was achieved. Recovery yields were always higher than 70%, thus giving better results with respect to the previously applied clean-up procedure.

The results of the chromatographic analysis, carried out after hexane or diethyl ether extraction, are reported in **Table 4**.

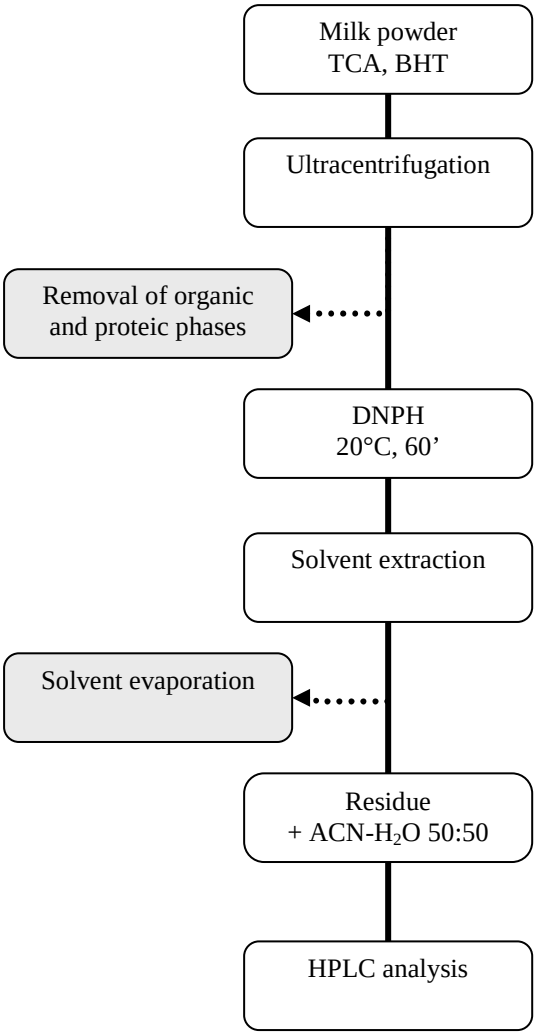

Figure 3. Clean-up for chromatographic analysis

The extraction with diethyl ether allowed reaching higher recoveries respect to those obtained with hexane. Even in this case, however, the different recovery yields do not represent a problem for the data reproducibility. HPLC analysis allowed lowering the limit of quantitation from 80 ng g-1 of the spectrophotometric method, to 30 ng g^{-1} and 5 ng g^{-1}, respectively for extraction with hexane and diethyl ether.

HPLC analysis gave better results in term of recovery yields and quantification limits; it was performed after a simplified and faster clean-up, even if a longer time for the analytical procedure was required. Moreover, the comparison between MDA levels found with TBA-test and DNPH derivatisation evidences that the results are in a good agreement to each other, so demonstrating a good accuracy for both methods, except in the case of sample **3**.
All the collected data, reported in **Table 5**, well evidence the accuracy and the reproducibility of the adopted procedures.

Table 5. Comparison among all obtained results

	1	***1a***	***2***	***3***	***4***	***4a***	***5***	***6***	***average recovery yield****
centrifugation + spectrophotometric analysis	384		1495	878	198		227	323	
rec%	57		60	63	55		52	47	56
ultracentrifugation + spectrophotometric analysis	370	429	1418	550	185	200	239	340	
rec%	66	83	63	82	93	72	62	63	73
hydrolysis + centrifugation + spectroph. analysis	386			791	170	182	250	355	
rec%	71			75	67	80	84	80	76
hydrolysis + ultracentrifugation + spectroph. analysis		425					223	337	
rec%		87					88	86	87
hexane extraction + HPLC-DAD analysis	363		1417	563	175		238	343	
rec%	81		91	80	71		76	69	78
diethyl ether extraction + HPLC-DAD analysis		371				186	221	322	
rec%		100				96	95	98	97
average ppb MDA	376	408	1443	----	182	189	233	337	
*rsd%***	2.9	7.9	3.1		6.8	5.0	4.8	3.7	

****for each adopted procedure; *****for each analysed sample***

The average recovery yield value reported for all the methods shows an increase from 56% to 97%, passing from centrifugation steps combined with spectrophotometric analysis to ultracentrifugation steps combined with diethyl ether extraction and chromatographic analysis.

Moreover, for all the samples, an average level of MDA content is reported. The comparison among these data demonstrates that, independently of the applied procedure and the analytical method, an identical MDA value is found with very high reproducibility (rsd% 2.9-7.9%), showing the validity of both the analytical procedures.

The only significant exception is represented by the LC-PUFA added formula, sample ***3***, in which not comparable levels of MDA were found when different procedures were adopted. In this case, the clean-up seems to play a more relevant role respect to the other milk samples. It is likely that this kind of formula enriched with LC-PUFA was deeply modified during the two centrifugation steps of 45' at not controlled temperature even in the presence of the antioxidant BHT. When these steps were substituted with ultracentrifugation (two steps of 15' at controlled temperature of 10°C), the found level of MDA decreased to 550 ng g^{-1} (Table 5, ultracentrifugation + spectrophotometric analysis). This value agrees with the level of 563 ng g^{-1} found with the ultracentrifugation clean-up procedure followed by the HPLC-DAD analysis, where the heating at 70°C is not required.

Analogous results should be expected for the human milk samples where the LC-PUFA contents are comparable to those of infant formula ***3***. Surprisingly in this case the same MDA levels were found adopting all the six procedures, so demonstrating the higher stability of human milk compared to the formulated milk. Maybe natural substance present in human milk could play a protective role towards autoxidation process. In any case much more samples should be analysed in order to confirm results and hypothesis.

Overall it is evident that, if the clean-up of milk is correctly applied, the analytical procedure does not affect the final result so that the same MDA levels are gained, with the only exception of LC-PUFA enriched infant formulae.

As a consequence, if an accurate clean-up is applied, the spectrophotometric method could be safely used for routine analysis of milk samples when HPLC analysis is not available.

CONCLUSION

Derivatization with DNPH followed by HPLC-DAD analysis, respect to spectrophotometric with third derivative analysis, is an excellent procedure for MDA traces detection in milk, because it does not need heating treatment and allows obtaining a lower limit of quantitation and a better recovery yields, especially when diethyl ether is used as extracting solvent.

However, the results obtained in the present work show that also the quick and versatile spectrophotometric method can be used for MDA detection in milk with a good accuracy.

In particular, in order to get accurate results with the TBA-test, great attention must be paid to the nature of the analysed samples, choosing the appropriate clean-up procedure and avoiding long treatment at uncontrolled temperature, especially when the organic phase is still present and milk is reach in polyunsaturated lipids. Indeed, the reported data suggested that the applied clean-up influences the validity of the spectrophotometric method for milk analysis, with the best pre-treatment represented by the fast ultracentrifugation step at low temperature. Obviously, this method could be applied to other food matrices only after an accurate study of the appropriate clean-up, that depends on the complexity of the analysed matrix.

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