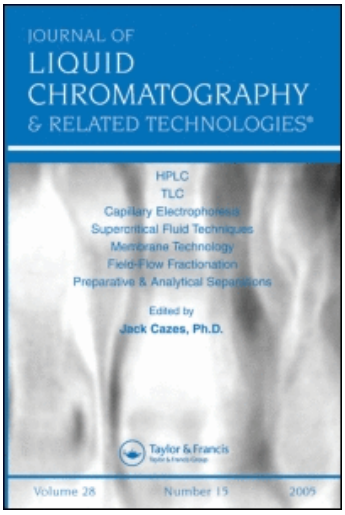

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DETERMINATION OF FUROSINE IN HYDROLYZATE OF PROCESSED MILK BY HPLC USING A NARROW BORE COLUMN AND DIODE-ARRAY DETECTOR

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ABSTRACT

A simple, rapid, sensitive, reproducible, and accurate HPLC procedure employing a narrow bore reversed phase column and a diode-array detector is proposed for the determination of ε-N-2-furoylmethyl-L-lysine (furosine) in acid hydrolyzed of milk and powdered milk samples.

After optimization of the variables involved, the method was characterized and validated in terms of selectivity, repeatability, sensitivity, and accuracy and applied to determine furosine content in hydrolyzed milk samples.

INTRODUCTION

Heat treatment of milk determines significant chemical changes that can result in losses of nutritional quality as well as in organoleptic proprieties of

milk and milk products. The Maillard reaction is one of the most important reactions involved during thermal processing and storage of milk. In the initial step of this reaction the ε-amino group of the lysine residues of milk proteins reacts with lactose to form ε-N-deoxylactulosyl-lysine (known as Amadori product), making lysine nutritionally unavailable.[1] Knowledge of the extent of the Maillard reaction and the level of nutritionally unavailable (blocked) lysine facilitates the evaluation of nutritional quality of processed milk such as pasteurized milk, ultra-high temperature (UHT) milk, in-bottle sterilized milk, or milk powder, both immediately after processing and during storage.

Current analytical methods to evaluate the amount of blocked lysine in processed milk require the transformation of ε-N-deoxylactulosyl-lysine into ε-N-2-furoylmethyl-lysine (furosine) by acid hydrolysis and subsequent determination of this analytical artifact.[2] The amount of furosine has been accepted as a quantitative indicator of the Maillard reaction and as an indicator to study nutritional changes in food by evaluation of the concentration of reactive and blocked lysine.[3]

Several chromatographic methods have been reported for the determination of furosine in the acid hydrolyzed samples. These include methods based on gas chromatography,[4-5] ion-exchange chromatography with commonly used amino acid analyzers,[6-7] and ion-pair reversed phase HPLC.[8-10]

In a recent paper we developed a RP-HPLC method to separate and quantify furosine in concentrated milk,[11] as well as, in milk-based commercial products,[12] employing a microbore column (1.0 mm I.D.) and a UV detector with a flow cell having a volume of 300 nL. With the aim to introduce further improvements directed mainly to increase the sensitivity and the suitability of the use of HPLC column with reduced I.D. coupled to a detector equipped with a conventional flow-cell, we present here a simple and accurate analytical HPLC method using a narrow bore reversed phase column (2.0 mm I.D.) and diode-array detection, for the rapid separation and quantification of furosine in processed milk samples, demonstrating its suitability for routine determinations.

EXPERIMENTAL

Instrument and Column

The chromatographic experiments were carried out using a Shimadzu (Milan, Italy) HPLC system consisting of a Model LC-10AD pump, a Model SPD-M10A diode array detector equipped with a 8 μL flow cell, and a Rheodyne (Cotati, CA, USA) Model 9125 nonmetal poly ether ether ketone

(PEEK) injection valve with a peek 5 μL sample loop. The diode-array detector was operated at 280 nm and spectra were recorded in the range 200-400 nm. Data acquisition and evaluation were performed by a Shimadzu Class 10A data system. The column employed in the experiments was a SGE (Ringwood, Victoria, Australia) glass lined tubing (250 x 2.0 mm I.D.) packed with a 5 μm C-18/P-8/5 polymer coated silica-based octadecyl sorbent.

Chemicals and Samples

All reagents (from Carlo Erba, Milan, Italy) were of analytical or HPLC grade, as required. Furosine of purity ≥99% was supplied by Neosystem Laboratoire (Strasbourg, France). Sample of dried milks and whey were kindly provided by SAGIT (Division of Unilever Italia S.p.A., Cisterna di Latina, Italy). High quality pasteurized milk, UHT milks, and in-bottle sterilized milk were purchased from a local market.

Procedures

HPLC mobile phase was prepared by adding 50 mL of 1 M phosphoric acid to a 250 mL volumetric flask containing HPLC grade water. The pH of this solution was adjusted to pH 3.05 adding the requested volume of 1 M sodium hydroxide aqueous solution, measuring the pH with a glass electrode Model HI 1131 and Model HI 9017 Microprocessor pH-meter, both from Hanna Instruments (Woonsocket, RI, USA). Then the volume was brought to the mark with water and the pH of the solution was controlled again. All solutions were filtered through a type HA 0.45 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sparging with helium before use. Isocratic elution at a flow rate of 150 μL/min, was used throughout the entire study. Furosine was identified by chromatographic comparison with an authentic standard and by its specific UV spectrum.

Sample Preparation

A weighted aliquot of milk or milk powder corresponding to 50 mg of protein (determined by Kjeldhal method) was subjected to acid hydrolysis by adding 6 mL of 10.6 N hydrochloric acid in liquid samples or 8 mL of 8 N hydrochloric acid in solid samples in a screw-cap glass tube. After purging with nitrogen for 2 min, the glass tube was capped and heated at 110°C for 23 hours. The hydrolyzed sample was then filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and injected after diluting 50-fold with HPLC grade water.

Quantitative Analysis

The quantification of furosine was achieved by using the external standard method. The calibration curve constructed from the peak area versus furosine concentration was linear (r = 0.99998) from the quantification limit to at least 0.52 μg/mL of furosine. Recalibration was performed regularly.

For the recovery test, known amounts of furosine were added to the samples which were subjected to the chromatographic analysis. The furosine was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate.

RESULTS AND DISCUSSION

In a previous work[11] we demonstrated that furosine can be efficiently and selectively analyzed in hydrolyzed samples of concentrated milk by a RP-HPLC method, which was accomplished employing a polymer coated silica-based octadecyl stationary phase packed in a 250 x 1.0 mm I.D. microbore column.

The objective of this work was directed towards the suitability of a similar RP-HPLC method for the routine analysis of furosine either in UHT or dried milk samples subjected to acid hydrolysis, using a narrow bore (250 x 2.0 mm I.D.) column. Transfer of the proposed RP-HPLC method from the 1.0 mm I.D. column to the 2.0 mm I.D. column was chosen to evaluate the compatibility of the narrow-bore packed column with a diode array detector fitted with a conventionally size flow cell (8 μL).

The use of the narrow bore column and a mobile phase consisting of a net aqueous solution containing 20 mM phosphate buffer at pH 3.05 produced, under isocratic conditions, a good elution pattern of furosine and its separation from unknown peaks detected in the hydrolyzed samples.

Identification of furosine was performed by characterizing the sample peak in terms of retention time and UV spectrum. A typical chromatogram of a hydrolyzed milk sample in which furosine was detected at 1.48 μg/mL is depicted in Figure 1A. The peak component with a retention time of 13.18 min was compared with a standard sample of furosine (Figure 1B). The two spectra were almost identical (Figure 1C), confirming that the peak component eluting at 13.18 min was furosine. The excellent agreement between standard and sample spectra found in all analyzed sample of milks subjected to acid hydrolysis, indicates that, under the proposed conditions, separation of furosine is not subjected to interference by other components in milk.

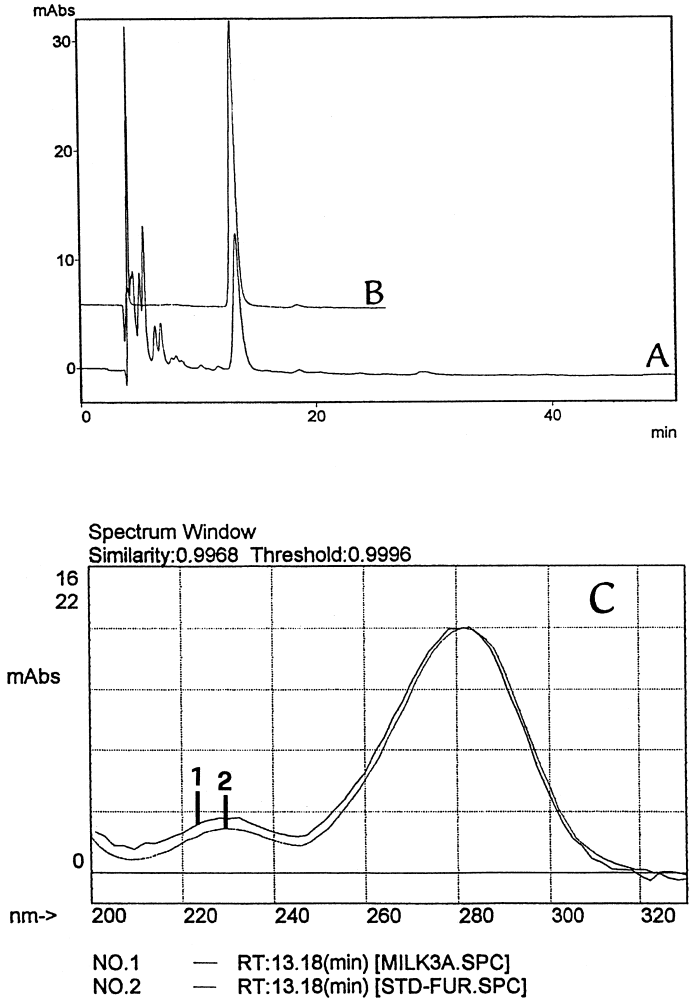

Figure 1. Chromatogram of an acid hydrolyzed sample of powdered milk (A) and of a solution of standard furosine (B). Panel C, UV spectra from 200 to 400 nm of furosine in milk sample (spectra 1) and UV spectra from 200 to 400 nm of furosine standard (spectra 2). Chromatographic conditions: column SGE 5 mm C-18/P-8/5 (250 x 2.0 mm I.D.); mobile phase, 20 mM phosphate buffer pH 3.05; flow rate, 150 mL/min; temperature, 25°C; detection, 280 nm.

Quantitative Determination

Dosage of furosine in hydrolyzed milk samples was performed by the method of the external standard. The calibration graph was obtained by triplicate determinations of the furosine standard at five different concentrations. The peak area values (arbitrary units) were plotted as average value, and the relative percent average deviations of triplicates were less than 2.5% in all cases. The linear regression analysis provided the equation $y = 0.01224 + 69.187x$ with a correlation coefficient of 0.99998, where y is the peak area and x is the concentration of furosine expressed as μg/mL.

Samples of two powdered milks, a powdered milk whey, a high-quality pasteurized milk, three UHT milks, and an in-bottle sterilized milk, subjected to acid hydrolysis, were injected into the HPLC and the average (n = 5) peak areas were computed. The corresponding concentrations of furosine were calculated from the calibration curve and the results are reported in Table 1.

As expected, powdered milk and milk whey show a higher content of furosine than liquid milk samples. Furosine was determined in UHT and sterilized milk samples, whereas, it was not detected in high quality pasteurized milk, which is a fresh selected milk treated under mild conditions in order to avoid or limit the formation of ε-N-deoxylactulosyl-lysine. In liquid milk samples the maximum concentration of furosine was found in the in-bottle sterilized milk, whereas UHT milks showed a lower furosine content ranging from 6.0 to 8.3 mg per 100 g of product.

Table 1

Quantitative Determination of Furosine in the Analyzed Samples

Product	Furosine mg/100 g Product ± S.D.
High Quality Pasteurized Milk	n.d.*
UHT_1 Milk	8.3 ± 0.28
UHT_2 Milk	6.0 ± 0.12
UHT_3 Milk	6.6 ± 0.25
In Bottle Sterilized Milk	18.8 ± 0.05
Powdered Milk 1	96.4 ± 1.27
Powdered Milk 2	170.2 ± 1.67
Powdered Whey	61.6 ± 0.39

* n.d. = not detected.

Validation

Validation parameters such as specificity and linearity have been discussed above and documented. The limit of detection value (LOD) was calculated as the amount of the injected sample which gave a signal-to-noise ratio of 3. The limit of quantitation (LOQ) of furosine was set at the concentration of the lowest calibration standard with a RSD better than 3% and a signal-to-noise ratio of at least 10:1. The LOD and LOQ values obtained were 0.4 ng and 0.15 μg/mL, respectively.

In order to determine the suitability of the HPLC system using the narrow bore column with the diode-array detector equipped with conventional size cell, aqueous solutions of standard furosine at five different concentrations (5.2, 2.6, 1.3 0.65, 0.325 μg/mL) were prepared on three different days and analyzed ten times each day with the HPLC procedure. The relative standard deviations for the measured peak areas for the three days are reported in Table 2 and show that there is little variability in the instrumental response.

Table 2

Intraday and Interday Peak Area Repeatability of Furosine Standard at Five Different Concentrations

Concentration (μg/mL)	0.32	0.64	1.28	2.56	5.12
Day 1	2215[a]	4429	8841	17713	35425
S.D.	45.8	66.5	112.2	195.8	273.5
R.S.D.%	2.10[a]	1.50	1.27	1.10	0.77
Day 2	2268	4501	8972	17519	35155
S.D.	65.77	77.42	140.87	261.3	482.5
R.S.D.%	2.90	1.71	1.57	1.49	1.37
Day 3	2198	4387	8821	17649	35251
S.D.	59.4	94.32	161.42	278.25	405.40
R.S.D.%	2.7	2.15	1.83	1.58	1.15
Mean	2227	4439	8878	17627	35277
S.D.	36.51	57.65	82.01	98.85	136.86
R.S.D.%	1.64	1.30	0.92	0.56	0.39

[a]Entries in the upper part of the table are mean values of ten repeated injections. Summary statistics are included in the lower part of the table.

Table 3

Recovery of Furosine from a UHT Milk

Amount in Sample	Added[a]	Found[a]	Recovery	R.S.D. (%)
8.30	0.8	9.39	103.19	2.77
	1.6	10.19	102.93	2.81
	2.4	10.97	102.52	2.34

[a] mg of furosine/100 g of product.

Recovery experiments were carried out by adding known amounts of furosine to aliquots of two milk samples. Furosine was spiked at three different concentrations for a UHT milk and an in-bottle sterilized milk sample. Typical recoveries, determined by comparing the found and calculated amounts of furosine after spiking, are reported in Table 3 and in Table 4. It can be seen that the average recoveries lied between 97.15% and 103.19%, indicating that the method has an adequate degree of accuracy for the determination of furosine in the analyzed samples.

To evaluate the precision of the method, within-run and between-run relative standard deviations were calculated in samples having different concentrations of furosine. The within-run precision, performed by repeated injections (n = 5) of aliquots of the same milk sample with 8.31 mg of furosine per 100 gram of product was 2.85%. The between-run precision, obtained from analy-

Table 4

Recovery of Furosine from Bottle Sterilized Milk

Amount in Sample	Added[a]	Found[a]	Recovery	R.S.D. (%)
18.80	1.2	19.43	97.15	2.87
	2.4	21.75	102.60	2.61
	3.6	21.95	97.99	2.15

[a] mg of furosine/100 g of product.

Table 5

Inter-Day Precision for the Determination of Furosine in Processed Milk Samples

Day	UTH Milk (Mean Value[a,b] ± S.D.)	R.S.D. (%)	In Bottle Sterilized Milk (Mean Value[a,b] ± S.D.)	R.S.D. (%)	Powdered Milk[a] (Mean Value[a,b] ± S.D.)[a]	R.S.D. (%)
1	8.31[b] ± 0.28	3.36	18.80 ± 0.05	0.27	96.40 ± 1.27	1.31
2	8.62 ± 0.29	3.36	18.47 ± 0.11	0.59	98.11 ± 1.38	1.41
3	7.97 ± 0.27	3.39	19.37 ± 0.08	0.41	94.11 ± 1.32	1.40
4	8.43 ± 0.29	3.44	19.31 ± 0.22	1.14.	97.87 ± 01.69	1.73
5	8.69 ± 0.30	3.45	19.32 ± 0.08	0.41	97.61 ± 1.38	1.41
Mean	8.38		19.05		96.82	
S.D.	0.263		0.40		1.65	
R.S.D. (%)	3.14		2.1		1.70	

[a] mg of jurosine/100 g of product. [b] Mean values of five repeated injections.

ses of the same sample repeated on five subsequent days was 3.14% It is evident from the results reported in Table 5 that the assay method is sufficiently precise for quantitative analysis of furosine in hydrolyzed milk samples.

CONCLUSION

A precise, accurate, and rapid HPLC method using a narrowbore RP-column under isocratic conditions and employing diode-array detection, has been developed for the determination of furosine in hydrolyzed of processed milks. The narrowbore format of the column advantageously resulted in detecting furosine by a diode-array detector having a conventional 8 μL flow-cell.

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