Determination of Furosine in Milk Samples by Ion-Pair Reversed Phase Liquid Chromatography

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Key Words

Column liquid chromatography
Ion-pairing
Furosine
Pyridosine
Milk

Summary

An ion-pair, reversed-phase, liquid chromatographic procedure using UV detection for quantitation of furosine is described. The standard plot was linear ($r > 0.999$) over a 5 ng range. An authentic synthesised sample of furosine was used for calibration. Commercial milk samples were analyzed by the described procedure.

Introduction

Heat treatment of milk causes significant changes in milk proteins that can result in losses of their nutritional quality as well as changes of their functional properties. Estimation of the extent of protein damage induced by heating has been the subject of many papers. Some methods are based on the evaluation of the extent of the Maillard reaction by measuring the furosine present in acid hydrolyzed milk.

Most of the first reported methods for furosine analysis are based on the use of ion-exchange chromatography [1–7]. Some of these methods give satisfactory results but are time-consuming due to the tedious column regeneration and post-column derivatization. This inconvenience is eliminated when furosine is analyzed by reverse-phase high-performance liquid chromatography (HPLC).

Recently, Resmini et al. [8] have reported an ion-pair, reversed phase method under gradient elution conditions which allows the determination of furosine at concentrations as low as those present in pasteurized milks. These authors suggested that under previously reported isocratic conditions [9–11] furosine is unlikely to be eluted as a pure peak.

This paper describes the results of a study to find a rapid quantitative isocratic HPLC method for the determination of furosine in milk.

Experimental

Furosine and pyridosine were obtained by acid hydrolysis of ε-N-deoxyfructosyl-lysine (I) according to the procedure of Finot et al. (1968) [12]. 500 mg of (I) were hydrolyzed by refluxing in 500 mL 6M HCl for 24 h, and then evaporated under vacuum to dryness. The dried mixture was chromatographed on a Dowex 50WX4 column (110 × 2.5 cm) in the H^+ form. Elution was with 2M HCl and 20 mL fractions were collected. Fractions were analyzed by HPLC and those showing a single peak were combined, evaporated to dryness at room temperature and then lyophilized.

Nuclear Magnetic Resonance

^1H NMR spectra were from a Varian XL-300 spectrometer operating at 300 MHz using D_2O as solvent and DSS as internal standard. Typical conditions were: pulse angle, 77°; acquisition time 4 s; sweep width 4000 Hz and data size 32 K.

High-Performance Liquid Chromatography (HPLC)

The chromatographic system consisted of a Model 510 pump (Waters Assoc.), a Model 7125 Rheodyne injector and a variable-wavelength UV detector Model SM 4000 (LDC Analytical) connected to a System Gold (Beckman) for data output. A Spherisorb ODS2 5 µm column (0.46 × 25 cm) (Phenomenex) operated at ambient temperature was used in the analysis. The mobile-phase system consisted of 5 mM sodium heptanesulphonate with 20 % acetonitrile as organic modifier and 0.2 % formic acid. The flow rate of elution was 1.2 ml min^{-1}. The UV detector was set at 280 nm.

Calibration curves were constructed by plotting absorbance, expressed in units of area versus µg pure furosine or pyridosine in aqueous solution. A third calibration curve was constructed for furosine by adding to a previously hydrolyzed sample of raw milk increasing quantities of

0009-5893/92/4 0374-03 $ 03.00/0

furosine within the range expected in milk for this compound.

Precision of the whole assay procedure was tested by subjecting seven portions of each milk sample to the entire assay procedure and determining the coefficent of variation (CV%).

Treatment of Milk Samples

Dry samples corresponding to 15–20 mg protein were hydrolyzed in the presence of 3 ml 6M HCl (for liquid milk samples 7M HCl was used to obtain a 6M concentration in the mixture). Hydrolysis was performed at 110 °C for 24 h in evacuated and sealed tubes. The hydrolyzate was evaporated to dryness in a SpeedVac Concentrator A-160 (Savant). The dried sample was dissolved in 0.5 ml water and this solution was passed through a pre-wetted Sep-pak C_{18} cartridge (Millipore) and washed with 4.5 ml water : acetonitrile : formic acid (95 : 5 : 0.2) prior to HPLC analysis.

Results and Discussion

Isolation of Furosine and Pyridosine

Furosine was eluted in the 170–195 fractions of the Dowex 50WX4 column and pyridosine was found in the 225–240 fractions. The ^{1}H NMR spectrum of furosine showed multiplets corresponding to 14 protons. The assignment is straightforward. At low field three multiplets appear showing chemical shifts and coupling constants in concordance with a 2-substituted furan ring: δH3 7.64 ppm [^{3}J (H3, H4) = 4.2 Hz ^{4}J (H3, H5) = 0.8 Hz], δH4 6.82 ppm [^{3}J (H3, H4) = 4.2 Hz ^{3}J (H4, H5) = 1.7 Hz] and δH5 7.97 ppm [^{3}J (H4, H5) = 1.7 Hz ^{4}J (H3, H5) = 0.8 Hz]. The remaining signals are due to the chain protons of the molecule: 4.70 ppm (CO-CH_2, singlet), 4.05 ppm (CH-α triplet), 3.28 ppm (CH_2-ε triplet), 2.06 ppm (CH_2-δ multiplet), 1.91 ppm (CH_2-β doublet of triplets) and 1.6 ppm (CH_2-γ multiplet). In the same way the ^{1}H NMR spectrum of pyridosine showed multiplets corresponding to 14 protons. Signals at 8.13 ppm (H3, singlet) and 7.18 ppm (H6, singlet) are assigned to the two uncloupled protons in the ring and signal at 2.66 ppm (CH_3, singlet) to the 2-methyl substituent. Signals at 4.36 ppm (CH_2-ε, triplet), 4.23 ppm (CH-α, triplet), 2.1 ppm (CH_2-β and CH_2-δ, multiplets) and 1.6 ppm (CH_2-γ, multiplet) correspond to the chain protons of the lysine moiety. These results are in agreement with the previously reported data for furosine [12, 13] and pyridosine [14].

The UV spectrum of furosine in water showed two maxima at 280 and 224 nm and that of pyridosine showed maxima at 276 nm in neutral solution, 276 and 246 nm in acidic solution and 330 nm in basic solution.

The ratio of absorbance in water, 280/254, nm was 2.9 for furosine and 1.2 for pyridosine.

Figure 1 demonstrates the chromatograms obtained by injection of furosine and pyridosine standards as well as from the hydrolysis products of milk samples. As shown, the furosine standard in this system was found to have a retention time of 10.5 min. and pyridosine was found to have a retention time of 1.5 min.

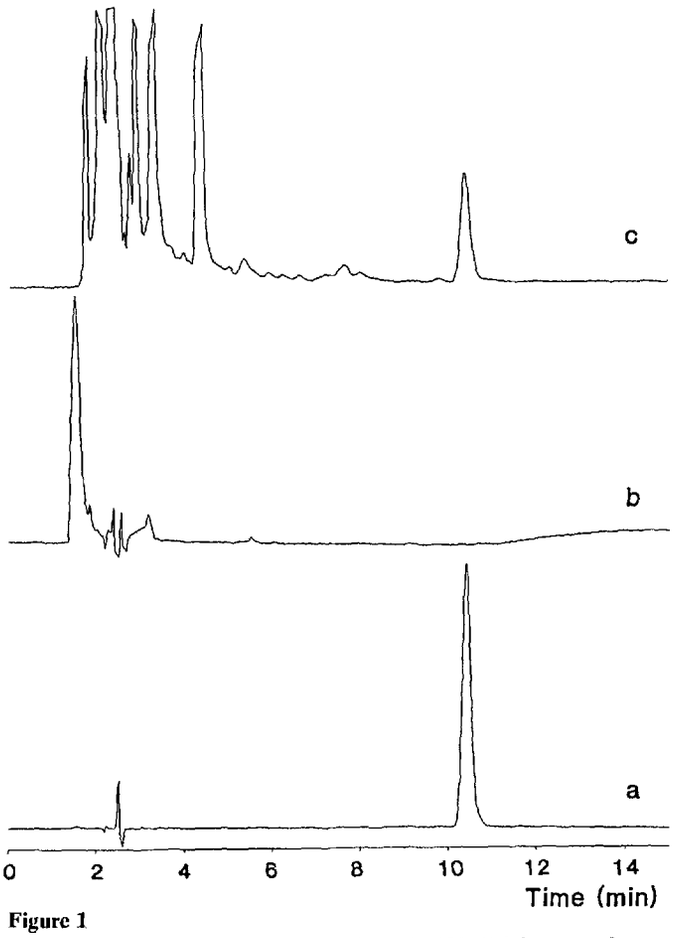

Figure 1

HPLC chromatograms: a) furosine eluted in 170–195 fractions from Dowex 50 W × 4 column (retention 10.5 min.); b) pyridosine eluted in 225–240 fractions from same column (retention 1.5 min.); c) acid hydrolyzate of indirect UHT milk sample.

Response linearity for aqueous solutions of pure furosine and pyridosine was evaluated. A plot of peak area versus μg furosine or pyridosine injected was found to obey the following equations:

$y = 71.93 \times - 0.1446$

($r = 0.999$; standard error 0.0024) for furosine

$y = 2.971 \times - 0.0776$

($r = 0.998$; standard error 0.0298) for pyridosine

where y is integration units, and x is the amount of compound injected (μg). The equation indivcate that, for equal amounts injected, the peak area of furosine is about 25 times that of pyridosine. These results explain the presence of pyridosine as a trace peak in previously reported HPLC determinations of furosine [8, 9] in spite of the high values found by other methods [15, 16]. In this system, the minimum detectable levels of furosine and pyridosine injected were 5 and 60 ng respectively, defined as twice the standard error.

The precision of the instrument was determined with both small (30 ng) and large amounts (80 ng) of furosine. The coefficient of variation (CV%) for seven replicates was determined to be in the range 0.95–0.68 %.

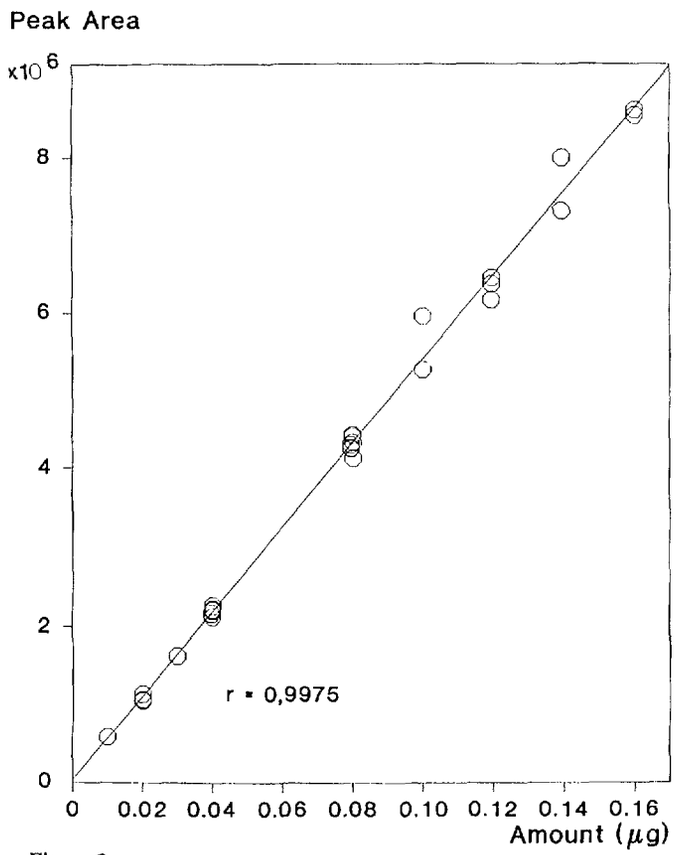

Figure 2

Calibration for peak area response in range 10–160 ng furosine injected. Regression equation: y = 51.1408 x + 0.04058; r = 0.9975.

Table 1 Repeatability of complete assay procedure for same-day analysis in three different types of commercial milk (n = 7)

Sample	Furosine (mg L^{-1})	CV (%)*
Direct UHT	30.21	8.21
Indirect UHT	39.44	4.37
Sterilized	76.97	7.05

* Statistical significance level: 95 %.

Results of calibration carried out by adding increasing quantities of furosine to a hydrolyzed raw milk sample is shown in Figure 2. A linear response was obtained with high correlation coefficient. The object of constructing a third calibration curve was to see the effect of the treatment of hydrolyzed milk sample on the slope and other parameters of the calibration curve. As shown in Figure 2, the slope of the calibration curve is lower than the slope in pure water solutions of furosine. This indicate that some of the furosine may be lost during treatment of the hydrolyzed milk sample before being injected.

Repeatability of matrix-dependent imprecision of the whole assay procedure for the same day analysis expressed as CV (%) is shown in Table I.

Three samples containing 29, 38 and 72 mg L^{-1} furosine analyzed every day for five days showed a CV (%) of 1.83, 1.06 and 2.10 respectively.

This study demonstrates that the present method can be used for determination of furosine in milk for routine work. Quantitative determination of pyridosine in milk was not possible due to the raised amount at minimum detectable level and interference with other reaction products.

Acknowledgement

This work was supported by Comision Interministerial de Ciencia y Tecnología (CICYT), Project number Ali-88-0158-CO2-02.

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Received: Nov. 29, 1991
Revised manuscript received: Jan. 29, 1992
Accepted: Jan. 31, 1992