JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Malondialdehyde Contents in Infant Milk Formulas

STEFANIA CESA*

Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università degli Studi di Roma "La Sapienza", P. le Aldo Moro, 5, 00185 Rome, Italy

Malondialdehyde (MDA) levels in infant milk formulas have been monitored by using an aqueous acid extraction method combined with the thiobarbituric acid method (TBA-test). Vegetable oils, with a remarkable content of polyunsaturated fatty acids (PUFA) are used to enrich the infant milk formulas. As PUFA are more susceptible to autoxidation, it becomes of great interest to have information about the safety and preservation of these products. We monitored MDA content in twenty of the most popular infant milk formulas and in some commercial cow milk samples and compared the obtained data. Levels of MDA ranged between 200 and 1200 ppb: all values but one were higher, up to five times, than those found in cow milk samples. To evaluate the accuracy of the data obtained from the TBA-test, some samples were also analyzed with an HPLC derivative method: preliminary results show a good agreement between the two analytical techniques.

KEYWORDS: Infant milk formulas; lipid peroxidation; malondialdehyde; thiobarbituric acid method; derivative spectrophotometry

INTRODUCTION

Autoxidation represents a limit for all fat/oil-containing foods. The deterioration of lipid-rich foods is correlated with the initial formation of peroxides, the unstable and reactive primary products of lipid oxidation, and with their breakdown that leads to a mixture of secondary products (mainly aldehydes and ketones) that impart off-flavors and are in correlation with loss of nutrients.

This problem acquires a particular interest for infant milk formulas for two main reasons: (i) these are the only food for bottle-fed newborns, (ii) the recent tendency to add vegetable oils (with a high content of polyunsaturated fatty acids, PUFA) to all the infant milk powders and long chain (C_{20}, C_{22}) polyunsaturated fatty acids (LC-PUFA) to formulas devoted to the premature babies increases the risk of autoxidation.

The most important secondary product of autoxidation is the malondialdehyde (MDA) that is usually used as an indicator of the lipid peroxidation process, both for the early appearance as peroxidation occurs and for the sensitivity of the analytical method.

Although many studies about the importance of PUFA and LC-PUFA in the correct development of neuronal cells of newborns (*1*−*3*) and about the determination of MDA levels in biological samples (*4*, *5*) have been carried out, only a few studies are related to the stability to oxidation of infant milk powders (*6*−*9*).

Granot et al. (*4*) report contradictory results about the protective role of PUFA and LC-PUFA with respect to the oxidative stress: their own results are difficult to explain, as breast fed infants show an increased peroxidative injury when compared with formula fed infants. So they conclude that "it is intriguing to speculate as to the physiological role of oxidants and PUFA in early life".

In any case, PUFA and LC-PUFA show a natural tendency to undergo autoxidation and the same oxidation products could give serious undesired biological effects as in the case of MDA; MDA is in fact known to be a mutagen species, a suspected carcinogen (*10*), and it can react with DNA to generate mutagenic adducts.

Because of the tender age (immaturity of enzymatic systems), the low weight of the consumers, and the evidence that they have no alternative, we considered of particular importance the detection of the oxidative level of some marketed infant milk powders. For this purpose, we chose the TBA-test (*6*), the simplest and fastest available method. The samples were extracted with aqueous trichloroacetic acid (*6*) and MDA was quantified on the basis of the third derivative absorption spectrum of the pink adduct between MDA and TBA.

Besides MDA, other products of lipid oxidation are considered of primary importance for their toxicological implications (*11*); some of these could also interfere with the TBA-test, producing an overestimation of MDA levels. For this reason, we compared the results obtained from infant milk formulas with those obtained from some cow milks selected as references.

Moreover, some samples were also analyzed with an HPLC derivative method according to Fenaille et al. (*9*) to evaluate the overestimation deriving from the TBA-test.

MATERIALS AND METHODS

Reagents and Samples. All reagents and solvents were of analytical or HPLC grade.

* To whom correspondence should be addressed. E-mail: stefania.cesa@uniromaI.it.

10.1021/jf034446l CCC: $27.50
Published on Web 03/04/2004

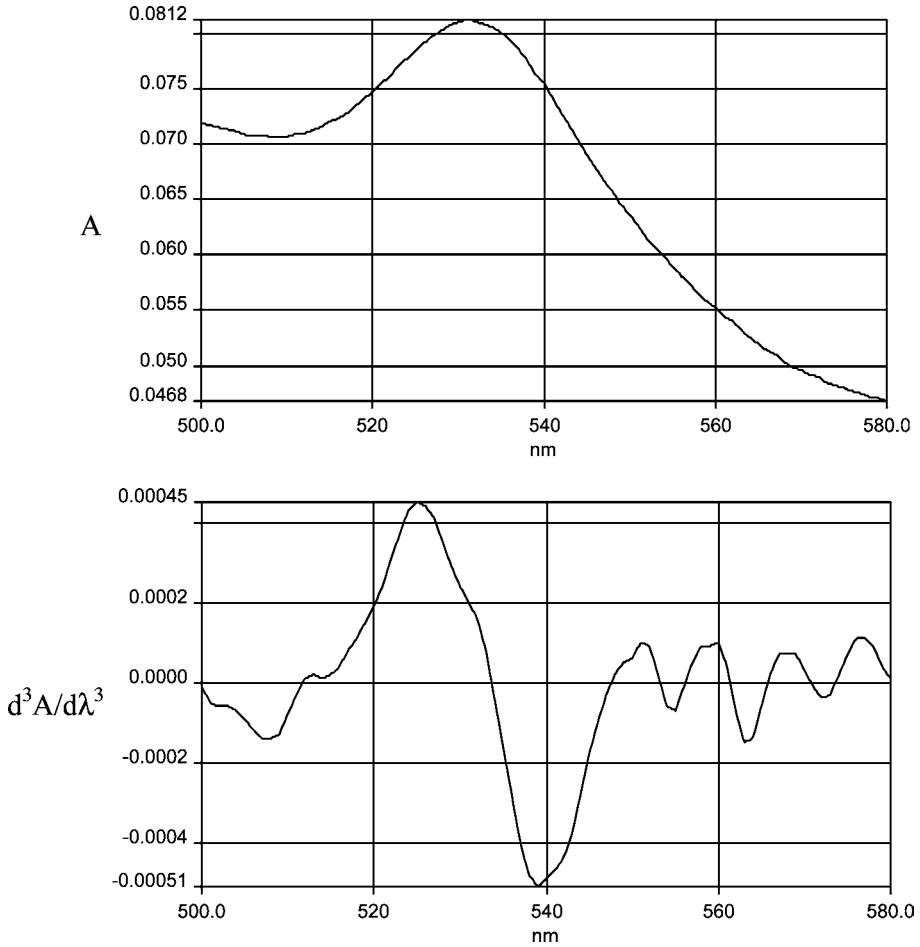

Figure 1. Normal and third derivative spectra of a MDA-TBA reaction mixture.

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

The 20 tested infant milk formulas and the cow milks were commercially available on the market.

All samples were tested before the expiring date of the preparation, and the containers, kept in a dry and cool place, were opened for the first time just before the analysis.

Preparation of MDA Standard and Recovery Experiments. Solutions of TMP (70 mg) in 0.1 N aqueous HCl (10 mL), were stirred for 5 min at 100 °C and cooled at room temperature. Working MDA solutions were prepared by diluting aliquots (1 mL) of the stock solution to increasing volumes with water. These solutions were used for the calibration curves and for recovery experiments.

The calibration curve (**Figure 1**) was obtained by plotting the value of peak height at 526 nm versus MDA concentration, and the obtained straight line (with correlation coefficient $R^2 = 0.9997$) can be expressed by: $y = 2.4\ 10^{-3}x + 4.0\ 10^{-5}$, where the MDA concentration was in μmolesL^{-1}. Quantisation was carried out by third derivative spectrophotometry.

Aliquots of the solutions, prepared as mentioned above, were used for the recovery experiments: recovery yields were calculated as the ratio (ppb found in the spiked sample) − (initial ppb found in the sample)/(added ppb) (*9*).

Hydrolysis Experiments. Solutions of trypsin (25 mg) in water (2 mL) were added to 2.0 g of sample and stirred for 1 h at 37 °C in a centrifuge tube: the sample was then treated as described in the sample preparation.

Sample Preparation. A 2.0-g sample was weighted (cow milk was previously freeze-dried) in a centrifuge tube and 5% aqueous TCA (8 mL) and 0.8% BHT in hexane (5 mL) were added. The mixture was stirred 10 min at room temperature and centrifuged.

After the top hexane layer was discarded, the cloudy mixture was heated for 20 min at 70 °C and then centrifuged again. 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.

To 1 mL of this solution, 0.8% TBA in *n*-hexane (1.5 mL), and 5% aqueous TCA (1.5 mL), were added: after incubation for 30 min at 70 °C in a screw capped bottle, the solution was analyzed by third derivative spectrophotometry against a blank reaction mixture.

All experiments were carried out at least in triplicate.

HPLC Experiments. HPLC experiments were carried out according to Fenaille et al. (9). The external calibration curve, obtained in the range 100−2000 ng/g, was expressed by: $y = 3.5156x - 1.696$ (with correlation coefficient $R^2 = 0.9827$) and used for the quantisation of MDA.

Instrumentation. A Perkin-Elmer UV spectrophotometer λ40, with 1-cm absorption cell and a scanning speed of 120 nm min^{-1}, was used for all measurements.

A lyophilizer Edwards, Modulyo 4K was used for the freeze-dried cow milk samples.

A Perkin-Elmer HPLC apparatus, made up from a Series 200 pump and a Series 200 diode array detector, equipped with Higgins Analytical Kromasil 100, C18 column (250- × 4.6-mm ID, 5-μm) was employed for HPLC analyses; data acquisition and processing were carried out with Perkin-Elmer Totalchrom software.

RESULTS AND DISCUSSION

Twenty infant milk formulas (samples **1**−**20**, **Table 1**) and five cow milk (samples **1a**−**5a**, **Table 2**), used as reference, were analyzed according to the above-described procedure.

Table 1. MDA Levels Found in Infant Formula Powder

sample	MDA[a] (ppb) ± SD	*n*	RSD %	recovery %[b]
1	723 ± 14	3	2.0	41
2	1211 ± 34	3	2.8	45
3	661 ± 4	3	0.6	70
4	624 ± 66	3	10.5	40
5	1086 ± 24	3	2.2	51
6	906 ± 54	3	6.0	62
7	465 ± 20	3	4.3	60
8	198 ± 6	3	2.9	50
9	313 ± 7	3	2.2	62
10	278 ± 22	4	8.0	41
11	383 ± 52	3	13.7	52
12	375 ± 22	3	5.8	55
13	452 ± 18	3	3.9	46
14	1027 ± 46	4	4.4	52
15	1094 ± 34	3	3.1	52
16	1077 ± 51	3	4.8	40
17	799 ± 83	4	10.4	78
18	761 ± 49	3	6.4	66
19	418 ± 31	3	7.6	65
20	835 ± 26	3	3.2	80

[a] MDA levels are corrected taking into account the recovery %. [b] Recoveries % are calculated according to Fenaille et al. (*9*).

Table 2. MDA Levels Found in Freeze-Dried Cow Milk

sample	MDA[a] (ppb) ± SD	*n*	RSD %	recovery %[b]
1a	238 ± 15	3	6.4	53
2a	163 ± 12	3	7.3	52
3a	174 ± 22	4	12.5	55
4a	362 ± 23	3	6.3	42
5a	309 ± 44	3	14.3	58

[a] MDA levels are corrected taking into account the recovery %. [b] Recoveries % are calculated according to Fenaille et al. (*9*).

According to the Italian and European laws, all the infant milk formulas were prepared and stored in agreement with the good manufacturing practice (GMP) from powder demineralized whey milk, vegetable oils, powder skim milk, milk proteins, maltodextrins, lactose, minerals, and vitamins.

Nevertheless, great differences in the oxidative status of the samples were found, and these cannot simply be justified by the different expiring date or by the different compositions.

Samples **1**−**13** are standard infant formulas, all added with vegetable oils, samples **14**−**16** are formulas enriched with LC-PUFA in addition to vegetable oils and samples **17**−**20** are hypoallergenic (HA) formulas in which proteins were partially hydrolyzed.

The recovery results for all the analyzed samples are reported in **Tables 1** and **2**. We had highly reproducible results within each sample but remarkably different recovery yields among the various samples, as evidenced in **Table 3**. In this table are reported the experiments on three samples (**10**, **11**, and **17**) that showed different recovery yields (41, 52, and 78%, respectively); the chosen samples spiked with two different levels of MDA gave a high reproducibility.

On these basis and because we had few available data in the literature (*6*−*9*), we decided to repeat these experiments in triplicate on all the analyzed samples, using only one level of MDA.

The found recovery yields are scattered between 40 and 80%. Testing hypoallergenic (HA) formulas in which the proteic fraction is partially hydrolyzed, we had recoveries between 65 and 80% (samples **17**−**20**, **Table 1**), while other standard infant milk formulas gave recoveries of 40−62%, with an exception

Table 3. Recovery Experiments on Samples **10**, **11**, and **17**

sample	added MDA (ppb)	found MDA (ppb)	recovery %[a]
10	0	114	
	811	463	43
	833	447	40
	4727	2042	41
	4750	2132	39
11	0	184	
	563	471	51
	561	470	51
	5525	3229	55
	4877	2655	51
17	0	668	
	815	1305	78
	892	1354	77
	5473	4815	76
	5386	4905	79

[a] Recoveries % are calculated according to Fenaille et al. (*9*).

only for sample **3**, (70%, **Table 1**); testing cow milk, we had 52−58% of recovery, with the exception of sample **4a** (42%, **Table 2**), but if we reproduced all procedures without sample, we raised recovery to 92−95%.

The differences among the various samples should depend on the proteic composition of the analyzed milk; this is demonstrated by the higher recovery yields obtained from HA-formulas, in which a modified proteic composition makes the filtration step easier.

In fact, with respect to the data in the literature (*6*), we introduced a step (70°C, 20 min), that leads to obtain the coagulation of the whey proteins, followed by a filtration, to avoid their precipitation when the TBA-test is carried out.

Hydrolysis experiments were carried out on the sample **2** to support this theory (a new packaging was opened, 1080 ppb were found). We had 45% with the standard procedure and 76% recovery yields after hydrolysis.

Results relative to MDA contents are listed in **Tables 1** and **2**. All values are recalculated on the basis of the obtained recoveries and are completed by the standard deviation (SD), the number of replicates (*n*) and the relative standard deviation (RSD%). The data show a good reproducibility (RSD < 8% in 80% of the samples) taking into account the complex matrix and the concentration of the analyzed substance.

The MDA levels found in the infant milk formulas range approximately between 200 and 1200 ppb, while in the few data reported in the literature, ranges between 101 (*6*) and 1080 (*8*) ppb are given.

From the obtained data, three groups of samples not completely corresponding to the three different classes of infant formulas can be evidenced. The first one is characterized by values comparable with those obtained from the cow milk used as references (198−465 ppb, in samples **7**−**13**, **19** versus 163−309 ppb found in cow milk samples **1a**−**5a**). The second class shows 2-fold or 3-fold values (624−906 ppb, samples **1**, **3**, **4**, **6**, **17**, **18**, **20**), and the third one has MDA levels higher than 1000 ppb (samples **2**, **14**−**16**), up to a maximum of 1211, 5-fold the mean value (∼250 ppb) found in the cow milk samples. Although the maximum value was found in a standard formula (**2**), three of the highest values, as expected, were those from the formulas enriched with LC-PUFA (sample **14**−**16**).

All obtained data were statistically treated (*t*-test): infant formulas (sample **1**−**20**) with respect to cow milk samples (**1a**−**5a**) gave $p < 0.001$; standard infant formulas (sample **1**−**13**)

with respect to cow milk (**1a**−**5a**) gave $p < 0.001$; LC-PUFA added infant formulas (**17**−**20**) with respect to standard infant formulas (**1**−**13**) gave $p < 0.001$; HA-formulas (**14**−**16**) with respect to standard formulas (**1**−**13**) gave $p < 0.01$; and LC-PUFA added (**14**−**16**) with respect to HA-formulas (**17**−**20**) gave $p < 0.001$.

Finally, formulas analyzed near (the last two months) to the expiring date (**1**−**5**, **8**, **9**) with respect to formulas analyzed far (one year or more) from expiring date (**6**, **7, 10**−**13**) gave $p < 0.001$.

A significant difference can be seen between all the groups of samples, and it demonstrates that not only the presence of high content of vegetable oils but also the hydrolysis treatment, the addition of LC-PUFA, and the long storage time of the samples deeply influence the oxidative status of these marketed products.

Because several authors (*9*, *11*) demonstrated the risk of an overestimation for the TBA-test, two other samples were analyzed to compare the results of the adopted spectrophotometric method with those from an HPLC derivative method (*9*).

The recovery yields for the first sample (a standard formula) were 58% (spectrophotometric method) and 81% (HPLC method): the absolute values of MDA, taking in account the recoveries, were 384 ppb (RSD = 0,54%, $n = 4$) and 363 ppb (RSD = 5.2%, $n = 2$), with a difference of only 5.6%.

The recovery yields for the second sample (an HA formula) were 60 and 91%, and the absolute values of MDA were 1495 (RSD = 0.7%, $n = 4$) and 1417 (RSD = 5.6%, $n = 2$), with a difference of only 5.4%.

These are only preliminary data, and the HPLC analyses are surely possible of improvement; however, on the basis of the data in the literature and of our experience, we think that a great care must be put in the cleanup of the sample to evaluate with accuracy the goodness of the adopted analytical technique.

Particular attention should be put in the steps of the coagulation and centrifugation of proteins, in the filtration and in the washing of the precipitates for the spectrophotometric analysis, and in the extraction of the derivative with *n*-hexane, for the HPLC analysis.

Moreover, it is of absolute importance to know exactly the recovery yield for each sample with every adopted analytical technique, because significant differences exist among samples.

The obtained results, although in perfect agreement, must be considered only preliminary data. It would be of great importance to estimate with major accuracy the real risk for newborns of exposition to MDA by using other more sophisticated analytical techniques and taking into account the daily allowance of milk powder for only bottle-fed newborns, also considering that they have not alternative from the birth up to four to six months of life.

The are not many studies (*10*, *12*−*14)* on the toxicology of MDA. Draper et al. showed that its administration in a range of 0.1−10 μg/g/day to mice produces dose-dependent neoplastic changes in the liver (*14*). More recent studies demonstrated its reactivity toward DNA and the other macromolecules.

Therefore, it becomes of importance to show the risk associated with a diet completely based on only one food containing this toxic aldehyde. This problem acquires more and more importance if we consider the worldwide impact of the infant milk formulas. In fact, the tested products, although brought from the Italian market, are actually produced by multinational industries and are exported in numerous countries around the world, directed to that cluster of people mostly exposed to toxic effects.

In conclusion, it would be of great importance to have regulations that could lead the analyst toward a correct interpretation of the estimated levels of MDA and other toxic substances deriving from the autoxidation process.

LITERATURE CITED

(1) Koletzko, B.; Rodriguez-Palmero, M.; Demmelmair, H.; Fidler, N.; Jensen, R.; Sauerwald, T. Physiological aspects of human milk lipids. *Early Hum. Dev*. **2001**, *65 Suppl.*, S3−S18.

(2) Lapillonne, A.; Carlson, S. E. Polyunsaturated fatty acids and infant growth. *Lipids* **2001**, *36*, 901−911.

(3) Uauy, R.; Mena, P.; Peirano, P. Dietary polyunsaturated fatty acids for optimal neurodevelopment: recommendations for perinatal nutrition. *Prev. Nutr*. **2001**, 415−431.

(4) Granot, E.; Golan, D.; Rivkin, L.; Kohen, R. Oxidative stress in healthy breast fed versus formula fed infants. *Nutr. Res*. **1999**, 869−879.

(5) Draper, H. H.; Csallany, A. S.; Hadley, M. Urinary aldehydes as indicators of lipid peroxidation in vivo. *Free Radic. Biol. Med.* **2000**, *29*, 1071−1077.

(6) Botsoglou, N. A.; Fletouris, D. J.; Papageorgiou, G. E.; Vassilopoulos, V. N.; Mantis, A. J., Trakatellis, A. G. Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *J. Agric. Food Chem*. **1994**, *42*, 1931−1937.

(7) Giammarioli, S.; Lammardo, A. M.; Sanzini, E.; Bellomonte, G. Preliminary study on lipid oxidation in infant formulas during storage. *Riv. Sci. Aliment*. **1997**, *26*, 80−88.

(8) Angulo, A. J.; Romera, J. M.; Ramirez, M.; Gil, A. Effects of storage conditions on lipid oxidation in infant formulas based on several protein sources. *J. Am. Oil Chem. Soc*. **1998**, *75*, 1603−1607.

(9) Fenaille, F.; Mottier, P.; Turesky, R. J.; Ali, S.; Guy, P. A. Comparison of analytical techniques to quantify malondialdehyde in milk powders. *J. Chromatogr. A* **2001**, *921*, 237−245.

(10) Aubourg, S. P. Interaction of malondialdehyde with biological molecules − new trends about reactivity and significance. *Int. J. Food. Sci. Technol*. **1993**, *28*, 323−335.

(11) Kosugi, H.; Kato, T.; Kikugawa, K. Formation of yellow, orange and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid. *Anal. Biochem.* **1987**, *165*, 456−464.

(12) Dooley, P.; Zhang, M.; Korbel, G. A.; Nechev L. V.; Harris, C. M.; Stone, M. P.; Harris, T. M. NMR determination of the conformation of a trimethylene interstrand cross-link in a oligodeoxynucleotide duplex containing a 5-d(GpC) motif. *J. Am Chem. Soc.* **2003**, *125*, 62−72.

(13) d′Ischia, M.; Costantini, C.; Prota, G. Lipofuscin-like pigments by autoxidation of polyunsaturated fatty acids in the presence of amine neurotransmitters: the role of malondialdehyde. *Biochim. Biophys. Acta* **1996**, *1290*, 319−326.

(14) Draper, H. H.; McGirr, L. C.; Hadley, M. The metabolism of malondialdehyde. *Lipids* **1986**, *21*, 305−307.

Received for review April 30, 2003. Revised manuscript received January 28, 2004. Accepted January 30, 2004.

JF034446L