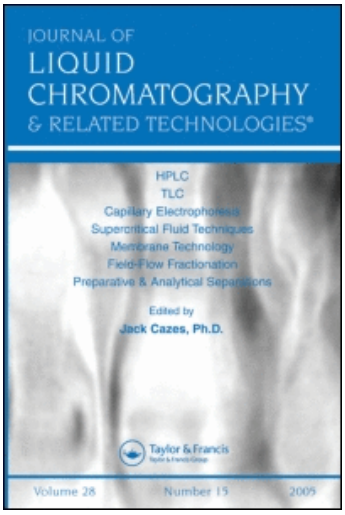

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I. Nicoletti[a]; E. Cogliandro[a]; C. Corradini[a]; D. Corradini[a]
[a] Istituto di Cromatografia del C.N.R. Area della Ricerca di Roma, Rome, Italy

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ANALYSIS OF ε-N-2-FUROYLMETHYL-L-LYSINE (FUROSINE®) IN CONCENTRATED MILK BY REVERSED PHASE CHROMATOGRAPHY WITH A MICROBORE COLUMN

I. Nicoletti,* E. Cogliandro, C. Corradini, D. Corradini

Istituto di Cromatografia del C.N.R.
Area della Ricerca di Roma
P.O. Box 10
I-00016 Monterotondo Stazione, Rome, Italy

ABSTRACT

This paper reports the results of a study aimed to develop a rapid, accurate and sensitive HPLC method for routine analysis of ε-N-2-furoylmethyl-L-lysine (furosine) in acid hydrolyzed of processed food products. The method uses a microbore reversed phase HPLC column packed with a polymer coated silica-based octadecyl sorbent (5 μm, 250 x 1 mm I.D) eluted with a mobile phase consisting of 20 mM phosphate buffer, at pH 2.5. The identification of furosine was performed by UV detection at 280 nm in a micro flow cell of 300 nL. The limit of detection was 0.3 ng based on a signal to noise ratio of 3 with a sample volume of 0.5 μL. The separation was achieved by isocratic elution within 15 min. This method was successfully applied to the identification and quantitative determination of furosine in a sample of concentrated milk hydrolyzed with hydrochloric acid. Dosage was performed by the method of external standard.

INTRODUCTION

The Maillard reaction (non-enzymatic browning) represents a complex series of degradative reactions involving carbonyl and amino functionalities which lead to the production of polymeric pigments, many flavor and aroma constituents and other degradation products. In milk and dairy products, as well as other food products, the Maillard reaction is initialized by the reaction between the carbonyl group of a reducing carbohydrate and the amino functions of a amino acid or protein with the formation of a glycosamine which is then transformed to a ketosamine by Amadori rearrangement. One of the most stable Amadori compounds in these food products is represented by ε-deoxy-ketosyl-lysine.[1] The lysine blocked in this Amadori compound is not nutritionally available. Current analytical methods to evaluate the extent of the Maillard reaction and the level of nutritionally unavailable lysine in processed food require the transformation of ε-deoxy-ketosyl-lysine into ε-N-2-furoylmethyl-L-lysine (furosine) by acid hydrolysis and subsequent determination of furosine.[2] The level of furosine has been accepted as an indicator of the Maillard reaction and as an indirect parameter to study possible changes in food nutritional quality by evaluation of the concentration of reactive and blocked lysine.

Chromatographic methods have been proposed for the determination of furosine in the acid hydrolyzed samples. These techniques include gas chromatography,[3-5] ion-exchange chromatography (ICE) with commonly used amino acid analyzers,[6-7] and ion pair reversed phase HPLC.[8-11] These methods differ in sensitivity and reproducibility, are not free from interferences, and some of them are time-consuming due to pre- or post column derivatization, pretreatment of sample and gradient elution.

In recent years increasing attention has been given to the use of micro-HPLC columns in a variety of applications in food analysis.[12-15] This interest is due to several advantages that micro-HPLC has compared to conventional HPLC. Recognized advantages using miniaturized HPLC techniques are the increased mass sensitivity, the higher peak efficiency, the low consumption of mobile phase, the low heat capacity and the smaller sample volume required for analysis.[16-19]

This paper reports the results of a study performed to develop an accurate and straightforward chromatographic method for the identification and dosage of furosine in food employing a reversed phase microbore column with isocratic condition and multiwavelength UV detection. In order to select the optimum

chromatographic elution to perform a rapid and reproducible separation, the effect of pH and temperature on the retention time and selectivity were examined. The optimized method was applied to the identification and dosage of furosine in a sample of concentrate milk hydrolyzed with hydrochloric acid.

MATERIALS AND METHODS

Instrument and Column

The chromatographic experiments were carried out using an HPLC system equipped with a Model 421A microprocessor controller and a Model 114M single-piston reciprocating pump with the capability of delivering micro flow rates, all from Beckman Instruments, Inc. (Fullerton, CA, USA); a 7520 Rheodyne (Cotati, CA, USA) microsample injector with a 0.5 μL sample rotor in peek and a Model 433 variable-wavelength detector with a standard micro flow cell (300 nL), in conjunction with a Data System 450 software, both from Kontron Instruments (Milan, Italy). A circulating water bath (Haake, Berlin, Germany) with variable temperature control was used to thermostat the temperature of the column and the eluent reservoir, which were enclosed in 500 mL and 1 L water jacked, respectively.

The column employed in the experiments was a SGE (Ringwood, Victoria, Australia) glass lined tubing (250 x 1 mm I.D.) packed with 5 μm C-18/P-8/5 polymer coated silica-based octadecyl sorbent.

Chemicals

Furosine of purity >99% was supplied by Neosystem Laboratoire (Strasbourg, France). Reagent-grade phosphoric acid, acetic acid, hydrochloric acid, sodium hydroxide and HPLC-grade water were obtained from Carlo Erba (Milan, Italy). Sample of concentrated milk was purchased from a local store.

Mobile phases were prepared by adding the correct volume of either phosphoric or acetic acid to a volumetric flask containing HPLC grade water. The pH was adjusted to the appropriate value with 0.1 M sodium hydroxide solution and measured with a glass electrode, Model HI 1131, and Model HI 9017 Microprocessor pH-meter, both from Hanna Instruments (Woonsocket, RI). All solutions were filtered through a type HA 0.45 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sparging with He before use.

Sample Preparation

A weighted aliquot of concentrated milk corresponding to 50 mg of protein (determined by Kjeldhal method) was submitted to acid hydrolysis by adding, in a screw-cap glass tube, 8 mL of 8 N hydrochloric acid. After purging with nitrogen for 2 min, the glass tube was capped and heated at 110°C for 23 h.[9] The hydrolyzed sample was then filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and injected prior to diluting 50-fold with water.

Quantitative Analysis

A stock standard solution was prepared by dissolving in water the appropriate amount of furosine. Working standard solutions at five different concentrations ranging from 1.19 to 23.8 μg/mL were prepared by diluting the stock standard solution with water. The calibration graph was obtained by the peak-area method analyzing each working standard solution in quintuplicate.

RESULTS AND DISCUSSION

The goal of this study was to develop a method for the rapid, selective and accurate analysis of furosine by using a microbore reversed phase column packed with a polymer coated silica-based octadecyl stationary phase. In order to find optimal isocratic conditions, a study of the influence of the mobile phase composition and of the temperature on the chromatographic behavior of furosine was undertaken. Experiments were performed under isocratic conditions using as eluent neat aqueous buffer solutions at various pH values ranging from 2.5 to 5.8. In order to cover this range, either sodium phosphate or acetate buffer was employed in the pH region where they posses adequate buffering capacity.

The effect of varying the pH of the mobile phase on the retention behavior of furosine is depicted in Figure 1. It is observed that the retention time of furosine is not greatly affected up to pH 4.5. Above this pH value, the retention time dramatically increases in accordance with a corresponding lower degree of protonation of the lysine residue of furosine at higher pH values which is aspected to improve the hydrophobicity of the analyte.

The effect of the temperature on the chromatographic retention of furosine was investigated in order to find optimal analysis time with mobile phases containing 20 mM phosphate buffer at pH 2.5 3.5 or 5.8. Under this

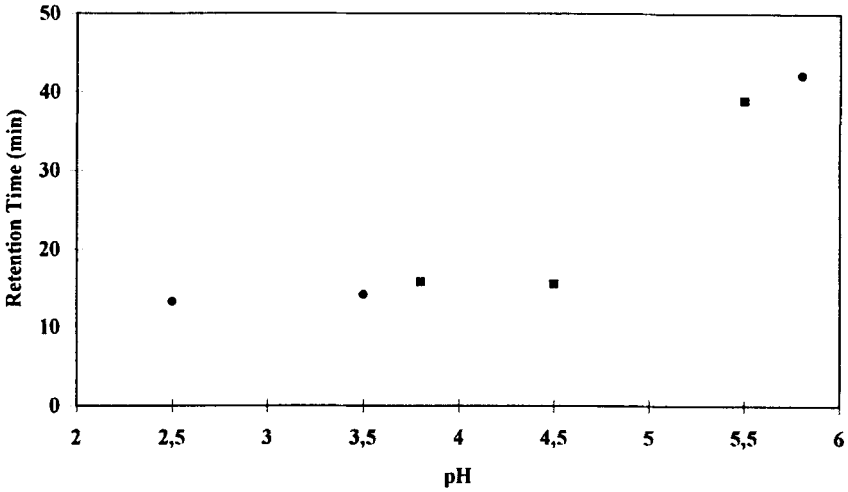

Figure 1. Dependence of furosine retention time on the pH of the mobile phase consisting of net aqueous solutions containing either 20 mM phosphate (●) or 20 mM acetate (■) buffer. Column SGE 5 μm C_{18}/P-8/5 (250 x 1 mm I.D.); flow rate 40 μl/min; temperature 25°C; detection, 280 nm.

experimental conditions the retention time decreased with increasing temperature and plots of the logarithmic retention factor versus reciprocal temperature yielded straight lines with correlation coefficients better than 0.9996, as it is shown in Figure 2.

In reversed phase chromatography the enthalpy changes associated with the reversible binding of the hydrophobic analyte by the hydrocarbonaceous stationary phase are in most cases aspected to be temperature independent and can be evaluated by the slope of the plots in Figure 2 which are referred to as van't Hoff plots.[20] Despite the relatively narrow temperature range (15°C) over which the van't Hoff plots of Figure 2 were obtained, we calculated the enthalpy of binding as reported above in order to gain some informations on the energetics of the furosine retention in the reversed phase column employed in this study. The values of the enthalpy of binding for the analyte eluted at pH 3.5 and 5.8 resulted to range from 1.52 to 1.54 kcal/mol, respectively. Such low values of enthalpy of binding are in accordance with the hydrophilic character of furosine which is eluted from the hydrophobic stationary phase with neat aqueous mobile phases. On the other hand, the almost identical value of the enthalpy of binding cannot explain the relatively large differences in the logarithmic retention factor obtained at pH 5.8. This may be related to changes

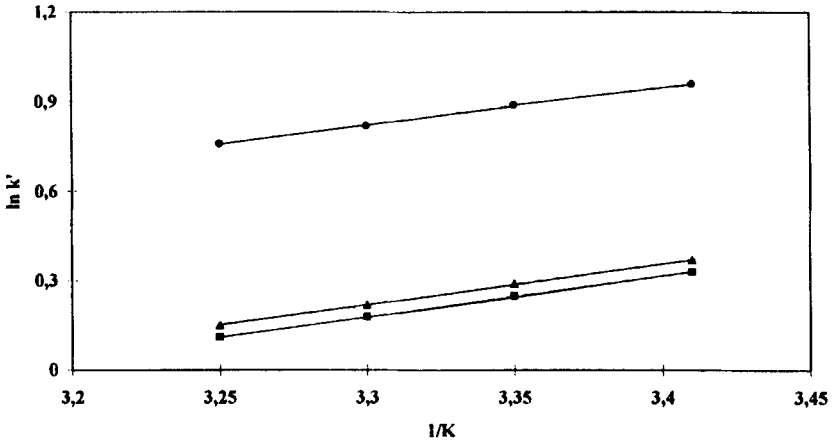

Figure 2. van't Hoff plots of furosine eluted at pH 2.5 (■), 3.8 (▲) and 5.8 (●). Experimental condition as in Figure 1.

in the enthalpy of binding which accompany the corresponding changes in the entropy of binding and that may be caused by the variation in the pH of the mobile phase. However, the evaluation of the corresponding variations of the entropy of binding is not possible owing to the unknown value of the phase ratio.

Further experiments were performed with the neat aqueous mobile phase containing 20 mM phosphate buffer at pH 2.5 and with column temperature controlled at 25°C. The precision of the method was evaluated in intraday and interday measurements of the retention time by repeated injections of an aqueous solution containing 0.01 mg/mL of standard furosine, using the same apparatus and column (see Table 1). Data obtained by two different operators were used for the evaluation of the interday precision. The interday data were acquired over a period of 10 days. The results presented in Tables 1 and 2 show that the intraday repeatability was 0.37 % whereas the interday precision was better than 0.75 %.

The same retention time with comparable repeatability was found in samples of concentrated milk subjected to acid hydrolysis, either with or without the addition of known amounts of standard furosine, ensuring sufficient peak identification. Dosage was performed by the method of external standard. The repeatability of the determination and linearity of the calibration graph were determined by analyzing solutions of standard furosine

Table 1

Retention Time, Standard Deviation (S.D.), and Relative Standard Deviation (R.S.D.) of Multiple Injections of a Standard Solution of Furosine. Chromatographic Conditions as in Figure 3.

Retention Time (min)				Mean (min)	S.D. (min)	R.S.D. (%)
13.21	13.18	13.24	13.38			
13.22	13.24	13.25	13.26			
13.26	13.21	13.31	13.30			
13.31	13.28	13.33	13.36	13.265	0.049	0.37

Table 2

Retention Time, Standard Deviation (S.D.), and Relative Standard Deviation (R.S.D.) of Multiple Injections of a Standard Solution of Furosine Performed in Ten Days. Chromatographic Conditions as in Figure 3.

Day	Mean Retention Time* (min)	S.D. (min)	R.S.D. (%)
1	13.427	0.100	0.75
2	13.219	0.052	0.39
3	13.235	0.031	0.23
4	13.224	0.048	0.36
5	13.237	0.054	0.41
6	13.360	0.047	0.35
7	13.392	0.082	0.62
8	13.371	0.073	0.56
9	13.332	0.086	0.65
10	03.307	0.036	0.27

* Mean of eight injections

at five different concentrations ranging from 1.19 to 23.8 μg/mL. The sample concentration was limited to the above range in order to avoid peak tailing and retention time shifting which occur when the sample size approaches the column sample load capacity,[21] which is a critical parameter for microbore columns.

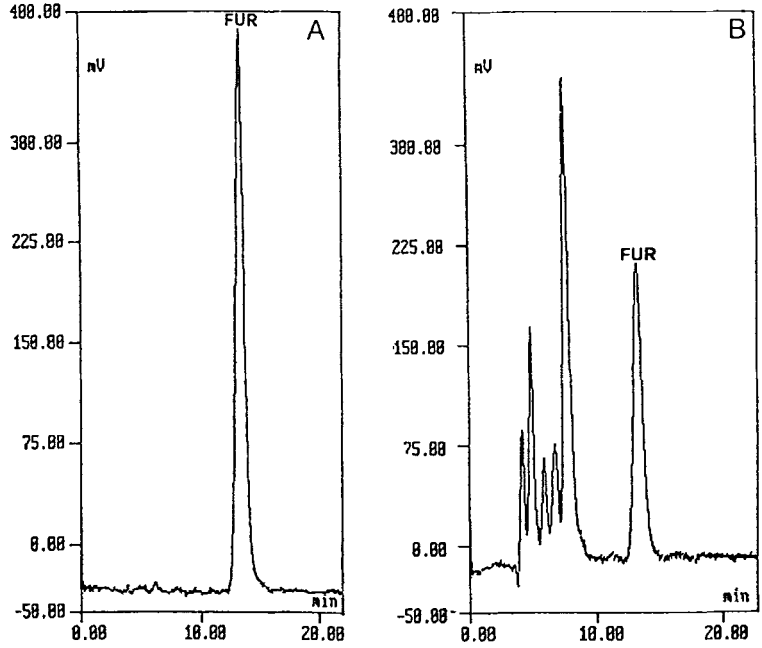

Figure 3. Chromatogram of a solution of standard furosine (panel A) and of an acid hydrolyzed sample of concentrated milk (panel B). Mobile phase, 20 mM phosphate buffer in water (pH 2.5); other conditions as in Figure 1.

Table 3

Reproducibility of Peak-Area and Peak-Height Mode

Concentration Furosine μg/mL	Mean Peak-Area mV×min	S.D. mV×min	R.S.D. (%)	Mean Peak-Height mV	S.D. mV	R.S.D. (%)
1.19	42.09	1.246	2.96	52.50	3.073	5.85
2.38	87.32	1.588	1.82	103.5	3.144	3.05
5.94	213.56	3.228	1.51	250.86	4.661	1.86
11.90	412.46	1.935	0.47	485.48	6.400	1.32
23.80	779.54	18.779	2.41	900.73	17.104	1.90

Table 4

Recovery of Furosine from a Concentrated Milk

Amount in Sample (ng)	Added (ng)	Found (ng)	Recovery (%)	R.S.D. (%)
2.484	0.476	2.948	99.58	0.256
	0.952	3.550	103.31	0.113
	1.428	4.062	103.84	0.112

The peak area mode gave a higher repeatability than the peak height mode (see Table 3) and for this reason was shown as the method of analysis. The linear regression analysis provided the equation $y = 12.9771 + 32.5167x$ with correlation coefficient of 0.9992, where y is the peak area and x is the concentration of furosine in μg/mL.

The limit of detection was defined as the amount of injected sample which gave a signal to noise ratio of 3 and was determinated to be 0.3 ng.

The accuracy of the method was evaluated by determining the recovery of furosine in a sample of concentrated milk of a known level of furosine. Three different amounts of standard furosine were added to the sample which was subjected to the chromatographic analysis. The recovery was calculated based on the difference between the total concentration determinated in the spiked samples and the concentration dose in the non spiked samples.

Results with the relative standard deviations are reported in Table 4. The relative standard deviation of the determinations for each concentration was better than 0.256 % and the mean recovery ranged from 99.6 to 103.8, indicating a high degree of accuracy.

The method was applied to the analysis of furosine in sample of concentrated milk subjected to acid hydrolysis. A typical chromatogram is displayed in Figure 3 and shows that unknown peaks detected in the sample did not interfere with the identification and dosage of furosine. The analysis in triplicate resulted in an average dosage of 1.38 g /100 g of proteins with repeatability of data better than 0.61 % (RSD).

CONCLUSIONS

This study has proven that furosine can be efficiently and selectively analyzed in samples of concentrated milk subjected to acid hydrolysis by a simple, rapid and straightforward method employing a polymer coated silica-based octodecyl stationary phase packed in a microbore column.

The microbore format of the column advantageously resulted in detecting furosine, which elutes as a very efficient and resolved peak with highly repeatable retention time and mass recovery, at level as low as 0.3 ng. It is also notable that no column pretreatment is necessary.

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