Liquid Chromatographic Determination of the Total Available Free and Intrachain Lysine in Various Foods

A. Hernández*, M.A. Serrano, M.M. Muñoz, and **G. Castillo**
Departamento de Nutrición y Bromatología, Universidad de Alcalá, 28871–Alcalá de Henares, Madrid, Spain

Abstract

A method for the quantitative determination of the total available lysine in various foods is developed. The method is based on the reaction of the amino groups on the lysine molecule with fluorodinitrobenzene and is capable of furnishing simultaneous determination of the available intrachain lysine (known as *N*-ε-[2,4-dinitrophenyl]-L-lysine) or the available free and/or *N*-terminal lysine (known as *N,N'*-di-[2,4-dinitrophenyl]-L-lysine). Optimum conditions for separation and quantitation are studied. The results show the proposed method to be both accurate and precise and suitable for food samples containing hydrolyzed proteins.

Introduction

Food proteins are highly reactive and may combine with other food components during processing and storage. Lysine is the amino acid most sensitive to damage because of the presence of the ε-amino group, which may react chemically with reducing sugars (Maillard reaction), polyphenols, or oxidizing fats. These newly formed compounds are not normally biologically available to an organism as a source of lysine and may also have specific physiological effects on an organism (1). Because lysine is an essential amino acid and is often limited in many diets, a measurement of the available lysine is extremely important from a nutritional standpoint.

Most direct chemical methods for determining available lysine are based on the reaction between the free ε-amino group on the lysine molecule and a chromophoric reagent. The treated protein is then hydrolyzed and the concentration of the lysine derivative determined.

The method most widely employed to date was established by Carpenter (2) using 1-fluoro-2,4-dinitrobenzene (FDNB) for derivatization. The *N*-ε-[2,4-dinitrophenyl]-L-lysine (ε-DNP-lysine) thus formed is measured spectrophotometrically after the acid hydrolysis and organic solvent extraction of the sample. Recently, chromatographic methods have been proposed for separating ε-DNP-lysine from interfering components with the intent of increasing the exactness of the determinations (3–5).

One limitation of these methods is that they do not measure lysine units with both the free α-amino group and free ε-amino group (*N*-terminal or free lysine), although these units are nutritionally available (2). During derivatization, such units form the derivative *N,N'*-di-[2,4-dinitrophenyl]-L-lysine (α,ε-diDNP-lysine), for which no method of analysis has been published.

Nevertheless, because most conventional protein foods are composed of whole proteins and contain only minor amounts of free and *N*-terminal lysine, the possible error of the existing methods of analysis for samples of this type is negligible (2,6,7).

However, more and more foodstuff containing smaller or larger proportions of hydrolyzed proteins are becoming available in the marketplace (i.e., foodstuff containing oligopeptides and free amino acids or free amino acids only) (8–10). Enteral formulas intended for persons with nutrient absorption problems, hypoallergenic baby formulas, and nutritional protein supplements are examples of this type of foodstuff.

In samples of this type, determination of ε-DNP-lysine alone underestimates the available lysine (11), because the ε-DNP-lysine accounts for the available intrachain lysine only. In such cases there is a need to determine both the available free lysine and the available *N*-terminal lysine, including the α,ε-diDNP-lysine determination. The two values combined (i.e., ε-DNP-lysine and α,ε-diDNP-lysine) yield the total available lysine.

Consequently, the object of this study was to develop the conditions for the simultaneous separation and quantitation of ε-DNP-lysine and α,ε-diDNP-lysine taking as a basis the chromatographic method for determining ε-DNP-lysine developed by Castillo et al. (5) and also to study the accuracy and precision of the method thus developed.

Experimental

Chemicals and reagents

The FDNB, ε-DNP-lysine, α,ε-diDNP-lysine, and L-lysine monohydrochloride used were purchased from Sigma Chemical Company (St. Louis, MO). The HPLC-grade acetonitrile,

* Author to whom correspondence should be addressed: e-mail amelia.hernandez@uah.es.

methanol, and glacial acetic acid for HPLC were 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).

FDNB is a very potent irritant with toxic effects that may cause skin sensitization. The use of nitrile gloves offers the best protection for skin during this type of laboratory work (12).

Equipment

The HPLC apparatus consisted of two Model 110B pumps and a Model 210A injector from Beckman (Berkeley, CA) equipped with a 20-µL loop and a 168 diode array detector (Beckman). Peak areas were determined using a GOLD System (Beckman).

Total available lysine determination

Sample derivatization and hydrolysis

Derivatization of the lysine residues in the sample and subsequent hydrolysis of the sample were carried out using the method of Castillo et al. (5). Briefly described, a quantity of sample accurately weighed out containing approximately 2.5 mg of total lysine was poured into a 250-mL Pyrex screw-cap flask. Then, 10 mL of 8% $NaHCO_3$ was added, and the suspension was stirred for 10 min. Ethanol (15 mL) and then FDNB (0.1 mL) were added to the reaction mixture. This was followed by stirring the mixture at room temperature for 2 h while protecting it from light using aluminum foil. Finally, the ethanol was evaporated away completely in a water bath thermostatted at 90°C.

After derivatization, the protein was hydrolyzed using 6M HCl. The acid volume was calculated in order to achieve a ratio of 1 mL of HCl per mg of protein in the sample and neutralize the added $NaHCO_3$. The mixture was sonicated for approximately 20 min to remove the CO_2 generated by the neutralization reaction—an essential procedure in preventing excess pressure from building up during hydrolysis that could cause the flask to break. It should be noted that the flask should have sufficient empty headspace as a safety measure. Hydrolysis was carried out in a nitrogen atmosphere at 110°C for 24 h. Three hydrolyses of each sample were performed.

After the hydrolysate had cooled to room temperature, 50 mL of acetonitrile was added to the flask and the mixture was sonicated for 5 min. It was then filtered through No. 52 Whatman paper, and the screw-cap flask was washed with 50 mL of acetonitrile and then 50 mL of Milli-Q water. The volume was then increased to 250 mL with Milli-Q water. All sample hydrolysates were stored chilled at 4°C in hermetically sealed brown-tinted bottles.

An aliquot of this solution was evaporated to dryness in a rotary evaporator at 40°C. The dry residue was reconstituted with Milli-Q water–acetonitrile (1:4), taking care to add the acetonitrile first and dissolve the residue by sonication before adding the water. The mixture was injected on the chromatograph without any further filtration.

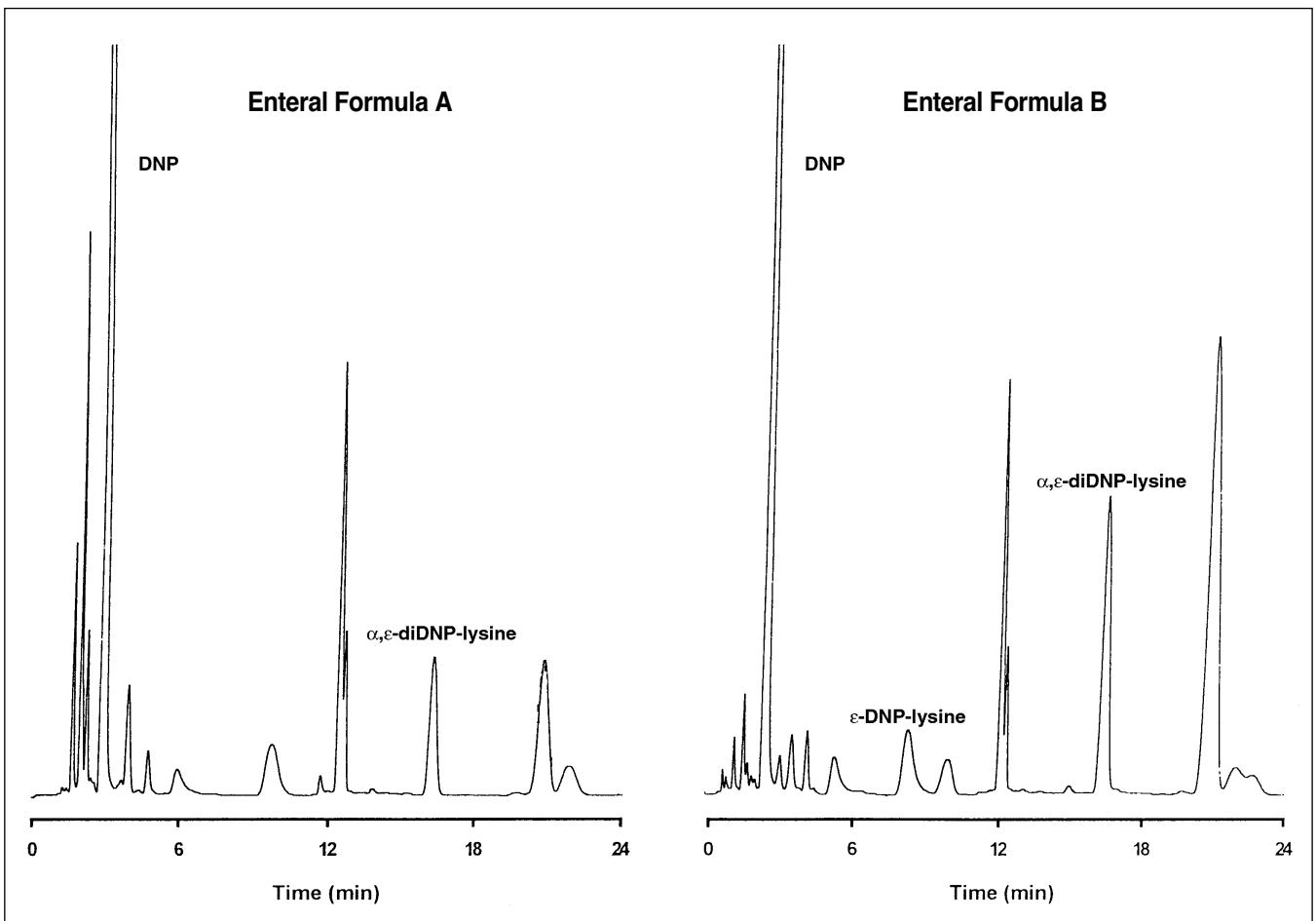

Figure 1. Chromatograms for two enteral formulas using a new guard column.

Quantitative analysis

Quantitation was performed using two external standards, one for the ε-DNP-lysine and another for the α,ε-diDNP-lysine. The standard solution was prepared by dissolving an appropriate quantity of α,ε-diDNP-lysine in acetonitrile. When dissolved, an appropriate quantity of ε-DNP-lysine in 10 mL of Milli-Q water for proper solubilization was added, and the mixture was increased to 200 mL with acetonitrile. This standard solution was diluted with Milli-Q water to concentrations ranging from 1 to 30 µg/mL for ε-DNP-lysine and from 1 to 18 µg/mL for α,ε-diDNP-lysine. The calibration curve was obtained by plotting the peak areas against concentration.

An L-lysine monohydrochloride standard was used to assess recovery because of its ability to form α,ε-diDNP-lysine upon derivatization. Three different assays were performed to verify the recovery level achieved using the method. Three replications of each assay were performed. The amount of standard was calculated to ensure that the final measurement would be within the calibration interval.

All standard solutions and samples were injected two times.

Chromatographic conditions

Separations were carried out on µ-Bondapak C_{18} 300- × 3.9-mm-i.d. columns (particle size 10 µm) (Waters, Milford, CT) using a guard column of the same characteristics. The columns were thermostatted at 50°C. Separation was performed using the following gradient: 20% B for 8 min, 20–35% B in 1.5 min, 35% B for 14.5 min, 35–20% B in 1.5 min, and an 11-min step at 20% B to re-equilibrate the column to the initial conditions between runs. Mobile phases for the gradient were for phase A, 0.01M acetate buffer (pH 5), and for phase B, acetonitrile. The flow rate was 2 mL/min, and detection was carried out at 360 nm.

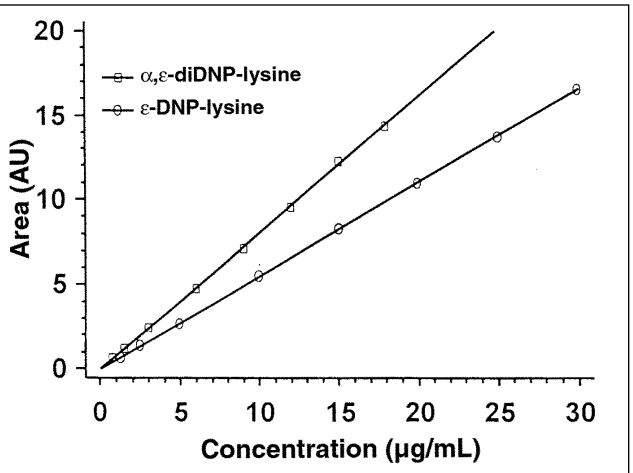

Figure 2. Calibration curves for ε-DNP-lysine and α,ε-diDNP-lysine.

Results and Discussion

Chromatographic method

Castillo et al. (5) carried out chromatographic separations in which ε-DNP-lysine eluted after a retention time of 6.5 min using a mobile phase consisting of acetonitrile–0.01M acetate buffer (pH 5) (22:78) at a flow rate of 2 mL/min and a column temperature of 50°C. Employing these same conditions, the chromatograms displayed peaks that eluted after extended retention times—one such peak was suspected to be α,ε-diDNP-lysine, which as a more liposoluble component (2,11) was retained on the column for a longer time. The chromatographic peak was located and identified using a standard solution of α,ε-diDNP-lysine when increasing the proportion of acetonitrile in the mobile phase to 35%.

Conditions for separating the two peaks of interest (ε-DNP-lysine and α,ε-diDNP-lysine together) were then tested using two enteral formulas—one (formula A) containing only free amino acids as the sole source of protein and the other (formula C) containing both oligopeptides and free amino acids. In order to achieve optimal separation from the other peaks present on the chromatograms, it was necessary to employ an elution gradient consisting of 20% B for 8 min, 20–35% B in 1.5 min, 35% B for 14.5 min, 35–20% B in 1.5 min, and an 11-min step at 20% B to reequilibrate the column to the initial conditions between runs. In these conditions, the retention time for the ε-DNP-lysine was 9 min and the retention time for the α,ε-diDNP-lysine was 18 min.

Castillo et al. (5) reported that nylon filters (0.22 µm) did not retain ε-DNP-lysine; therefore, they recommended using such filters to extend the mean working life of the column. The effect of the same filtration conditions on α,ε-diDNP-lysine was studied by comparing the peak areas of filtered and unfiltered standard solutions of α,ε-diDNP-lysine. The results showed that between 40% and 60% of that substance was retained by the filter. For that reason, it was decided not to filter the solutions and instead use a guard column to prolong the mean column life.

Guard-column age has an appreciable influence on both the peak resolution and the retention times of chromatographic separation, even without altering the system back-pressure. The retention time for α,ε-diDNP-lysine was particularly affected by as much as 2 min. Elution gradient conditions (in which retention times are less precise than under isocratic conditions) may also contribute to that effect (13). As a result, replacement of the guard column after approximately 200 injections is recommended. Figure 1 depicts the chromatograms for two enteral formulas with a new guard column.

By using the proposed HPLC method, linear calibration curves for ε-DNP-lysine and α,ε-diDNP-lysine were obtained (Figure 2). In Table I, the values of the linear working concentration range, slope, intercept, standard error, squared

Table I. Calibration and LOD of ε-DNP-Lysine and α,ε-diDNP-Lysine

Compound	Linear concentration range (µg/mL)	Slope*	Intercept*	Standard error	$r^{2\dagger}$	LOD (µg/mL)
ε-DNP-lysine	1.25–30	0.5545 (0.002)	–0.0597 (0.028)	0.0470	0.9999	0.25
α,ε-diDNP-lysine	0.75–18	0.8072 (0.007)	–0.0440 (0.068)	0.1148	0.9996	0.43

* Errors in the slope and intercept of the regression line are given in parentheses.
† r^2, Squared correlation coefficient.

correlation coefficient, and detection limit for every calibration are shown. The correlation between the concentrations of each standard and its peak area were close to 1.0 in the concentration range assayed. The limits of detection (defined as the concentration calculated from the calibration curve corresponding to a signal equal to the intercept of the regression line plus three times its standard error) were 0.25 µg/mL for ε-DNP-lysine and 0.43 µg/mL for α,ε-diDNP-lysine.

Sample derivatization and hydrolysis conditions

The derivatization and hydrolysis conditions employed were as described by Castillo et al. (5) who had previously established that derivatization of lysine with FDNB was complete and that ε-DNP-lysine remained stable during hydrolysis. The effect of these conditions on the α,ε-diDNP-lysine determination was therefore studied, though it was necessary to modify the conditions of hydrolysate filtration.

In their ε-DNP-lysine determination, Castillo et al. (5) filtered the hydrolysate through No. 52 Whatman paper, washed the residue in water, and then added a sufficient amount of acetonitrile to completely solubilize the 2,4-DNP precipitate in the filtered solution. However, when filtration was performed in those same conditions during the α,ε-diDNP-lysine determination, it was found that the chromatogram contained no peak for the α,ε-diDNP-lysine. Dissolving the residue resulting from filtration in acetonitrile for injection on the chromatograph showed that nearly all the α,ε-diDNP-lysine had been retained in the residue, probably because of its greater insolubility in water. Therefore, in order to completely solubilize the α,ε-diDNP-lysine, acetonitrile was added directly to the hydrolysate after cooling to room temperature and sonicated for 5 min before filtering the solution and then washing the residue with acetonitrile followed by water.

Table II. Recovery of α,ε-diDNP-Lysine from Standard Lysine Solutions

Lysine (mg)		Recovery (%)	
Used	Recovered*	Mean	RSD
Derivatized (unhydrolyzed)			
0.6208	0.6172 ± 0.01	99.4	1.0
1.2417	1.2428 ± 0.09	100.1	6.9
2.4822	2.3024 ± 0.16	92.8	6.8
Derivatized and hydrolyzed			
0.6202	0.5997 ± 0.03	96.7	4.7
1.2405	1.2036 ± 0.03	97.0	2.3
2.4810	2.5300 ± 0.04	102.0	1.4

* Values are the means of three replications ± standard deviation.

Table III. Recovery of α,ε-diDNP-Lysine in Enteral Formulas

	Lysine (mg)		Recovery (%)	
Enteral formula	Added	Recovered*	Mean	RSD
A	1.035	1.008 ± 0.05	97.4	5.2
C	1.035	1.085 ± 0.00	104.8	0.3

* Values are the means of three replications ± standard deviation.

Table IV. Available Lysine Content (Means of Three Replications) in Enteral Formulas and Peas

	Available lysine				
	Intrachain lysine		Free and/or *N*-terminal lysine		
Food sample	Mean	RSD	Mean	RSD	Total
Enteral formula					
A*	–	–	0.85	1.78	0.85
B*	–	–	2.15	2.46	2.15
C*	0.67	2.69	0.91	3.55	1.58
D†	0.13	2.33	0.19	1.41	0.32
E†	0.25	1.32	–	–	0.25
F†	0.35	0.12	–	–	0.35
Peas*	1.57	1.99	0.02	6.12	1.59

* Solid samples, values expressed as grams of lysine per 100 g of dry matter.
† Liquid samples, values expressed as grams of lysine per 100 g of sample.

Recovery assays

An L-lysine monohydrochloride standard was used in the recovery assays in order to evaluate the reliability of the method proposed. Three assays were performed.

The first assay examined lysine recovery after derivatization without hydrolysis. Aliquots of standard solutions of L-lysine monohydrochloride were derivatized as previously described and injected directly on the chromatograph without undergoing hydrolysis. The second assay followed both derivatization and hydrolysis in order to test both the completeness of the derivatization reaction and the component stability during hydrolysis. Thus, other aliquots of standard solutions of L-lysine monohydrochloride were derivatized and hydrolyzed using the method previously described. Because pure lysine was employed, all the lysine present had two free NH_2 (α and ε) groups, and consequently only α,ε-diDNP-lysine formed during derivatization. For this reason, a comparison of the values obtained using the proposed method and the theoretical values should provide an indication of the accuracy of the method.

Table II gives the recovery values obtained during the two assays. The table shows the values to be close to 100% with relative standard deviation (RSD) values smaller than 7%. Derivatization in those conditions can therefore be regarded as complete, and the α,ε-diDNP-lysine can be considered stable during hydrolysis.

The presence of carbohydrates in the sample may affect quantitation of the available lysine because of the reducing conditions that arise during acid hydrolysis, which brings about reduction of the nitro groups to amino groups on the dinitrophenylated derivatives (14). Therefore, after verifying that the derivatization reaction was complete and the α,ε-diDNP-lysine was stable during hydrolysis in the absence of carbohydrates in the sample, the next step was to consider recovery in the presence of carbohydrates. Recovery was tested using two types of enteral formulas, one (formula A) containing only free amino acids (i.e., only free lysine) and the other (formula C) containing both oligopeptides and free amino acids (i.e., both intrachain and *N*-terminal and/or

free lysine). A standard solution of L-lysine monohydrochloride was added to the samples of enteral formulas at the beginning of analysis prior to derivatization and hydrolysis. The analysis then proceeded in the conditions described previously. These same formulas were also assayed without the addition of the standard, and recoveries were estimated.

Table III presents the results for α,ε-diDNP-lysine recovery. Recovery was close to 100% for both the enteral formulas tested, with no differences between the formula containing both oligopeptides and free amino acids and the formula containing free amino acids only. It can therefore be concluded that the extent of hydrolysis of the sample did not affect percentage α,ε-diDNP-lysine recovery and α,ε-diDNP-lysine was stable during hydrolysis in the presence of carbohydrates, as was found for ε-DNP-lysine in the method developed by Castillo et al. (5).

Quantitative analysis of food samples

Finally, the method tested was applied to determine the total available lysine content of six enteral formulas with differing levels of hydrolysis of the source protein and a sample of peas. The results are given in Table IV and show that formulas A and B (made from free amino acids) contained only free lysine (α,ε-diDNP-lysine); no ε-DNP-lysine (indicative of the presence of intrachain lysine) was detectable. Formulas C and D (prepared from both oligopeptides and free amino acids as the protein source) contained both intrachain lysine (ε-DNP-lysine) and free and/or *N*-terminal lysine (α,ε-diDNP-lysine), mainly the latter. Formulas E and F were made from whole protein, as indicated by the single form of lysine detected (ε-DNP-lysine), which is indicative of intrachain lysine. Both intrachain lysine and free and/or *N*-terminal lysine (mainly the former) were present in the peas. As a measure of the precision of the method, the table shows that the maximum RSD value was approximately 6%.

Injections of the sample hydrolysates stored were performed at regular time intervals (10, 15, 21, and 45 days), showing the hydrolysates to remain stable at least over that time period.

Conclusion

The method described here is accurate and precise and suitable for the simultaneous determination of both the available intrachain lysine and the available free and/or *N*-terminal lysine present in all kinds of food samples, irrespective of the hydrolysis level of the protein in the sample.

Acknowledgments

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