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CONTENT OF FUROSINE IN INFANT FORMULAE AND FOLLOW-ON FORMULAE

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The determination of the furosine (FUR) indicator of the Maillard reaction in commercial infant formulae (IF), follow-on formulae (FF), human milk (N=10) and raw cow milk (N=7) was performed using high performance liquid chromatography with ultraviolet detection (HPLC/UV). A high FUR content was confirmed that ranged from 1320±102.2 to 1550.9±166.5 mg/100 g protein in infant formulae IF and from 931.9±153.8 to 1156.7±104.5 mg/100 g protein in follow-on formulae FF (human milk – at the average below 6 mg/100 g protein). Such a significant difference between FUR values of commercially available formulas is accounted for imperfection of different technologies of manufacturing IF and FF. In dairy products damage caused by heat treatment could be greater as a result of manufacturing processes and storage conditions. Furosine content was used in order to calculate the concentration of blocked lysine. In infant formulas IF's the blocked lysine levels were found to range from 19.6 to 34% of total lysine. Taking into consideration harmful for health, toxic products of Mallard reaction, the content of FUR should be labelled. In the Authors' opinion, the content of furosine tolerance should make compromise between that what is theoretical demanded and that what is practical reached (fresh milk powder for all purposes – about 120 mg FUR/100 g protein, FF of producer C – 930 mg FUR/100 g protein). The authors' suggestion is that the maximum allowable tolerance of FUR should not exceed 700 mg/100 g protein of IF and FF.

INTRODUCTION

The technology of production of infant formulae (IF) and follow-on formulae (FF) includes a range of operations and processes in which the product is heat treated. These include sterilization of milk, homogenization, drying and redrying the finished powdered product. The action of high temperature, necessary to obtain a certain technological effect, may cause a number of unfavourable changes decreasing the nutritive value and health quality of a food product.

One of the most significant changes caused by high temperature in food products is the reaction of nonenzymatic browning (Maillard reaction), which consists in a reaction between a carbonyl group of reducing sugars and a free amino group of amino acids, peptides and proteins [O'Brien & Morrissey, 1989; van Boekel, 1998; Rufian-Henares *et al.*, 2002].

Maillard reactions have a negative influence on the nutritive and health value of food. They cause loss and degradation of essential amino acids, especially lysine, which is very susceptible to denaturation due to an amino group in the ε position, but also arginine, methionine, tryptophan and histidine. In addition, products of the Maillard reaction limit the digestibility of proteins by blocking the availability of a peptide bond for trypsin and carboxypeptidase, and are inhibitors of digestive enzymes (proteases and disaccharides). They show an ability to chelate metal ions, which causes, among other effects, inhibition of metalloenzymes and urinary trace metal excretion. Some products of the non-enzymatic browning reaction are toxic substances. They accumulate in the liver, kidneys and pancreas, causing pathological changes in these organs in laboratory animals [O'Brien & Morrissey, 1989; Lee & Shibamoto, 2002].

In products based on cow's milk, such as infant formula (IF) and follow-on formula (FF), Maillard reactions take place mainly between the ε-amino group of lysine and lactose, which leads to the appearance of lactosyllysine (a Schiff's base) and, from that – as a result of Amadori rearrangement – lactulosyllysine (ε-N-deoxylactulosyl-L-lysine). In the analytical process, ε-N-deoxylactulosyl-L-lysine is partially converted by acid hydrolysis to furosine [Resmini *et al.*, 1990; van Boekel, 1998; Delgado *et al.*, 1992; Rufian-Henares *et al.*, 2002]. The concentration of furosine (FUR) in a product has been assumed as an indicator of the degree of a product degradation resulting from its heating [Ferrer *et al.*, 2000; Kuncewicz *et al.*, 2000; Rufian-Henares *et al.*, 2002]. On the basis of increased furosine content, it is possible to detect even a small addition of reconstituted milk in sterilised milk [Resmini *et al.*, 2003; Villamiel *et al.*, 1999].

Despite scientific proof of the negative effect of the Maillard reaction on the nutritive and health value of food, there is no EU legislation defining the allowable level of protein degradation, determined on the basis of furosine content of food products meant for infants.

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The purpose of this research was to determine furosine content of commercially available infant formulas (IFs), in human milk and in raw cow's milk, as well as to determine the degree of protein degradation in IF and FF.

MATERIAL AND METHODS

MATERIALS

The following materials were used in the study: (i) commercially available IF and FF, in warranty period, from four different manufacturers marked as A, B, C, D. From among many available IFs produced from cow's milk, those purchased most often were selected; (ii) human milk (N=10), between the 3rd and 5th month 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; and (iii) fresh, untreated cow's milk (N=4) originating from a farm in the Żuławy region, stored no longer that 24 h at a temperature of -18°C.

METHODS

Analytical procedure. Furosine content (FUR) was determined by Ion-Pair Reversed Phase High-Performance Liquid Chromatography with UV detection. The analytical procedure was performed on the basis of methods used by Delgado *et al.* [1992] and Ferrer *et al.* [2000].

Acid hydrolysis. A sample of milk powder, equivalent to 40–50 mg of protein (or 1.5 mL human milk) was put in a hermetically closed vial, to which 8 mL of 8 mol/L HCl was added. The sample was blown out for 1 min by a stream of N_2, hermetically closed and heated for 23 h at 110°C. After that time, the hydrolysate was cooled, filtrated and filled up with water to 8 mL.–Directly before HPLC analysis 1 mL of hydrolysate was taken and diluted with 3 mL of distilled water then neutralized with 0.4 mol/L NaOH to pH of 7. Woman's milk, hydrolysed, following cooling and filtration, was filled up to 8 mL. The solution was neutralised by adding 1.6 mol/L NaOH at a 1:4 ratio.

RP-HPLC conditions. Furosine content (FUR) was determined using the Ion-Pair Reversed Phase High-Performance Liquid Chromatography with UV detection. A Shimadzu, model LC-4A liquid chromatograph was used. It was equipped with an SPD 2AS spectrophotometric detector with a deuterium discharge lamp and a gradient pump. The chromatograph had also a Rheodyne 7725i injector, with a 20-μL loop injector. A Superspher® 100 RP-18 (Merck), 5 μm (250 × 4.6, I.D.) chromatographic column was used. The mobile phase was: 5.5 mmol/L sodium heptanesulfonate with 15% acetonitrile and 0.2% formic acid. The flow rate of the mobile phase was 0.8 mL/min.

Using a SPECTRONIC® Milton Roy 1001 Plus spectrophotometric detector, the UV spectrum of furosine was obtained. On the basis of the furosine UV spectrum, the analytical wavelength was determined at 280 nm (Figure 1).

Statistical parameters of the method. Standard furosine solutions were obtained by adding appropriate quantities of pure furosine (Neosystem Laboratories, Strasbourg, France) to samples of cow's milk, in which lactulosyllysine was not found. Further action was accordant with the analytical procedure. A calibration curve was plotted for the dependency for the chromatographic peak area as a function of FUR concentration in cow milk samples (λ_{anal}=280 nm) y=22252 x–348.8; R^2=0.995, RSD=6.925%, N=9. By determining the significance of the calibration curve coefficients it was possible to confirm the linearity of the analytical method in the concentration range from 0 to 2 μg FUR/L. Limit of Detection (LOD) for the ion-pair RP-HPLC method of determining FUR was 6.06 μg FUR/mL, Instrument Detection Limit IDL=0.8 ng FUR, Method Detection Limit MDL=6 mg FUR/100 g of protein. Precision of the analytical method was CV=3.996 % [Konieczka *et al.*, 2004].

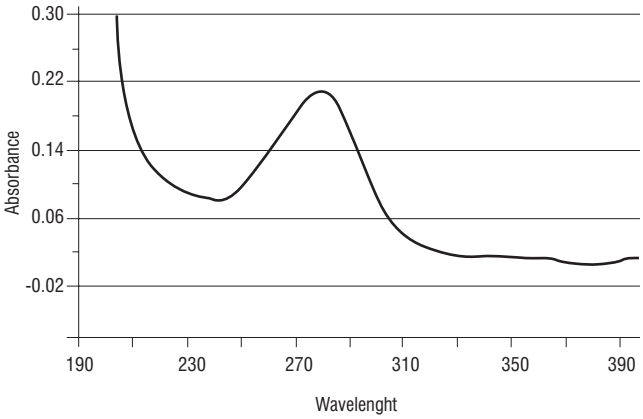

FIGURE 1. UV-VIS spectrum of furosine standard.

The analytical method applied in the paper allowed us to obtain over 97% recovery of the analyte (Table 1). The average recovery of the chromatographic FUR determining method for fresh cow's milk samples was 97.15%, while for samples of infant feeding formulas in powders, it was 97.27%.

TABLE 1. Recovery of furosine from samples.

Sample	Added FU (mg)	Found FU (mg)	Recovery (%)
Cow milk I	0.5065	0.4932	97.37
	1.0313	1.0065	97.59
	2.0267	1.9573	96.58
Cow milk II	0.5065	0.5009	98.89
	1.0313	1.0015	97.11
	2.0267	1.9329	95.37
Infant formulae A	0	1.2189	
	0.2510	1.4378	97.82
	0.5065	1.6564	96.00
	1.0313	2.1862	97.16
Follow-on formulae C	0	0.9097	
	0.2510	1.1245	96.88
	0.5065	1.3881	98.02
	1.0313	1.8967	97.72

Determination of total protein. Total nitrogen content in the samples was determined using the Kjeldahl method [AOAC, 1990]. To convert nitrogen values to protein a factor of 6.38 was applied.

RESULTS AND DISCUSSION

The contents of FUR in the investigated IFs and in the human and cow milks are presented in Table 2. For comparison in Tables 3 and 4 there are shown results of FUR and total lysine content reported by other authors [Henle *et al.*, 1995; Kuncewicz *et al.,* 2000; Rufian-Henares *et al.,* 2002; Van Renterghem *et al.,* 1996]. Blocked and available lysine content in samples as above are shown in Table 5.

Exemplary chromatograms of (a) furosine standard, (b) infant formulae, and (c) human milk, are presented in Figure 2. In food products made from milk, lactulosyllysine content, and so, the content of furosine appearing as a result of the hydrolysis of the amino acid, increases together with the

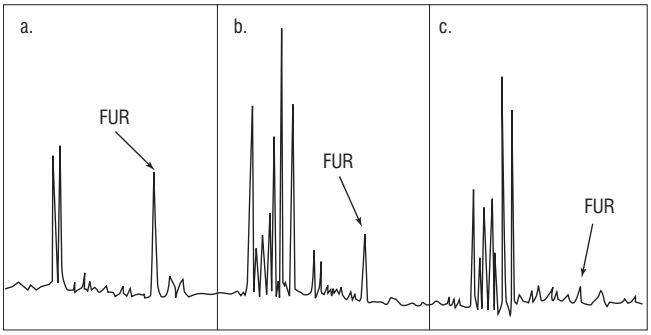

FIGURE 2. HPLC chromatograms: (a) furosine standard, (b) infant formula, (c) human milk.
Column: Superspher® 100 RP-18, 5μm (250x4.6, I.D.), mobile phase: 5.5 mmol/L sodium heptanesulfonate with 15% acetonitrile and 0.2% of formic acid. The flow rate: 0.8 mL/min. Detection: 280 nm.

temperature of the production process, duration of exposure to the temperature, as well as during storage of the finished product [Baptista & Carvalho, 2004; Ferrer *et al.,* 2002; Garcia-Banos *et al.*, 2005]. Raw cow's milk contains from 3 to 5.5 mg of furosine per 100 g of protein. Freshly produced, skimmed powdered milk contains approximately 100–120 mg FUR per 100 g of protein, but this increases even to 600 mg/100 g of protein, if critical parameters are used for drying the milk powder (roller drying) and the product is stored for a long time [Henle *et al.*, 1995; Kuncewicz *et al.*, 2000; Van Renterghem & De Block, 1996; Garcia-Banos *et al.*, 2005]. In powdered milk, Maillard reactions take place much quicker than in liquid milk. This is due not only to the low activity of water, but also to the increased concentration of reagents caused by the removal of water from the medium [van Boekel, 1998]. During research, Mauron [1981] noted that in dairy products and milk, lactulosyllysine was a stable product. Further stages of the non-enzymatic browning process require a drastic increase and long duration of the heating temperature. This means that even if there are no changes in the flavour, taste or colour of the product made from milk, losses of available lysine in the product may be considerable.

In the samples tested, FUR content reached – depending on the manufacturer – from 1320 to 1550 mg FUR/100 g of protein in IF, and from 930 to 1160 mg FUR/100 g of protein in FF (Table 2). For comparison fresh produced powdered milk contains about 120 mg FUR/100 g of protein [Van Renterghem & deBlock, 1996]. In the examined powdered IFs for infants, and small children, more than ten times higher FUR content was noted. The results obtained are in accordance with data presented by other authors (Table 3) [Henle *et al.*, 1995; Kuncewicz *et al.*, 2000; Rufian-Henares *et al.*, 2002; Van Renterghem & De Block, 1996].

TABLE 2. Furosine contents (expressed as mg FUR /100 g protein) of the samples analysed.

Manufacturer	FUR (mg/100 g protein) ±SD	N	RSD (%)
Infant formula IF			
A	1550.9±166.5	3	10.73
B	1443.5±101.4	4	7.02
C	1320.0±102.2	3	7.74
D	1372.8±104.9	3	7.64
Follow-on formula FF			
A	1053.8±94.6	3	8.98
B	985.7±84.8	4	8.60
C	931.9±153.8	3	16.50
D	1156.7±104.5	4	9.03
Human milk			
-	Below 6 mg/100 g protein	10	-

TABLE 3. Content of furosine (mg/100 g protein) in commercially available milk product reported by different authors.

Sample	FUR (mg/100 g protein)	References
Raw milk	3.5–5.5	[Henle *et al.,* 1995]
	3–5	[Kuncewicz *et al.*, 2000]
	4–5	[Van Renterghem & De Block, 1996]
Milk powder	180–1200	[Rufian-Henares *et al.*, 2002]
	150–600	[Kuncewicz *et al.*, 2000]
	170–300	[Van Renterghem & De Block, 1996]
Infant baby food (powdered)	930–1890	[Henle *et al.,* 1995]
	660–880	[Van Renterghem & De Block, 1996]
	930–1550	This study
Infant baby food (liquid)	730–1250	[Henle *et al.,* 1995]

In the case of dairy products, as a result of Maillard reaction, mainly amino groups of lysine (which is a component of proteins in milk) are reduced. High furosine content is a symptom of advanced degradation of proteins, and therefore a considerable decrement of lysine and lactose in the products examined. This decrease of nutritive values is especially unfavourable in the case of IF's, as they are the only source of proteins and carbohydrates during the early infant stage of children nourished by bottle.

Research conducted in 1998 in Germany, by the Institute of Chemistry and Physics [Product Quality of Milk and Milk Products – Investigation of Furosine. Report 1998] showed that human milk may also contain certain amounts of furosine, about 20–25 mg FUR/100 g of protein in milk from women in Germany. Single cases were noted, where women's milk contained over 160 mg FUR/100 g of protein. Such a high FUR content in raw human milk probably resulted from a high intake of highly processed foods by these women.

Very high FUR contents were not noted in milk from women in Poland (N=10). The content of FUR in the examined samples of human milk was below the detection limit of the applied analytical method, *i.e.* below 6 mg FUR/ 100 g of protein (Table 2).

Considering the technological processes applied in the production of IF and FF (using skimmed powdered milk as a raw material, exposure to high temperature, drying and re-drying of the product) it currently seems impossible to obtain modified milk for children, containing furosine at the level at which it is present in human milk (maximally 160 mg FUR/100 g of protein [Product Quality of Milk and Milk Products – Investigation of Furosine. Institute of Chemistry and Physics. Report 1998]). However, we should strive to limit the content of Maillard reaction products to the lowest attainable level, through, *e.g.* checking the storage time of the finished product.

In accordance with EU Commission Directive 91/321/EEC regarding the basic composition of infant foods, IF must contain at least 3.5 g of lactose/100 kcal of milk, while FF 1.8 g/100 kcal. Furthermore, IF should contain an available amount of every essential and relatively essential amino acid at least in the same amounts as in the standard protein (human milk protein), while for IFs the limiting amino acid index should be at least 80% of the standard protein (casein). This means that IF must contain no less than 6.7 g of available lysine/100 g of protein, and FF – at least 6.48 g of available lysine/100 g of protein. In most cases, manufacturers introduce into their IFs protein containing more essential and conditionally essential amino acids than the EU Commission Directive requires (Table 4).

As mentioned, the main product of the Maillard reaction in dairy foods, where it takes place mainly between lysine and lactose, is lactulosyllysine, a product of Amadori. As noted by Bujard & Finot [1978], as a result of acid hydrolysis in strictly determined conditions, lactulosyllysine forms 40% of molar mass of lysine, 32% of furosine and about 28% of pyridosine. Therefore, on the basis of furosine content, it is possible to calculate the content of blocked lysine and available lysine in the product [Baptista & Carvalho, 2004].

In the examined IFs, as a result of Maillard reaction, between 19.6% and over 34% of lysine occurs in a form unavailable for digestive enzymes and therefore unavailable for children. The real content of available lysine is, therefore, between 5.41 and 7.21 g/100 g of protein in IF and between 6.21 and 6.83 g / 100 g of protein in FF (Table 5). The recommendations of Commission Directive 91/321/EEC are: 6.7 g of lysine/100 g of protein in IF and 6.48 g of lysine/100 g of protein in FF. Therefore, three out of the four tested IF and two out of the four tested FF, selected on the basis of consumer preferences from a wide range of IF's and FF's available on the market, have shown decreased nutrient value for lysine (an essential amino acid). This is especially dangerous in the case of IFs, which are, for bottle-fed children, their only source of nutrients.

TABLE 5. Blocked lysine content (grams per 100 g of protein; % of total lysine) and available lysine content in milk-based infant formulas.

Sample	Blocked lysine (g/100 g protein)	Blocked lysine (% of total lysine)	Available lysine
Infant formula IF			
A	2.79	33.94	5.41
B	2.59	26.46	7.21
C	2.37	28.20	6.03
D	2.47	29.38	5.93
Follow-on formula FF			
A	1.89	23.26	6.21
B	1.77	21.01	6.63
C	1.67	19.57	6.83
D	2.08	24.50	6.42

It should also be remembered that lactulosyllysine is one of the more stable substances resulting from non-enzymatic browning process in dairy products, but not the only one. Further progress of the Maillard reaction leads to degradation of Amadori products (and other substances appearing from Schiff's bases), as a result of which, a range of decomposition products appear, such as amino compounds or sugar fragments bound to polymerised proteins, compounds with possible allergenic properties and even toxic compounds [Van Boekel, 1998; Lee *et al*., 2002].

CONCLUSIONS

1. Furosine content in IF and FF coming from various manufacturers differs to a great extent. It ranges from 932 mg FUR/100 g of protein for FF of producer C, to more than 1550 mg FUR /100 g of protein for IF of producer A.

2. In human milk (N=10) from the City of Gdańsk region, furosine content was not noted above its detection limit, *i.e.* above 6 mg FUR/100 g of protein.

3. As a result of Maillard reaction, most of the IFs tested showed an insufficient content of lysine, compared to the content of this essential amino acid required by international regulations regarding infant formulas.

4. As a result of uncontrolled Maillard reaction, a significant decrease of nutritive value of IFs occurs during their production, relative to the nutritive value declared by the manufacturers.

TABLE 4. Content of total lysine in commercially available infant formulas [reported by Jabłoński & Stolarczyk, 2002].

Infant formulae	Lysine (g/100 g protein)	Human milk [Commission Directive 91/321/EEC]	Follow-on formulae	Lysine (g/100 g protein)	Casein [Commission Directive 91/321/EEC]
NAN 1	9.8	6.7	NAN 2	8.4	80% from 8.1 6.48
Bebiko 1	8.4		Bebiko 2R	8.5	
Bebilon 1	9.9		Bebilon 2	8.5	
Humana 1	8.2		Humana 2	8.1	

5. Bearing in mind the harmfulness of products of Maillard reaction, it would be advisable to supplement the quality regulations in power, regarding IF and FF, with tolerance limits for the content of this type of substance. In the opinion of the authors, FUR content tolerances should be a compromise between what is theoretically required (no FUR), and what is practically obtainable (general purpose fresh powdered milk – around 120 mg FUR/100 g of protein, FF of producer C – 930 mg FUR/100 g of protein).

6. Furthermore, the authors suggest that finished products should be stored not on store shelves at room temperature (the guarantee period for IFs is 24 months!), but in cold store at a temperature of 4–6ºC.

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FUROZYNA W PREPARATACH DO POCZĄTKOWEGO I NASTĘPNEGO ŻYWIENIA NIEMOWLĄT

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Techniką wysokosprawnej chromatografii cieczowej z detekcją w zakresie ultrafioletu (HPLC/UV) oznaczono zawartość furozyny, będącej wskaźnikiem reakcji Maillarda, w handlowo dostępnym mleku do początkowego i następnego żywienia niemowląt; w mleku ludzkim (N=10) oraz w surowym mleku krowim (N=7). W żywości przeznaczonej dla niemowląt stwierdzono wysoką zawartość FUR, od 1320±102,2 do 1550,9±166,5 mg/100 g białka mleka początkowego oraz od 931,9±153,8 do 1156,7±104,5 mg/100 g białka mleka następnego (w mleku ludzkim – poniżej 6 mg/100 g białka) (tab. 2). Tak znaczące różnice w zawartości FUR w komercyjnie dostępnych preparatach do żywienia niemowląt wynikają ze stosowania różnych, niedoskonałych procesów technologicznych wytwarzania tego typu produktów. W produktach mlecznych degradacja składników żywności wywołana działaniem wysokiej temperatury może dalej postępować w zależności od parametrów procesu produkcyjnego oraz warunków przechowywania. Na podstawie zawartości furozyny można obliczyć stężenie lizyny w formie niedostępnej biologicznie. Stwierdzono, że w badanych preparatach do żywienia niemowląt od 19,6 do 34% ogólnej zawartości lizyny występuje w formie zablokowanej. Biorąc pod uwagę toksyczność niektórych produktów reakcji Maillarda poziom FUR w żywności przeznaczonej dla dzieci powinien być limitowany. W przekonaniu autorów pracy limitowany poziom FUR powinien stanowić kompromis pomiędzy teoretycznym zapotrzebowaniem a najniższą wartością technologicznie możliwą do uzyskania (w świeżym mleku w proszku zawartość FUR wynosi około120 mg FUR/100 g białka, w mleku następnym producenta C – 930 mg FUR/100 g białek). Autorzy proponują aby limit dopuszczalnej zawartości FUR nie przekraczał 700 mg/100 g białka mleka modyfikowanego.