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# CONTENT OF MALONDIALDEHYDE (MDA) IN INFANT FORMULAE AND FOLLOW-ON FORMULAE

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The content of malondialdehyde (MDA), considered as a product of oxidation of lipids harmful to health in commercial infant formulae (IF) and follow-on formulae (FF) (N=13), human milk (N=7), cow's milk (N=7), soybean oil and rapeseed oil was studied. The MDA content was confirmed to be high, ranging from 40.50±13.77 to 89.60±15.26 $\mu$g/100 mL infant milk formulae, compared to 19.35±5.14 $\mu$g/100 mL in human milk on average. Such a great difference between MDA contents of commercially available formulae and human milk is accounted for imperfection of different technologies of manufacturing IF and FF. The major quantity of MDA is formed during the production, packing in the air and storage of IF and FF. It is suggested that the content of MDA should be labeled. The authors' suggestion is that the maximum allowable tolerance of MDA should not exceed 48.0 $\mu$g MDA/ 100 mL liquid IF and FF.

## INTRODUCTION

Oxidation of lipids is one of the reasons of quality deterioration of high-fat food products during their processing and storage. Lipid oxidation results in the formation of a number of useless or even detrimental to health substances [Esterbauer, 1993; Halliwell & Chirico, 1993]. In addition, products of lipid oxidation react with non-lipid food components, *e.g.* proteins, thus triggering further unfavourable changes, including a loss of essential amino acids [Ziemlański & Budzyńska-Topolowska, 1991].

Investigations carried out on experimental animals have demonstrated that the intake of oxidized fats leads to the incidence of multiple pathological changes, including growth inhibition, intestinal irritation, enlarged liver and kidney, hemolytic anemia and decreased concentration of vitamin E in liver and serum [Esterbauer, 1993]. Oxidized lipids induce pathological changes in mucosa of the gastrointestinal tract, inhibit the activity of a number of enzymes and disturb lipid metabolism in the organism [Ziemlański & Budzyńska-Topolowska, 1991].

One of the secondary products of lipid oxidation, detrimental to health, is malondialdehyde (MDA) formed as a result of the oxidation process of fatty acids containing at least three double bonds. Measurement of MDA content is a generally accepted method for determining the degree of fat degradation in food [Fenaille *et al.*, 2001]. It has been applied as an indicator of the degree of lipid oxidation in meat [Marcinčak *et al.*, 2003; Abdel-Kader, 1996; Sun *et al*., 2001], cured meat products [de las Heras *et al*., 2003] as well as in edible fats and oils [Kosugi *et al*., 1991].

Direct acute toxicity of MDA administered with a diet is not high. Lethal dose $LD_{50}$ for rats accounts for 632 mg/kg body weight [The Registry of Toxic Effects of Chemical Substances]. Far more detrimental, however, is continuous administration of MDA with a diet that triggers a variety of pathological changes, including neoplastic ones, in experimental animals [Esterbauer, 1993; Spalding, 1989; Siu *et al.*, 1982].

Products of lipid oxidation supplied to an organism are partly inactivated by antioxidants. A decrease in the content of antioxidants in the body results in the disturbance of the peroxidation-antioxidation balance with a tendency for oxidative reactions, which in turn leads to the occurrence of oxidative stress, *i.e.* overproduction of Reactive Oxygen Species (ROS) in the body. Oxidation stress is a pathogenic factor in a number of human diseases. Being extremely reactive compounds, ROS can damage the cell membranes or DNA and disturb the lipid metabolism of the organism [Halliwell & Chirico, 1993]. They attack, among others, polyunsaturated fatty acids (PUFA) being a constituent of cell membranes, which consequently leads to the formation of typical products of lipid oxidation, including MDA [Halliwell & Chirico, 1993; Moore & Roberts, 1998]. The MDA produced in cells upon ROS activity having direct contact with cell organelles is a mutagenic factor [Niedernhofer *et al*., 2003]. It forms specific adducts with DNA. Hence, carcinogenicity of a high-fat diet has been postulated to be linked with interactions of lipid oxidation products, *e.g.* MDA, with DNA molecules [Fang *et al.*, 1996].

Infant formulae (IF) and follow-on formulae (FF) contain from 21 to 27% wt of fat rich in polyunsaturated fatty acids,

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which makes them susceptible to oxidative changes. Fat occurring in IF and FF is exposed to oxidation during production, packaging and storage of the products [Przygoński *et al*., 2000; Adamczyk & Bednarski, 1998; Ulberth & Roubicek, 1995].

Nowadays, requirements for IF and FF do not specify any restrictions as for the presence nor the level of any substance being a product of lipid oxidation in those formulae. A lack of comprehensive control of the health quality of IF and FF potentially endangers infants to the activity of lipid oxidation products detrimental to health that might occur in IF and FF due to imperfect production technology and storage conditions.

The present study was aimed at determining the presence and level of MDA in commercial IF and FF as well as at suggesting the limit of that lipid oxidation product detrimental to health in food products for infants.

## MATERIAL AND METHODS

**Materials.** The studies were carried out using:

(1) IF and FF, originating from four different producers (denoted with letters A, B, C, D), in warranty period, 12 | 16 months from packaging into a consumer package. Amongst a variety of commercially available preparations for infant nutrition, those purchased most often were selected for the study. Number 1 was used to denote IF preparations and number 2 to denote FF preparations. Out of 13 different preparations, 3 soybean preparations (denoted with S) and 2 hypoallergenic products (denoted with HA) were analysed.

(2) Human milk (N=7), mature *i.e.* after 15$^{th}$ day of lactation, provided voluntarily by women from the City of Gdańsk staying on an average diet; the milk was sampled between 7 and 12 a.m. and stored not longer than for 24 h at a temperature of -18°C.

(3) Fresh, untreated cow's milk (N=7) originating from a farm from the Gdańsk region, stored not longer that for 24 h at a temperature of -18°C.

(4) Refined soybean and rapeseed oils, constituents of IF and FF, being a source of polyunsaturated fatty acids.

**Analytical procedures.** Standard MDA solution was obtained by acid hydrolysis of TMP (1,1,3,3-tetramethoxypropane) in 0.01 mol/L hydrochloric acid. The analytical procedure with some alterations was based on methods described by Lappen *et al*. [2001] and Fenaille *et al*. [2001].

A 2.5-g portion of the infant formula examined (weighed exact to 0.001 g) was solubilised in distilled water at a temperature of *ca.* 40°C to the final volume of 25 mL. A 1-mL portion of the solution obtained (human milk, cow's milk) was collected into centrifuge tubes. The reaction mixture (1 mL) containing equal volumes of 15% TCA (trichloroacetic acid), 0.25 mol/L HCl and 0.375% TBA (thiobarbituric acid) as well as 0.1 mL of a 0.4% solution of BHT (butylated hydroxytoluene) in 96% ethanol were added. The mixture was homogenized and centrifuged for 5 min (2000 × g /min). The upper phase was transferred to twist test tubes. The precipitate left was again homogenized with 1 mL of the reaction mixture and centrifuged. The combined upper phases were heated for 30 min at a temperature of 95°C under nitrogen atmosphere. Then, the reaction was stopped through rapid chilling of the test tube in water with ice.

**Determination of MDA with the HPLC technique.** A quantitative analysis of the MDA as MDA:2TBA complex was carried out by HPLC. The HPLC set up consisted of: Dionex P 580 pump, Dionex column thermostat, Rheodyne 7725i injector and spectrophotometric detector SpectraSYSTEM UV3000HR (Thermoseparation®). The column Hypersil® BDS C18 (250 × 4.6 mm, $d_p$ 5 $\mu$m) (Agilent Technologies), at temperature of 20°C together with Rheodyne 5 $\mu$L loop injector were used. The mobile phase was 5 mmol/L phosphate buffer pH 7.0 : ACN, 85:15 (v:v). Using standard solutions of MDA, calibration curve was plotted for the relationship between chromatographic peak area and a concentration of MDA ($\lambda_{anal}$=534 nm). The calibration curve obtained was as follows: y=9591x+380; $R^2$=0.9986. Determination of the significance of calibration curve coefficients enabled confirming linearity of the analytical method in the concentration range from 0 to 306.28 $\mu$g MDA/L. Detection limit (LOD) of MDA with the use of described technique accounted for 9.72 $\mu$g MDA/L, instrument detection limit (IDL) – 0.04 ng MDA, whereas method detection limit (MDL) – 0.38 mg/kg. Precision of the analytical method reached CV=4.65 % [Konieczka *et al.*, 2004].

## RESULTS AND DISCUSSION

As a highly reactive compound, MDA attaches itself to functional groups of food components, *e.g.* -SH and -$NH_2$ of proteins [Marcinčak, 2003]. Release of MDA from bonds with proteins and proper course of protein coagulation as well as their removal from a solution are one of the more important stages of MDA determination in milk samples [Cesa, 2004]. Of crucial importance is also quantitative recovery of MDA from the other milk fractions. It is common knowledge that fat occurs in milk in the form of globules with diameter accounting for 1–2 $\mu$m in human milk and for 2–4 $\mu$m in infant formulas (IFs). As demonstrated in our study, not less than 10% of total MDA content in milk is accumulated inside fat globules. The application of acidic hydrolysis and double rinsing the sample with the reaction mixture enabled 74.70±2.98% recovery of the analyte in the case of IFs and 85.16±2.69% in the case of natural milk sample (Table 1).

TABLE 1. MDA recovery.

| Sample | Added MDA (μg) | Found MDA (μg) | Recovery (%) |
|---|---|---|---|
| Cow's milk 1 | 0 | 10.85 | |
| | 30.6 | 36.12 | 87.14 |
| | 61.27 | 61.62 | 85.44 |
| | 122.54 | 112.22 | 84.13 |
| Cow's milk 2 | 0 | 15.92 | |
| | 30.6 | 41.27 | 88.71 |
| | 61.27 | 64.93 | 84.12 |
| | 122.54 | 112.69 | 81.39 |
| Infant formulae B1 | 0 | 112.25 | |
| | 30.6 | 111.84 | 78.28 |
| | 61.27 | 126.51 | 72.91 |
| | 122.54 | 165.74 | 70.59 |
| Follow-on formulae D2 | 0 | 48.25 | |
| | 30.6 | 60.91 | 77.25 |
| | 61.27 | 82.39 | 75.22 |
| | 122.54 | 126.25 | 73.92 |

One of the basic methods used to determine the degree of lipid oxidation in food products is the TBA test [de las Heras *et al.,* 2003; Nanua, 2000; Turoli, 2004]. It consists in the spectrophotometric determination of the content of secondary products of lipid oxidation reacting under specified conditions with thiobarbituric acid (Thiobarbituric Acid Reactive Substances, TBARS). Under analytical conditions, thiobarbituric acid forms colour complexes with a number of substances, not all of them identified. Turoli *et al.* [2004] have postulated that the higher TBARS content in human milk than in IF and FF, as observed in their study, was likely to result from the presence of substances naturally occurring in milk, not being products of lipid oxidation. Taking this into account, we have decided to use the HPLC technique that affords the possibility of selective determination of malondialdehyde (MDA) content [de las Heras *et al.,* 2003; Fenaille *et al.*, 2001]. Chromatograms of: (a) standard solution of MDA and (b) isolate of initial milk, are presented in Figure 1 (retention time of MDA:2TBA complex was 8.76 min).

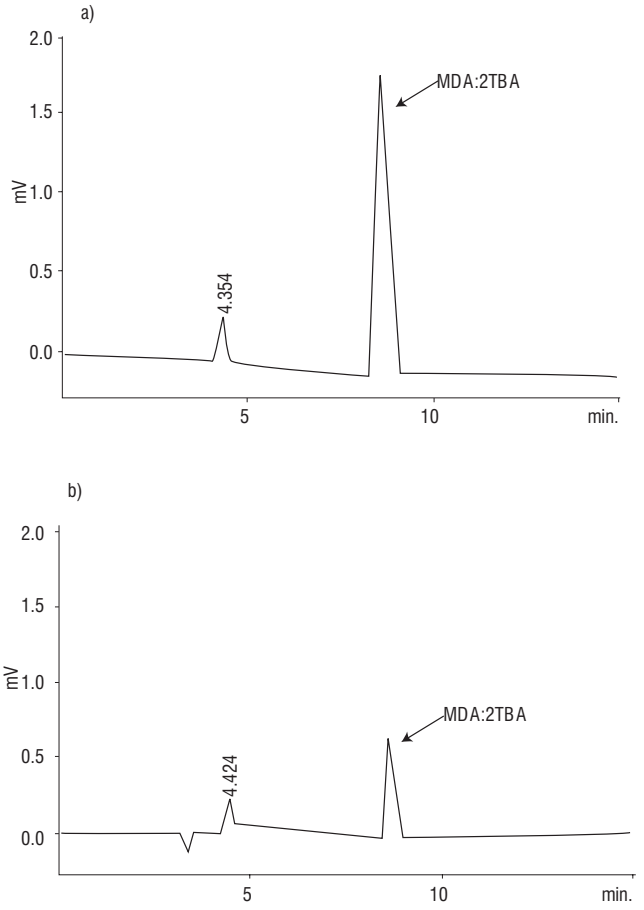


FIGURE 1. HPLC chromatograms: (a) MDA standard, (b) infant formula. Column: Hypersil® BDS C18 (250 × 4.6 mm, $d_p$ 5 $\mu$m), mobile phase: 5 mmol/L sodium phosphate buffer (pH 7.0) and acetonitrile (85:15, v/v). Flow rate: 07 mL/min. Detection: 532 nm.

**MDA content of human milk**

To an infant, human milk constitutes, between other components, a source of Long Chain Polyunsaturated Fatty Acids (LC PUFA) highly important for child's development [Koletzko, 2003]. The content of LC PUFA in human milk is related, to some extent, to the composition of fatty acids in women's diet [Smit *et al*., 2002]. In addition, human milk contains enzymes, *e.g.* lipoprotein lipase, that may substantially affect the degree of fat oxidation in milk by an increased presence of free fatty acids. As a result, human milk is far more susceptible to oxidative changes than IFs [Turoli, 2004]. Determination of lipid oxidation products in natural milk should, therefore, be carried out immediately after the collection of milk sample. In cases when immediate analysis is impossible, the sample should be frozen to at least -18°C and the period of storage should not be longer than 48 h. Storage of the milk sample at 4±6°C does not guarantee the inhibition of the oxidation processes [Miranda, 2004].

The analyses carried out in this work demonstrated a considerable dispersion of MDA content of human milk, *i.e*. from 9.20 to 27.10 $\mu$g/100 mL of milk (Table 2). For comparison, in milk of Spanish women the content of malondialdehyde has been reported to range from 2.16 to 182 $\mu$g [Miranda *et al*., 2004]. In both cases, the results refer to milk of women staying on an average diet, representing the same geographical area but not under uniform living conditions.

Such distinct differences in MDA contents might indicate the influence of environmental conditions (diet, lifestyle, physical activity, health condition) on the level of the above-mentioned secondary products of lipid oxidation in human milk. Undoubtedly, the level of MDA in human milk is affected by smoking. Even in the case of passive smokers, MDA content of human milk is considerably higher than in the non-smoking women [Bahri *et al*., 2005]. To some extent, human milk is protected against oxidation of lipids present in it. Milk contains both vitamins and enzymes that demonstrate anti-

TABLE 2. MDA content of infant milk formulas, human milk and cow milk (mean ±Δx).

| Sample | MDA ($\mu$g MDA/100 mL liquid milk) | MDA (mg MDA/kg milk powder) |
|---|---|---|
| A 1 | 48.44 ± 7.79 | 3.67 ± 0.59 |
| A 2 | 46.23 ± 11.59 | 3.35 ± 0.84 |
| A HA1 | 46.20 ± 6.34 | 4.56 ± 0.26 |
| A HA2 | 47.33 ± 8.56 | 3.50 ± 0.48 |
| A S | 64.30 ± 3.67 | 3.43 ± 0.62 |
| B 1 | 82.69 ± 8.64 | 6.41 ± 0.67 |
| B 2 | 89.60 ±15.26 | 6.40 ± 1.09 |
| C 1 | 40.50 ± 13.77 | 3.00 ± 1.02 |
| C 2 | 42.48 ± 6.62 | 2.95 ± 0.46 |
| C S 1 | 55.19 ± 4.16 | 4.38 ± 0.33 |
| C S 2 | 73.01 ± 11.81 | 5.07 ± 0.82 |
| D 1 | 47.12 ± 2.84 | 3.49 ± 0.21 |
| D 2 | 45.60 ± 5.28 | 2.85 ± 0.33 |
| Human milk (N=7) | | |
| Mean ± SD | 19.35 ± 5.14 | - |
| Min. – max. | 9.20 ÷ 27.10 | |
| Cow's milk (N=7) | | |
| Mean ± SD | 4.07 ± 1.08 | - |
| Min. – max. | 3.41 ÷ 5.21 | |

oxidative activity. Such an enzyme is glutathione peroxidase (GPX), the selenium-dependent enzyme decomposing peroxides. The activity of GPX is determined by the presence of selenium in the body. In their study into GPX, Moore *et al.* [2000] suggested the occurrence of some type of biochemical adjustment in women, *i.e.* the use of selenium reserves by mother's organism in the case of selenium deficiency in a diet and concomitant high demand for GPX. As a consequence of this, the level of GPX in human milk is adjusted to the level of oxidative stress that has occurred. This way, mother's organism automatically protects the child against the intake of lipid oxidation products with milk. Despite such "preservations" human milk contains secondary products of lipid oxidation [Bahri, 2005; Martysiak-Żurowska & Stołyhwo, 2005; Miranda, 2004; Moore, 2000]. In addition, a high level of TBARS in human milk does not correspond with a low activity of antioxidants in milk [Turoli *et al*., 2004]. Hence, at least a part of secondary products of lipid oxidation occurring in mother's milk have been claimed to originate directly from her body (they are not produced as a result of oxidation processes of human milk fat).

In an overview of literature referring to the subject of the presented study, no data have been found that would report on any direct correlation between the content of TBARS, secondary products of lipid oxidation in a diet and MDA content of human milk.

Our investigations aimed at comparing the content of MDA in milk of women staying at uniform hospital conditions in contrast to milk of women on individual diets seem to pre-confirm the occurrence of the phenomenon of direct MDA transfer to diet from human milk.

In cow's milk a low content of MDA was affirmed, *i.e.* 4.0±1.08 $\mu$g MDA/100 mL of milk (Table 2). Al-Mabruk *et al.* [2004] reported from 0 to 0.8 $\mu$g TBARS, expressed as MDA, in 1 mL fresh cow's milk depending on the type of feed applied.

**MDA content of infant formulae**

In order to compare contents of MDA in selected samples of human milk and infant formulae, the results were expressed in $\mu$g MDA/100 mL of ready-to-eat milk (Table 2) prepared according to manufacturer recommendations. The expression of MDA content per 100 mL of ready-to-eat milk enables determining the actual amount of the above-mentioned lipid oxidation products supplied to a child with the diet.

A 100 mL portion of solubilized IF and FF appeared to contain from 40.50±13.77 to 89.60±15.26 $\mu$g MDA, depending on the type of preparation and its producer. The highest MDA content, and consequently advanced degradation of lipid matrix as a result of the oxidation processes, was observed in IF and FF of producer B (Table 2). Amongst the IF and FF examined, the lowest content of MDA was reported for preparations of producer D (50% of MDA content in IF and FF of producer B). As a consequence, while feeding an infant with IF and FF preparations, the parent administers the child with nearly 90 $\mu$g of MDA or in the best case "only" 40 $\mu$g of MDA per each 100 mL of solubilized IF and FF. The study has demonstrated explicitly that commercial IF and FF contain at least 2 times more MDA than mature human milk. This explains results obtained by other researchers [Koletzko *et al.*, 2003] pointing to a considerably higher content of MDA in urine of children receiving IFs than in the breast-fed ones, *i.e.* 1.41 $\mu$mol MDA/L and 0.40 $\mu$mol MDA/L, respectively.

Despite the application of similar manufacturing technologies, specified parameters of production aimed at providing the optimal quality of the end product as well as production of IFs following principles of Good Manufacturing Practices (GMP), significant differences in MDA contents have been reported between the IFs examined. Similar results were obtained in the case of IFs produced *e.g.* in Italy [Cesa, 2004; Turoli, 2004].

**MDA content of edible oils**

The refining process of vegetable oils results in the formation of some amounts of secondary products of lipid oxidation, mainly at the stage of oil bleaching and deodorization which is carried out at temperature of 225–245°C [Stołyhwo, 1995]. In order to answer the question about the possible sources of MDA in IFs, the content of MDA was analysed in vegetable oils that are used as substrates for the production of IF and FF. Data obtained were compiled with MDA content per kg of lipid matrix of the preparations examined (Figure 2).

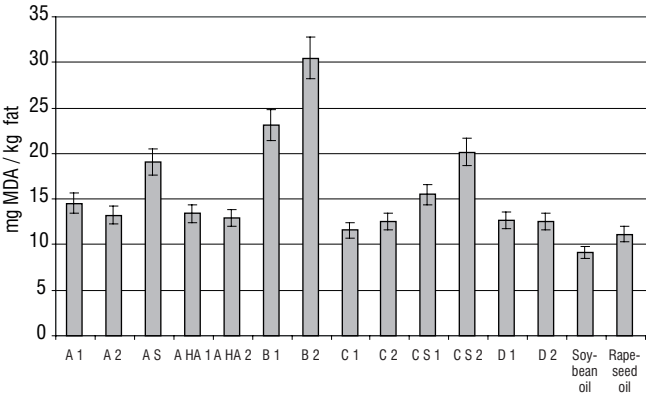


FIGURE 2. Content of MDA (mg MDA/kg fat) in fats from infant formulae, follow-on formulae, soybean oil and rapeseed oil.

The investigated soybean and rapeseed oils appeared to contain 9.17±0.46 and 11.12±0.53 mg MDA/kg of fat, respectively. When MDA content was expressed per kg of lipid matrix, the IFs examined contained from 12.50±1.45 to 30.48±5.19 mg MDA. Thus it may be concluded that the major part of MDA present in infant formulas does not originate directly from vegetable oil but is generated during the production of IF and FF. The most critical point is spray-drying of milk powder enriched in polyunsaturated soybean and rapeseed oil. Highly developed surface area and contact with oxygen results in accelerated oxidation processes which take place also during storage of IF and FF in large bulk containers till the re-packaging of the finished product into consumer packages under nitrogen atmosphere [Adamczyk & Bednarski, 1998; Przygoński *et al*., 2000; Ulberth & Roubicek, 1995].

Our previous investigations have demonstrated that apart from MDA and TBARS, the IFs contain also other secondary products of lipid oxidation, including fatty acids contain-

ing a system of three conjugated double bonds [Martysiak-Żurowska & Stołyhwo, 2005]. Effects of pasteurization and drying temperature of milk powder have also been confirmed by results obtained by Nanua *et al.* [2000]. Twice pasteurized high-fat milk powder (HFMP) produced under UHT conditions contains more TBARS than the low-heated HFMP pasteurized only once. In both cases the level of TBARS increases rapidly during storage of HFMP [Nanua *et al.,* 2000].

Nowadays, attempts are still made to search for new, natural, more active antioxidants to be used as food additives. One of such substances is oryzanol, naturally occurring in rice bran. Nanua *et al.* [2000] have demonstrated that oryzanol added to whole milk powder (WMP) substantially improves the oxidation stability of the product. It should be remembered, however, that infants belong to the most demanding consumers. Hence, the introduction to IFs of new antioxidants of natural origin, yet not occurring in human milk naturally, requires in-depth analysis and advanced research.

Likewise in the case of other food products (meat, cured meat products, edible oils), the MDA content of infant formulas enables concluding on the degree of fat oxidation and, consequently, on the decline in the nutritive value of a given product. Taking into account detrimental effects of lipid oxidation products – especially harmful in the case of a developing organism of a child, it is necessary to update qualitative recommendations for IF and FF with data on tolerance of MDA content. In the case of children nourished by bottle IFs constitute the only food to a baby up to 4 months of life. Currently, a lack of limits referring to the content of lipid oxidation products in preparations for infant nutrition is in fundamental contrast with food safety principles. In our opinion, such a situation is unacceptable and requires introducing tolerance levels of such substances in infants formulas.

In our opinion, tolerance of MDA content should refer to ready-to-use products for infants and be a compromise between what is required theoretically (lack or level in human milk) and what is practically attainable (from 2.85±0.33 to 6.41±0.67 mg MDA/kg of preparation). Setting strict requirements for an allowable level of MDA in IF and FF would cause a considerable improvement of the production process. Consequently, it would constitute a factor of technological and technical progress with evident benefit to the health of infants.

## CONCLUSIONS

1. Infant formulae commonly available on the Polish market contain considerable amounts of malondialdehyde (MDA), *i.e.* 56.05±9.77 $\mu$g MDA/100 mL of milk on average, whereas in human milk its content accounts for 19.35±5.14 $\mu$g/100 mL on average.

2. Major part of detrimental to health MDA occurring in IF and FF is generated during the production, packaging and storage of preparations for infant nutrition.

3. Differences in MDA content of particular IF and FF examined (at a level from 2.85±0.33 to 6.41±0.67 mg/kg of preparation in the form of powder) result from the application of various IF and FF production technologies by producers.

4. A lack of limits reducing the presence of secondary products of lipid oxidation detrimental to health in IF and FF is in contrast with fundamental principles of health safety of food for children.

5. The Authors suggest introduction of maximum allowable limits for MDA content in IF and FF at 48 $\mu$g MDA/100 mL of solubilized modified milk. The postulated level of MDA was reported in 7 out of 13 preparations examined.

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## ZAWARTOŚĆ DIALDEHYDU MALONOWEGO (MDA) W PREPARATACH DO POCZĄTKOWEGO I NASTĘPNEGO ŻYWIENIA NIEMOWLĄT

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Zbadano zawartość dialdehydu malonowego MDA, szkodliwego dla zdrowia produktu utleniania lipidów, w preparatach do początkowego MP i następnego MN żywienia niemowląt (N=13), w mleku ludzkim (N=7), nieprzetworzonym mleku krowim (N=7) oraz w oleju sojowym i rzepakowym – składnikach MP i MN. W preparatach MP i MN stwierdzono znaczne zawartości MDA od 40,50 ± 13,77 do 89,60 ± 15,26 $\mu$g MDA w 100 ml (mleko ludzkie – średnio 19,35 ± 5,14 $\mu$g/100 ml mleka), w zależności od stosowanej przez producenta technologii produkcji, sposobu pakowania oraz warunków przechowywania gotowego produktu. Mając na uwadze szkodliwe dla zdrowia działanie MDA, zwłaszcza dla niemowląt i małych dzieci, zasugerowano wprowadzenie limitu zawartości MDA w mleku początkowym i następnym dla niemowląt. Na podstawie uzyskanych w ramach niniejszej pracy wyników autorzy zaproponowali limit zawartości MDA na 48,0 $\mu$g MDA / 100 mL roztworzonego mleka modyfikowanego.