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Studies on N-Terminal Glycation of Peptides in Hypoallergenic Infant Formulas: Quantification of α-*N*-(2-Furoylmethyl) Amino Acids

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To obtain information about the extent of the early Maillard reaction between the N-termini of peptides and lactose, α-*N*-(2-furoylmethyl) amino acids (FMAAs) were quantified together with ϵ-*N*-(2-furoylmethyl)lysine (furosine) in acid hydrolyzates of hypoallergenic infant formulas, conventional infant formulas, and human milk samples using RP-HPLC with UV-detection. FMAAs are formed during acid hydrolysis of peptide-bound N-terminal Amadori products (APs), and furosine is formed from the Amadori products of peptide-bound lysine. Unambiguous identification was achieved by means of LC/MS and UV-spectroscopy using independently prepared reference material. The extent of acid-induced conversion of APs to FMAAs was studied by RP-HPLC with chemiluminescent nitrogen detection (CLND). Depending on the corresponding α-*N*-lactulosyl amino acid, between 6.0% and 18.1% of FMAAs were formed during hydrolysis for 23 h at 110 °C in 8 N HCl. From ϵ-*N*-lactulosyllysine, 50% furosine is formed under these conditions. Whereas furosine was detectable in all assayed samples, five different FMAAs, α-FM-Lys, α-FM-Ala, α-FM-Val, α-FM-Ile, and α-FM-Leu, were exclusively detected in acid hydrolyzates of hypoallergenic infant formulas in amounts ranging from 35 to 396 μmol/100 g protein. Taking the conversion factors into account, modification of N-terminal amino acids in peptides by reducing carbohydrates was between 0.3% and 8.4%. This has to be considered within the discussion concerning the nutritional quality of peptide-containing foods.

KEYWORDS: **α-*N*-(2-Furoylmethyl) amino acids; furosine; hypoallergenic infant formula; N-terminal glycation; peptides; Amadori product; Maillard reaction**

INTRODUCTION

The Maillard reaction, also referred to as nonenzymatic browning or glycation, is responsible for the formation of desired flavors and colors but also for undesired derivatization of essential amino acids, thus leading to a decrease in the nutritional value of heated or stored foods (*1*). In the early stage of the Maillard reaction, reducing carbohydrates such as glucose or lactose react with amino groups from amino acids, peptides, or proteins to amino ketoses, the so-called Amadori products (APs). APs of the essential amino acid lysine are biologically not available during digestion (*2*). The analysis of Amadori products, therefore, is of importance to assess the impact of heating or storage on the nutritional quality of foods. The major problem in quantification of APs by amino acid analysis is the fact that these amino acid derivatives are degraded during acid hydrolysis to several reaction products, among which UV-active *N*-(2-furoylmethyl) amino acids are of particular importance (*3*, *10*, *11*) (**Figure 1**). The extent of glycation at the ϵ-amino group of peptide-bound lysine can be monitored by ϵ-*N*-(2-furoylmethyl)-lysine (furosine), which is formed in constant amounts together with lysine and pyridosine during acid hydrolysis from APs such as *N*-ϵ-lactulosyllysine or *N*-ϵ-fructosyllysine (*2*−*4*). Furosine is a widely used marker for the evaluation of the early Maillard reaction in a great variety of foods (*5*−*7*) and biological samples (*8*). Numerous studies have focused on the side-chains of peptide-bound lysine and arginine residues as main targets for a derivatization by carbohydrates or their degradation products (*1*). As compared to this, glycation reactions occurring at N-terminal amino acids of peptides or proteins have not received particular attention up to now, although in certain peptide-containing foods, α-amino groups of N-terminal amino acids may be quantitatively much more important than ϵ-amino groups of peptide-bound lysine. Furthermore, N-terminal glycation in vivo is well known. Quantification of glycated hemoglobin (HbA_{1C}), in which the N-terminal valine of the α-chain is modified by glucose, is used as a tool to monitor long-term glycation in diabetic patients (*1*, *9*).

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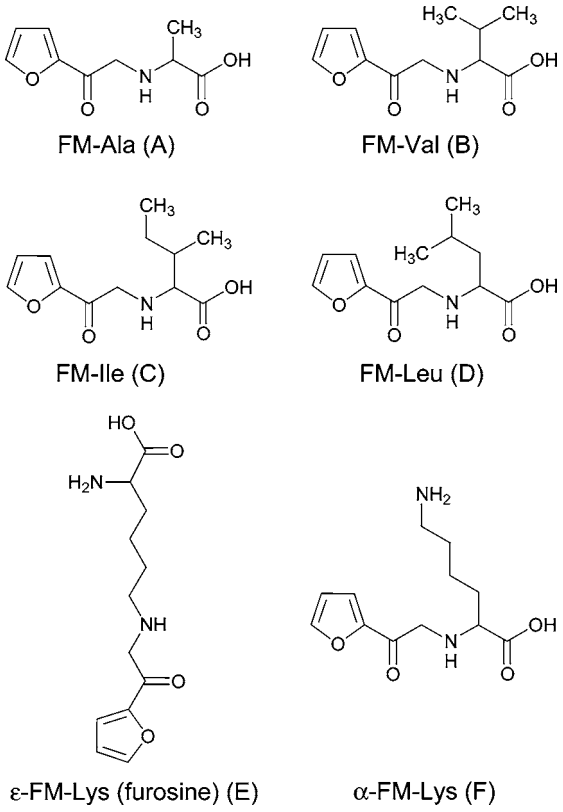

Figure 1. Structures of determined FMAAs (A–F).

In the present study, we address the question whether early Maillard reactions occurring at the N-termini of peptides are of importance in foods such as hypoallergenic formulas, which contain a multiplicity of peptides from hydrolyzed whey proteins or casein. Selected *N*-(2-furoylmethyl) amino acids, which are formed from Amadori products of N-terminal amino acids during acid hydrolysis (**Figure 1**), were analyzed by RP-HPLC with UV-detection in comparison to reference samples prepared from amino acids and lactose.

EXPERIMENTAL PROCEDURES

Preparation of Samples for Chromatographic Analysis. Seven samples of hypoallergenic infant formulas (peptide-containing) and four samples of conventional infant formulas (intact protein-containing) were obtained from local retail stores. Four human milk samples were obtained from healthy volunteers. Of the infant formulas, 100 mg was hydrolyzed with 1.2 mL of 7.95 N hydrochloric acid in screw-cap test tubes at 110 °C for 23 h. Of the human milk samples, 2.0 mL was hydrolyzed with 3.9 mL of 37% hydrochloric acid under the same conditions. After cooling to room temperature, hydrolyzates were centrifugated (10 000 rpm, 15 min), and the supernatant was dried under vacuum. The dry residues were dissolved in 500 μL of 0.2 N hydrochloric acid. After membrane filtration (0.2 μm), 50 μL was subjected to LC-ESI-TOF-MS or into the RP-HPLC with UV- or CLND-detection, respectively.

RP-HPLC with UV-Detection for Quantification of FMAAs in Acid Hydrolysates. For analysis of FM-Val, FM-Leu, and FM-Ile, an HPLC system consisting of a pump K1001, a gradient mixing system, a detector K-2501, a column oven, and an automatic injector Marathon (all from Knauer, Berlin, Germany) was used. The column was a Eurospher 100-C18 column (5 μm, 4.6 mm × 250 mm, Knauer, Berlin, Germany) protected by a guard column (8 × 5 mm) containing the same material. The injection volume was 50 μL, the flow rate was 0.7 mL/min, and the temperature was set at 25 °C. A gradient was applied with water (solvent A) and methanol (solvent B), each containing 2% formic acid. The gradient was as follows: 0% solvent B for 10 min, 0% to 10% solvent B in 5 min, 10% to 20% solvent B in 30 min, 20% to 80% solvent B in 5 min, 80% solvent B for 5 min, 80% to 0% solvent B in 5 min, 0% solvent B for 10 min. The detection wavelength was 280 nm.

For analysis of FM-Val and furosine, the HPLC system was as above, but a RP-8 column "furosine dedicated" (Alltech, Unterhaching, Germany) was used. A linear binary gradient was applied with water containing 0.4% acetic acid (solvent A) and water containing 0.4% acetic acid and 0.27% KCL (solvent B). The gradient was as follows: 0% solvent B for 13.5 min, 0% to 50% solvent B in 7 min, 50% solvent B for 1.5 min, 50% to 0% solvent B in 1 min, 0% solvent B for 9 min (*3*). The injection volume was 50 μL, the flow was 1.1 mL/min, and the temperature was set at 34 °C.

For quantification of FMAAs, an external standard of furosine (Neosystems, Strasbourg, France) was used.

LC-ESI-TOF-MS for Identification of FMAAs. An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, USA) consisting of a high-pressure gradient pump system, column oven, automatic injector, and diode array detector was coupled to a PerSeptive Biosystems Mariner time-of-flight mass spectrometer (TOF-MS) equipped with an electrospray ionization source (ESI) working in the positive mode (Applied Biosystems, Stafford, USA). RP-HPLC was performed as described above for the quantification of FM-Val, FM-Leu, and FM-Ile. MS conditions were as follows: quadrupole RF voltage 799.80, nozzle temperature 140.01 °C, reflector potential 1549.99, detector voltage 2299.99, first mass 100, last mass 700.

Preparation and Analysis of Reference Samples. 2 mmol of each amino acid and peptide (ε-*N*-acetyllysine, α-*N*-acetyllysine, alanine, valine, leucine, isoleucine, isoleucinylglycin, and isoleucinylglycylglycin, respectively; all from Merck, Darmstadt, Germany; peptides from Bachem, Bubendorf, Switzerland) and 4 mmol of lactose monohydrate were dissolved in a mixture of 11 mL of methanol and 9 mL of DMSO and were heated at 90 °C for up to 8 h under reflux. Solvents were removed under vacuum. The dried residues were dissolved in 20 mL of distilled water. After membrane filtration (0.45 μm), these solutions were used as reference samples containing the original amino acid plus the corresponding Amadori products ("lactulosyl amino acid", AP-solution). To obtain samples containing the corresponding FMAAs, 700 μL of each AP-solution was hydrolyzed with 1380 μL of 12 N HCL in screw-cap test tubes for 23 h at 110 °C. The complete hydrolyzates were centrifugated (10 000 rpm, 20 min), and the supernatant was dried under vacuum. The dry residues were dissolved in 500 μL of 0.2 N hydrochloric acid. After membrane filtration, these solutions were used as reference samples containing FMAAs (FMAA solution). 10 μL of each AP- or FMAA-solution, respectively, was analyzed using RP-HPLC with chemiluminescent nitrogen detector (CLND). For this, an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, USA) consisting of a high-pressure gradient pump system, column oven, automatic injector, and UV-detector was used in combination with a nitrogen-sensitive detector Antek 8060 (Baumel, Houston, Texas). A RP-18 column (Eurospher 100-C18 column, 5 μm, 3 mm × 250 mm, Knauer, Berlin, Germany) was used at a flow rate of 0.2 mL/min. Gradient elution was performed, using water (solvent A) and methanol (solvent B), each containing 2% of formic acid. The following gradients were applied for analyzing the AP-solutions: alanine (0% to 10% solvent B in 25 min, 30 °C), α-*N*-acetyllysine and ε-*N*-acetyllysine (0% to 10% solvent B in 25 min, 50 °C), valine (0% to 15% solvent B in 25 min, 50 °C), leucine and isoleucine (0% to 20% solvent B in 25 min, 50 °C). The following gradients were applied for analyzing the FMAA-solutions: FM-Ala (0% to 10% solvent B in 25 min, 50 °C), α-FM-Lys/furosine (5% to 20% solvent B in 30 min, 50 °C), FM-Val (5% to 40% solvent B in 30 min, 50 °C), FM-Leu and FM-Ile (5% to 50% solvent B in 30 min, 50 °C). A solution of caffeine (1 mg/mL; Fluka, Buchs, Switzerland) was used for external calibration. The concentrations of APs and corresponding FMAAs in the sample solutions were calculated using the theoretical nitrogen content of the corresponding compound. The identity of FMAAs and amino acids was verified by LC-ESI-TOF-MS (system described above).

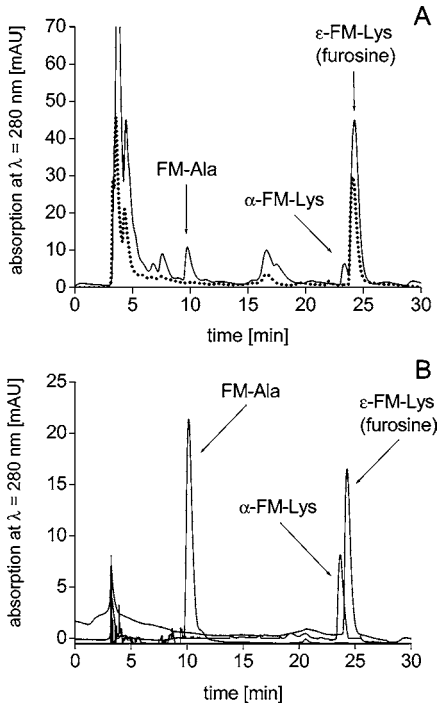

Figure 2. RP-HPLC with UV-detection using an RP-8 column (see Experimental Procedures). (**a**) Acid hydrolysates of a hypoallergenic infant formula (black) and a pre-infant formula (dotted). (**b**) Reference samples for FMAAs obtained after acid hydrolysis of heated mixtures of lactose with α-*N*-acetyllysine, ϵ-*N*-acetyllysine, or alanine, respectively.

RESULTS AND DISCUSSION

Our strategy to obtain information about the extent of N-terminal glycation occurring at the N-termini of peptides in hypoallergenic infant formula was based on the conversion of Amadori products to *N*-(2-furoylmethyl)amino acids (FMAAs) during acid hydrolysis, which could be analyzed and quantified via RP-HPLC with UV-detection using different columns and gradient systems. When using an RP-8 column and elution conditions established for the routine analysis of furosine (*5*), in addition to furosine, two new peaks eluting with retention times of 10.5 and 24.0 min, respectively, could be detected exclusively in acid hydrolyzates of hypoallergenic formulas (**Figure 2a**). Peaks showing identical retention times were detectable in the acid hydrolyzates (FMAA-solutions) of incubation mixtures containing lactose and alanine or ϵ-*N*-acetyllysine, respectively (**Figure 2b**). The two unknown peaks in the hydrolyzates of the milk formulas were unambiguously identified as FM-Ala (*m*/*z* 198.13) and α-FM-Lys (*m*/*z* 255.18) by comparing retention time, UV spectra (typical maximum at 278 nm) as measured via diode array detection, and mass spectra as measured by ESI-TOF-MS with the reference samples. For quantification of FM-Ala and α-FM-Lys, an external standard of furosine was used according to Sanz et al., who successfully applied furosine as a standard substance for the determination of FMAAs in processed tomato products (*10*) and dehydrated fruits (*11*). The amount of FM-Ala in acid hydrolyzed hypoallergenic formulas ranged from 119 to 310 μmol/100 g or 24 to 61 mg/100 g protein and that of α-FM-Lys from 35 to 167 μmol/100 g or 9 to 42 mg/100 g protein, respectively. Thus, the extent of derivatization at the α-amino group of lysine residues was significantly lower as compared to modification at the ϵ-amino group as indicated by a furosine content ranging from 1411 to 2410 μmol/100 g or 359 to 613 mg/100 g protein in hydrolyzates of hypoallergenic infant formulas and 1081 to 1458 μmol/100 g or 275 to 371 mg/100 g protein in conventional infant formulas, respectively (**Table 1**). Minor amounts of furosine up to 18 μmol/100 g or 5 mg/100 g protein have been determined in hydrolyzed samples of human milk. In a recently published study, clearly smaller amounts of FM-Ala (14 mg/100 g protein) have been quantified together with furosine (85−306 mg/100 g protein) and α-FM-Lys (18−24 mg/100 g protein) by Garcia-Banos et al. (*12*) in hydrolyzates of intact protein-containing powdered enteral formulas, indicating that a substantial amount of N-terminal amino acids is modified by reducing carbohydrates during Maillard reactions mainly in peptide-containing hypoallergenic infant formulas. To the best of our knowledge, this is the first report on glycated N-terminally modified peptides in foods. FMAAs resulting from free amino acids have been quantified by Sanz et al. (*10*, *11*) and del Castillo et al. (*13*), using the routine method for furosine analysis established by Resmini et al. (*5*). These authors have reported on the quantification of FM-Ala, α-FM-Lys, FM-Arg, and γ-*N*-(2-furoylmethyl)aminobutyric acid together with furosine in hydrolyzates of processed foods containing mainly polar free amino acids. In hypoallergenic infant formulas, containing a multiplicity of peptides, the main source for the formation of Amadori products are more hydrophobic N-terminally peptide-bound amino acids. For the determination of the more hydrophobic *N*-(2-furoylmethyl) amino acid derivatives, the hydrolyzed samples were analyzed using RP-HPLC on a RP-18 column with UV-detection. The FMAAs resulting from *N*-lactulosylvaline (FM-Val, *m*/*z* 226.16), *N*-lactulosylleucine (FM-Leu, *m*/*z* 240.18), and *N*-lactulosylisoleucine (FM-Ile, *m*/*z* 240.18) during acid hydrolysis were identified exclusively in hypoallergenic formula samples (**Figure 3a**) by comparing retention time, UV spectra (typical maximum at 278 nm), and mass spectra as measured by ESI-TOF-MS with the corresponding reference substances obtained after incubation of the said amino acids with lactose and subsequent hydrolysis (**Figure 3b**). For quantification of FM-Val, FM-Ile, and FM-Leu, an external standard of furosine (elution time 8.2 min, chromatogram not shown) was used. Values of FM-Leu as major derivative ranged from 163 to 396 μmol/100 g or 39 mg to 95 mg/100 g protein in the hydrolyzates of the hypoallergenic infant formulas (**Table 1**). The corresponding values for FM-Ile and FM-Val as further derivatives of essential amino acids were between 105−232 μmol/100 g or 25−56 mg/100 g protein and 69−214 μmol/100 g or 16−48 mg/100 g protein, respectively (**Table 1**). Taking the detection limit of 0.03 μmol/L into account, none of the α-FMAAs was detectable in the conventional infant formula or in the human milk samples. The phenomenon that mainly hydrophobic amino acids were derivatized in the hypoallergenic

Table 1. FMAAs (Means of Triplicates) in Seven Hypoallergenic (HA) Infant Formulas, Measured after Acid Hydrolysis (7.95 N HCL, 110 °C, 23 h), As Compared to Amount of Amino Acid in Whey Protein (*16*) and Calculated Range of Modification of Amino Acids Due to Formation of APs

FMAAs	range of FMAAs in HA infant formulas [μmol/100 g protein]	amount of AAs in whey protein [mmol/100 g]	resultant range of modification of AAs [%]
ϵ-FM-Lys (furosine)	1411–2410	65.7 (Lys)	4.3–7.3
α-FM-Lys	35–167	65.7 (Lys)	0.3–1.4
FM-Ala	119–310	61.7 (Ala)	3.2–8.4
FM-Val	69–214	64.0 (Val)	1.7–5.3
FM-Ile	105–232	53.4 (Ile)	2.8–6.2
FM-Leu	163–396	90.0 (Leu)	2.8–6.7

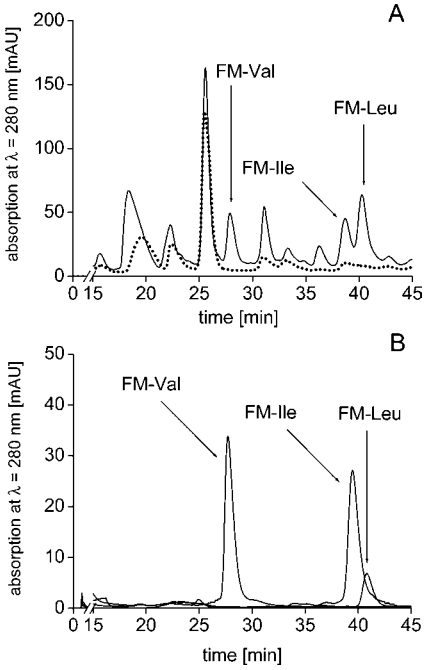

Figure 3. RP-HPLC with UV-detection on an RP-18 column (see Experimental Procedures). (**a**) Acid hydrolysates of a hypoallergenic infant formula (black) and a pre-infant formula (dotted). (**b**) Reference samples for FMAAs obtained after acid hydrolysis of heated mixtures of lactose with valine, isoleucine, or leucine, respectively.

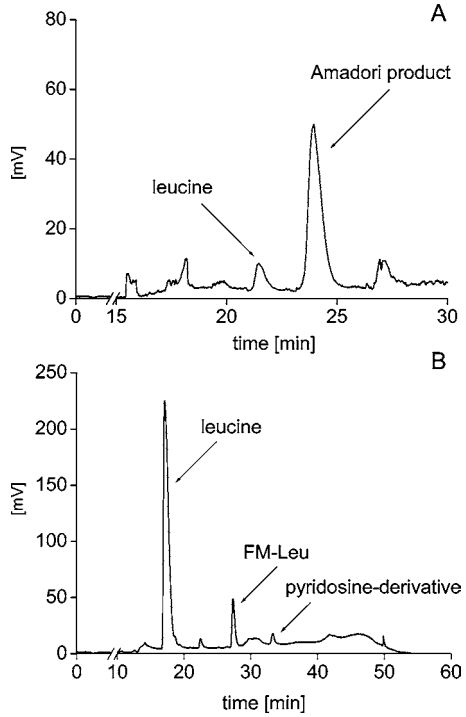

Figure 4. RP-HPLC with N-sensitive detection. (**a**) Heated mixture of lactose with leucine. (**b**) Heated mixture of lactose with leucine after hydrolysis with 7.95 N HCl. Note that elution conditions are different in (**a**) and (**b**) (see Experimental Procedures for details).

samples may be due to the fact that the whey proteins used as starting material were predominantly hydrolyzed in the hydrophobic regions of the proteins to avoid the formation of bitter peptides. Therefore, it can be expected that mainly peptides containing isoleucine, valine, and leucine as N-terminal amino acids are present in the hydrolyzed whey protein of the hypoallergenic infant formulas.

To assess the degree of derivatization due to formation of Amadori products at the N-termini of peptides, it is important to know to which extent APs are converted to FMAAs during acid hydrolysis. For studying this, the synthesis of pure APs and pure FMAAs is usually necessary (*3*). In our work, we used a new approach by comparing absolute nitrogen contents measured via RP-HPLC with chemiluminescent nitrogen detector (CLND) for an AP before hydrolysis and subsequently for the corresponding FMAA present after hydrolysis. The combination of HPLC with CLND is known as a specific and selective chromatography method for analyzing underivatized amino acids even in complex matrices or in peptide hydrolyzates (*14*, *15*). After the identity of FMAAs and amino acids in the sample solutions was verified by LC-ESI-TOF-MS, the concentrations of APs and corresponding FMAAs were calculated using the theoretical nitrogen content of the corresponding compound based on an external calibration with a caffeine standard solution (**Figure 4**). By comparing molar amount of AP before hydrolysis with the amount of the corresponding FMAA after hydrolysis, the conversion factors shown in **Table 2** could be calculated. Using this approach, we found that 50% of *N*-ϵ-lactulosyllysine, which represents the AP of α-*N*-acetyllysine, is converted to furosine during hydrolysis with 7.95 N HCl at 110 °C for 23 h. This value is in perfect agreement with data obtained by Krause et al. (*3*), who had found 46% yield of furosine for *N*-ϵ-fructosyllysine and 50% for *N*-ϵ-lactulosyllysine after hydrolysis of synthetically prepared APs. Compared to this, only 18% of the Amadori product of ϵ-*N*-

Table 2. Yields of FMAAs ± Standard Deviation (6-fold Determination) Formed during Acid Hydrolysis from APs As Measured via CLND, and Conversion Factors Calculated Therefrom

AP of	yield of FMAAs [%]	conversion factors
α-*N*-acetyl-lysine	49.9 ± 0.6	2.0
ϵ-*N*-acetyl-lysine	18.1 ± 0.6	5.5
alanine	6.0 ± 0.3	16.7
valine	6.3 ± 0.5	15.9
isoleucine	6.6 ± 0.1	15.2
leucine	7.0 ± 0.6	14.3

acetyllysine and only 6−7% of the Amadori products of valine, isoleucine, leucine, and alanine, respectively, are converted to the corresponding α-*N*-(2-furoylmethyl)amino acids, indicating significantly lower formation of α-FMAAs from corresponding APs as compared to the formation of furosine from *N*-ϵ-lactulosyllysine in strong acid solutions, probably due to pronounced instability of the α-*N*-glycated amino acids under these conditions. Additional hydrolysis experiments using peptide-bound APs formed from the dipeptides IleLeu or IleGlyGly, respectively, during incubation with lactose gave similar results for FM-Ile formation during acid hydrolysis. Taking these conversion factors into account, it was possible to calculate the amount of N-terminal Amadori products present in the samples before hydrolysis from the amount of FM-AAs. Therefrom, the extent of amino acid derivatization was estimated by comparing the amount of APs with the total amount of the corresponding amino acid, taking the average amino acid content in bovine whey protein into consideration (**Table 1**). Derivatization of N-terminal amino acids was between 1.7% and 8.4%. These data obtained for the essential amino acids alanine, valine, isoleucine, and leucine are comparable to values for lysine measured in conventional infant formulas, demonstrating that N-terminal glycation in peptide-containing foods and resulting

derivatization of essential amino acids must be taken into account as quality parameters to assess the extent of the early stage of the Maillard reaction in heated or stored foods. Special attention should be focused on hypoallergenic infant formulas, which represent the sole intake for infants in the first 3 months. For this food items, possible nutritional implications resulting from N-terminal derivatization of essential amino acids in addition to lysine should be investigated.

In conclusion, it was shown that the extent of early Maillard reactions occurring at the N-termini of peptides is of quantitative importance in foods such as hypoallergenic formulas, which contain a multiplicity of peptides from hydrolyzed whey proteins or casein. The quantification of FMAAs and the calculation of APs therefrom may be a valuable tool to monitor the impact of early Maillard reactions on the nutritional quality.

ABBREVIATIONS USED

AP, Amadori product; FMAA, *N*-(2-furoylmethyl)amino acid; CLND, chemiluminescent nitrogen detection.

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