

Influence of Hydrolysis, Purification, and Calibration Method on Furosine Determination Using Ion-Pair Reversed-Phase High-Performance Liquid Chromatography

M.A. Serrano, G. Castillo, M.M. Muñoz, and A. Hernández*

Departamento de Nutrición y Bromatología, Campus Universitario, Universidad de Alcalá, 28871-Alcalá de Henares (Madrid), Spain

Abstract

The influence of HCl concentration (6M, 8M, and 10M) and the ratio of sample protein to acid (1 or 5 mg of protein per mL of acid) on furosine formation during sample hydrolysis is studied. The conditions that maximize furosine formation are 10M HCl in the ratio of 1 mg of protein to 1 mL of acid. Purification of the hydrolysate by solid-phase extraction is also considered by examining the effect of hydrolysate volume and volume of 3M HCl used to elute the furosine. Furosine quantitation is carried out using the standard additions and external standard methods. The results indicate that there is no interference by the sample matrix and that external calibration is adequate.

Introduction

Furosine (ϵ -*N*-(2-furoylmethyl)-L-lysine) is an amino acid formed during the acid hydrolysis of such Amadori products as fructoselysine, lactuloselysine, and maltuloselysine, which are generated in the early stages of the Maillard reaction during the heat processing of foods (1). For that reason, estimates of the extent of protein damage caused by heating in the first stages of that reaction are often based on determinations of the amount of furosine that forms during the acid hydrolysis of foods.

Furosine determinations may be carried out by ion-exchange chromatography (IEC) (2–4), gas chromatography (5), and ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC) (6–12). Recently, capillary electrophoresis (CE) methods of furosine determination have also been developed (13–15).

The first step in furosine analysis is hydrolysis of the sample proteins. Although optimization of the hydrolysis step has

been considered in previous research (2,3,8–12,16), Resmini et al. (8) pointed out that more research on the effect of acid hydrolysis on furosine formation was needed. Additional issues associated with the assay include whether hydrolysate cleanup by solid-phase extraction improves assay performance and what calibration strategy produces optimum performance.

As a consequence of the foregoing, the object of this study was to examine the influence of HCl concentration and ratio on furosine formation and to establish the most suitable conditions for hydrolysate purification and furosine quantitation based on the chromatographic conditions developed by Delgado et al. (9).

Experimental

Chemicals and reagents

The furosine standard with a purity of approximately 70% was obtained from Neosystem (Strasbourg, France). HPLC-grade acetonitrile was from Scharlau (Barcelona, Spain). The other analytical reagent-grade chemicals were from Merck (Darmstadt, Germany). Water was quartz-distilled and deionized using the Milli-Q system (Millipore, Bedford, MA).

Equipment

The HPLC apparatus consisted of a Model 110B pump and a Model 210A injector from Beckman (Berkeley, CA) equipped with a 20- μ L loop and a KNK-029.757 UV-vis detector (Konik Instruments S.A., Barcelona, Spain). Peak areas were determined with the aid of an SP-4290 recorder-integrator (Spectra-Physics, San Jose, CA).

Samples

The trials were performed using two samples (A and B) of a powdered enteral formula and two samples (A and B) of

* Author to whom correspondence should be addressed: email amelia.hernandez@uah.es.

powdered whole milk. Caseinate was the protein source for the enteral formula, and the protein content was 20%. The protein content of the powdered whole milk was 25%.

Sample hydrolysis

In order to determine the influence of HCl concentration and ratio on furosine formation during hydrolysis, three HCl concentrations (i.e., 6M, 8M, and 10M) and two ratios of sample protein to acid volume (i.e., 1 and 5 mg of protein per mL of acid) were tested. A quantity of sample accurately weighed was poured into a 250-mL Pyrex screw-cap flask, and an appropriate quantity of acid was added. Hydrolysis was carried out in a nitrogen atmosphere at 110°C for 24 h. After the hydrolysate had cooled to room temperature, it was filtered through No. 52 Whatman paper (Whatman, Maidstone, U.K.), and the screw-cap flask was washed out with Milli-Q water. All the liquids were collected in a volumetric flask that was then topped off with Milli-Q water.

For the sample protein-to-acid ratio trials using 1 mg of protein per mL of acid, approximately 0.20 g of the powdered milk or 0.25 g of the enteral formula was weighed out and hydrolyzed with 50 mL of acid, and the volume was diluted to 100 mL. For the sample protein-to-acid ratio trials using 5 mg of protein per mL of acid, approximately 0.40 g of the powdered milk or 0.50 g of the enteral formula was weighed and hydrolyzed with 20 mL of acid, and the volume was diluted to 50 mL.

Hydrolysate purification

The different sample hydrolysates were purified using a Sep-Pak C₁₈ cartridge (WAT020515, Waters, Milford, MA). The cartridges were prewetted with 5 mL of methanol and 10 mL of Milli-Q water before use.

Different trials were performed to ascertain the optimal conditions for purification. Aliquots of 0.5 and 1 mL of filtered hydrolysate of an enteral formula were gradually loaded onto the cartridge, and the displaced liquid was collected in an evaporation flask, being careful not to allow air to enter the cartridge. Elution of the furosine on the cartridge was then carried out using 3 or 5 mL of 3M HCl, the eluate being collected in the same flask. The solution thus obtained was evaporated to dryness in a rotary evaporator at 30°C. The dry residue was reconstituted with acetonitrile–Milli-Q water–formic acid (20:79.8:0.2). Other aliquots of the same filtered hydrolysate were injected onto the chromatograph without undergoing purification.

Other parallel trials were performed using 0.5 mL filtered hydrolysate of the enteral formula and the powdered whole milk in the same conditions to corroborate the effect of collecting or discarding the displaced liquid when running the hydrolysate through the cartridge.

Quantitative analysis

Quantitation was performed using the external standard method. A stock solution with approximately 140 µg/mL of pure furosine standard was prepared by dissolving the total amount of a commercial vial in 0.1M HCl. This stock solution was stored under refrigeration at 4°C.

An appropriate aliquot of the stock solution was evaporated to dryness in a rotary evaporator, and the dry residue was reconstituted with an appropriate volume of acetonitrile–Milli-Q water–formic acid (20:79.8:0.2). Using that same solvent, eight standard dilutions ranging in concentration from 1 to 8 µg/mL were prepared from the solution that was obtained. A calibration curve was obtained by plotting the peak areas versus the micrograms per milliliter of furosine injected.

Calibration by the standard additions method was also tested. For this purpose, five solutions of each sample (powdered enteral formula and powdered milk) were prepared by taking a uniform quantity of hydrolysate and increasing quantities of furosine standard (ranging from 1 to 5 µg/mL). Thus, two standard additions curves were obtained.

All standard solutions and samples were injected twice.

Chromatographic conditions

Furosine was determined by ion-pair RP-HPLC according to the method of Delgado et al. (9). Separations were carried out on a Spherisorb ODS2 5-µm column (250- × 4.6-mm i.d.) (Phenomenex, Torrance, CA) thermostatted at 30°C. The mobile phase was 5mM sodium heptane sulfonate with 20% acetonitrile and 0.2% formic acid at a flow rate of 1.2 mL/min. Detection was carried out at 280 nm.

Results and Discussion

The chromatographic method of Delgado et al. (9) was selected because it was an isocratic method in which furosine was eluted after a short retention time, thereby reducing the analysis time. In view of the discrepancies concerning hydrolysate purification contained in the literature, the first question addressed was whether or not a purification stage was necessary and what the optimum purification conditions were.

Figure 1 presents the chromatograms for an unpurified and purified hydrolysate of an enteral formula. The furosine eluted after a retention time of 10 min. On the chromatogram for the unpurified hydrolysate, a series of small spikes can be observed along the entire baseline, with two spikes being located quite close to the furosine peak. The chromatogram for the purified hydrolysate presented a more stable baseline and a smaller number of peaks, which translates into better separation and integration. Furthermore, the lifetime of the chromatographic column was extended by purification of the hydrolysates.

Two trials were run to test the purification conditions. The first trial was carried out on a hydrolyzed enteral formula. Two different volumes of hydrolysate (0.5 and 1 mL) were purified, with the displaced liquid collected in both cases. In addition, two different volumes (3 and 5 mL) of 3M HCl for furosine elution were tested. Other aliquots of hydrolysate were injected without purification of any kind. Four replications were performed for each set of conditions.

The results are presented in Table I. There was no significant difference in the furosine values obtained under either set of conditions when 0.5 or 1 mL of the hydrolysate was run

through the cartridge. Thus, the step of solid-phase extraction can be used not only for purification of the hydrolysate but also to increase the amount of furosine in the dry residue obtained, provided that the displaced liquid is also collected. This advantage may be quite useful when analyzing samples containing smaller quantities of furosine, such as pasteurized milk.

In addition, the amount of furosine obtained on elution with 3 mL of HCl was 9–12% lower than the amount obtained in the unpurified hydrolysate injected directly onto the column. However, elution carried out using 5 mL of HCl yielded the same amount of furosine as in the unpurified hydrolysates. Thus, collecting the displaced liquid when running the hydrolysate through the cartridge and eluting with 5 mL of 3M HCl implies a furosine recovery of 100% and makes it unnecessary to use a correction factor.

Another trial was carried out to confirm the previously mentioned results and to try to elucidate the effect of discarding or collecting the displaced liquid when running the sample through the cartridge. In this trial samples of both an enteral formula and powdered milk were used. Two hydrolysates were obtained from each sample. Parallel trials were performed using 0.5 mL of each hydrolysate, discarding or collecting the displaced liquid from the cartridge, and eluting the furosine with 3 or 5 mL of 3M HCl. Table II summarizes the results, which shows that the furosine values in all cases were higher when the displaced liquid was not discarded than when it was discarded, though the difference was small (approximately 1% to 3%) and only statistically significant for the enteral formula. The values were also higher when the furosine was eluted using 5 mL of acid instead of 3 mL, but in this case the difference was higher (approximately 9%) and statistically significant for both samples. Use of a larger volume of HCl for elution in laboratory tests confirmed that the same results were obtained.

It can therefore be concluded that, in the conditions of the experiment, the effect of using a larger volume of HCl to elute the furosine was greater than collecting or discarding the displaced liquid. Nevertheless, if a larger volume of hydrolysate were to undergo purification,

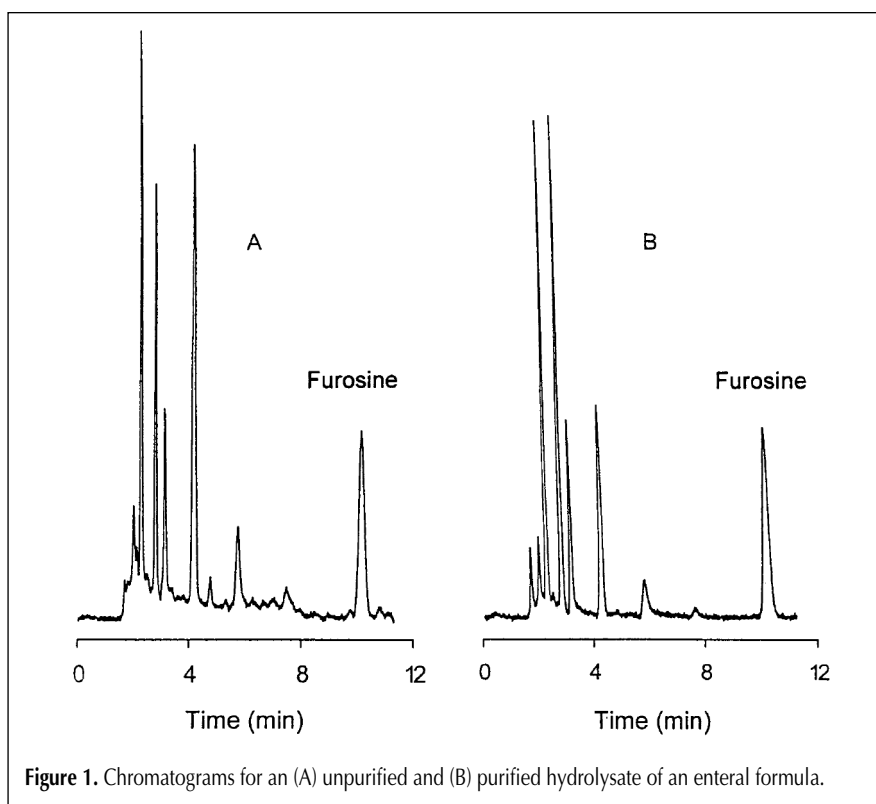


Figure 1. Chromatograms for an (A) unpurified and (B) purified hydrolysate of an enteral formula.

Table I. Effect of Hydrolysate Volume Purified and 3M HCl Volume Used as the Elution Solvent on the Furosine* Determination in a Powdered Enteral Formula†

Enteral formula A	Volume of hydrolysate run through the Sep-Pak cartridge	
	0.5 mL	1 mL
Unpurified hydrolysate	66.21 ± 2.48	66.70 ± 0.71
Purified hydrolysate		
Elution with 3 mL 3M HCl	58.22 ± 0.78	60.73 ± 0.95
Elution with 5 mL 3M HCl	66.29 ± 0.71	66.68 ± 0.01

* Milligrams per 100 g of product. Values are the means of four replications ± standard deviation.
† Hydrolysate prepared using 6M HCl at a ratio of 5 mg protein to 1 mL HCl.

Table II. Effect of Collecting or Discarding the Liquid Displaced from the Cartridge by Hydrolysate and the 3M HCl Volume Used as the Elution Solvent on the Furosine* Determination in a Powdered Milk† and a Powdered Enteral Formula†

Sample	Displaced hydrolysate	
	Discarded	Collected
<i>Powdered milk B</i>		
Elution with 3 mL 3M HCl	120.83 ± 6.00	124.05 ± 2.34
Elution with 5 mL 3M HCl	131.94 ± 2.87	132.86 ± 2.55
<i>Powdered enteral formula B</i>		
Elution with 3 mL 3M HCl	128.25 ± 4.56	133.12 ± 4.41
Elution with 5 mL 3M HCl	139.49 ± 4.99	143.53 ± 6.91

* Milligrams per 100 g of product. Values are the means of four replications ± standard deviation.
† Hydrolysate prepared using 10M HCl at a ratio of 1 mg of protein to 1 mL HCl.

the error produced by discarding the displaced liquid could become considerable. The rest of the trials were performed in the optimum conditions described previously.

Conflicting information has been reported in terms of the most appropriate calibration method to use with both the use of the external standards (8,17) and standard additions (9,12) mentioned. Because calibration by means of the standard additions method makes analysis both longer and more complicated because a separate calibration curve is required for every sample with a different composition, the calibration conditions were studied. Two calibration curves (one for the powdered milk and another for the enteral formula) were obtained by the standard additions method by adding increasing quantities from 1 to 5 µg/mL of the furosine standard to the corresponding previously hydrolyzed samples. Two other external standard calibration curves were also obtained.

Table III summarizes the results of the calibration study. The slopes of the two calibration curves, the external standard calibration curve, and the standard additions calibration curve were quite similar. A statistical comparison of the slopes of the two curves found no statistically significant differences between them ($P < 0.05$). Therefore, it can be concluded that

there were no effects attributable to the hydrolysate matrix of either of the samples tested, even though they differed considerably in composition.

The furosine concentration in the samples was also calculated using the two calibration curves, and the furosine recovery (%) was calculated by the ratio between furosine determined with standard additions and external standard curve equations (Table III). The recovery was 100%, which again confirmed that there were no differences attributable to the sample matrix.

One of the drawbacks to the standard additions method is that sample concentration is calculated by extrapolation rather than by interpolation while it is in the external standard calibration method. For this reason, the accuracy of the external standard method was also evaluated by calculating the recovery of furosine as the known (increasing quantities of the standard were added to the samples). The recoveries are shown in Table IV. Mean recovery values for the two samples were nearly 100%, with coefficients of variation lower than 0.2%. Thus, calculation error resulting from extrapolation would appear to be minimal, and calibration by the external standard method is adequate.

Table III. Calibration Curve Equations and Furosine Recovery for a Powdered Enteral Formula and a Powdered Milk

Sample	External standard calibration		Standard additions calibration		
	Curve equation	Furosine injected (µg/mL)	Curve equation	Furosine injected (µg/mL)	Recovery (%)
Enteral formula A	$y = 71526.1x - 2547.1$ s.e.* = 1454 $r^{2\dagger} = 0.9999$	3.39 ± 0.01	$y = 70428.9x + 239409$ s.e. = 941 $r^2 = 0.9998$	3.40	100.2
Powdered milk A	$y = 71481.4x - 2100.8$ s.e. = 2157 $r^2 = 0.9999$	2.10 ± 0.01	$y = 70304.2x + 147529$ s.e. = 1210 $r^2 = 0.9999$	2.09	99.7

* s.e., standard error of estimation.
† Determination coefficient.

Table IV. Percentage Furosine Recovery for a Powdered Enteral Formula and a Powdered Milk

Sample	Furosine (µg/mL)			Recovery (%)
	Initial	Added	Recovered	
Enteral formula A	3.39	0.95	4.30	99.08
		1.42	4.78	99.38
		1.89	5.25	99.43
		2.37	5.72	99.31
		Mean value		
Powdered milk A	2.10	0.95	3.02	99.02
		1.89	3.94	98.75
		2.84	4.89	98.99
		3.79	5.84	99.15
		Mean value		

Table V. Effect of HCl Concentration and Ratio During Hydrolysis on Furosine* Formation in a Powdered Enteral Formula and a Powdered Milk

Hydrolysis conditions	Enteral formula A [†]	Powdered milk A [†]
<i>1 mg protein to 1 mL acid</i>		
10M HCl	546.22 ± 4.39 (100)	322.95 ± 0.95 (100)
8M HCl	460.45 ± 2.41 (84.3)	289.37 ± 4.55 (89.6)
6M HCl	332.66 ± 1.24 (60.9)	195.63 ± 0.43 (60.6)
<i>5 mg protein to 1 mL acid</i>		
10M HCl	493.59 ± 4.40 (100)	306.39 ± 0.98 (100)
8M HCl	428.14 ± 5.56 (86.7)	268.89 ± 5.53 (87.8)
6M HCl	320.26 ± 2.56 (64.9)	161.66 ± 1.47 (52.8)

* Milligrams per 100 g protein. Values are the means of three replications \pm standard deviation.
† Difference in percentage furosine formation with respect to the values obtained using 10M HCl in brackets.

The effect of different hydrolysis conditions on the amount of furosine formed was tested under optimal purification conditions using external standard calibration. Three acid concentrations (6M, 8M, and 10M) and two quantities of protein per mL of acid (1 mg of dilute hydrolysis and 5 mg of concentrated hydrolysis) were tested. Samples of the enteral formula and the powdered milk were used, and the results were expressed as milligrams of furosine per 100 g of protein in the sample. The results of these trials appear in Table V.

The amount of furosine formed increased with increasing acid concentration in both the dilute and concentrated hydrolysis of both samples. Furosine formation was thus highest for the 10M HCl. The difference in the level of furosine formation was greater between the 6M and 8M acid concentrations than between the 8M and 10M acid concentrations for both the milk and the enteral formula.

The protein-to-acid ratio during hydrolysis also affects furosine formation. Furosine formation was in all cases higher for the ratio of 1 mg of protein to 1 mL of acid (in other words, for dilute hydrolysis). Nevertheless, the influence of HCl concentration was much greater than that of the sample protein-to-acid ratio during hydrolysis. The dilute ratio has customarily been recommended for the analysis of total amino acids (16).

The increase in furosine formation according to HCl acid concentration is in agreement with the results obtained for different foods by other researchers (2,3,11,12,17).

No information concerning the influence of the protein-to-acid ratio on furosine formation was found in the literature.

Based on the experimental results, it can be concluded that optimum hydrolysis conditions to maximize furosine formation are 10M HCl in the ratio of 1 mg of protein to 1 mL of acid. Because furosine is formed from the Amadori products during the hydrolysis of foodstuffs, its concentration was used to evaluate thermal damage sustained by foods during processing, and it will therefore in all cases be appropriate to try to maximize furosine formation to ensure the maximum correctness of the evaluation. This is even more important in samples that undergo milder heat processing.

In liquid foods the concentration of the HCl added to the samples should be higher, so that the final acid concentration during hydrolysis will be 10M. Although the concentration of commercial HCl acid concentrate is approximately 11.9M, concentrations higher than 10M were not tested in order to ensure comparability of the results for the solid and liquid samples.

Acknowledgments

This study was supported by a grant awarded by the Comisión Interministerial de Ciencia y Tecnología (Project

ALI97-0606-C02-02) of the Spanish Ministry of Education and Science.

References

1. H. Erbersdobler and H. Zucker. Untersuchungen zum gehalt an lysin und verfügbarem lysin in trockenmagermilch. *Milchwissenschaft* **21**: 564–68 (1966).
2. A. Brandt and H. Erbersdobler. Zur bestimmung von furosine in nahrungs- und futtermitteln. *Landwirtsch. Forsch, Sonderheft* **28/II**: 115–19 (1973).
3. I. Molnár-Perl, M. Pintér-Szakács, R. Wittmann, M. Reutter, and K. Eichner. Optimum yield of pyridosine and furosine originating from Maillard reactions monitored by ion-exchange chromatography. *J. Chromatogr.* **361**: 311–20 (1986).
4. J. Hartkopf and H. Erbersdobler. Stability of furosine during ion-exchange chromatography in comparison with reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **635**: 151–54 (1993).
5. W. Büser and H. Erbersdobler. Determination of furosine by gas-liquid chromatography. *J. Chromatogr.* **346**: 363–68 (1985).
6. E. Schleicher and O.H. Wieland. Specific quantitation by HPLC of protein (lysine) bound glucose in human serum albumin and other glycosylated proteins. *J. Clin. Chem. Clin. Biochem.* **19**: 81–87 (1981).
7. G.H. Chiang. A simple and rapid high-performance liquid chromatographic procedure for determination of furosine, lysine-reducing sugar derivative. *J. Agric. Food Chem.* **31**: 1373–74 (1983).
8. P. Resmini, L. Pellegrino, and G. Battelli. Accurate quantification of furosine in milk and dairy products by a direct HPLC method. *Ital. J. Food Sci.* **3**: 173–83 (1990).
9. T. Delgado, N. Corzo, G. Santa-Maria, M. L. Jimeno, and A. Olano. Determination of furosine in milk samples by ion-pair reversed phase liquid chromatography. *Chromatographia* **33**: 374–76 (1992).
10. T. Maroni and P. Lazzari. Determinazione di furosina in HPLC con metodo isocratico. *Ind. Aliment.* **33**: 964–66 (1994).
11. T. Henle, G. Zehetner, and H. Klostermeyer. Fast and sensitive determination of furosine. *Z. Lebensm. Unters. Forsch.* **200**: 235–37 (1995).
12. E. Guerra-Hernández and N. Corzo. Furosine determination in baby cereals by ion-pair reversed-phase liquid chromatography. *Cereal Chem.* **73**: 729–31 (1996).
13. A. Tirelli and L. Pellegrino. Determination of furosine in dairy products by capillary zone electrophoresis. A comparison with the HPLC method. *Ital. J. Food Sci.* **4**: 379–85 (1995).
14. L. Del Giovine and A. Bocca. Elettroforesi capillare applicata all'analisi della furosina nel latte. *Riv. Sci. Aliment.* **3**: 247–52 (1996).
15. A. Tirelli. Improved method for the determination of furosine in food by capillary electrophoresis. *J. Food Prot.* **61**: 1400–1404 (1998).
16. J.W. Finley. *Digestibility and Amino Acid Availability in Cereals and Oilseeds*. J.W. Finley and D. Hopkins, Eds. Am. Assoc. Cereal Chem., St Paul, MN, 1985, pp. 15–30.
17. F. Evangelisti, C. Calcagno, S. Nardi, and P. Zunin. Deterioration of protein fraction by Maillard reaction in dietetic milks. *J. Dairy Res.* **66**: 237–43 (1999).

Manuscript accepted November 26, 2001.