An Isocratic High-Performance Liquid Chromatographic Method for Determining the Available Lysine in Foods

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Abstract

A method for the quantitative determination of the available lysine in foods based on the reaction of the ε-amino group of lysine with fluorodinitrobenzene is developed. Derivatization, hydrolysis, and the chromatographic conditions for separation are studied. To establish the reliability of the proposed method, the accuracy and precision of the analysis are evaluated using pure proteins and food samples. The results show the proposed method to be both accurate and precise.

Introduction

Lysine (Lys) measurement has received much attention because lysine is the limiting essential amino acid in many foodstuffs, and the ε-amino group in that amino acid facilitates the participation of lysine in degradational reactions affecting the proteins in foodstuffs during heat processing and storage. The ε-amino group in lysine may react chemically with glutamic acid or aspartic acid (1–3), oxidized lipids (4), and reducing sugars (Maillard reaction) (4,5), and it can form crosslinks in the form of lysinoalanine (2). The Maillard reaction is the most important of the degradational mechanisms.

The nutritional changes induced by these reactions include lower protein digestibility, lower lysine availability, and the possible formation of growth inhibitors or toxic compounds (6,7), all of which make the measurement of available lysine important from a nutritional standpoint. The amount of available lysine can be determined by indirect or direct chemical methods.

The main indirect method is furosine determination in a hydrolyzed sample. However, it can only be used to assess the Maillard reaction, and even then it has two drawbacks. On one hand, the relation used to determine the quantities of blocked lysine from furosine is only correct when the product of the Maillard reaction is protein-bound lactulosyl-lysine, not for samples with qualitatively different carbohydrate fractions (8). On the other hand, because the more complex polymers formed in the advanced stages of the Maillard reaction do not break down to form furosine upon acid hydrolysis (9), the furosine method cannot be used as a quantitative indicator in all instances of protein–sugar interaction (10,11). Henle and Klostermeyer (12) have shown that the extent of lysine alteration calculated from furosine was three or four times lower than the level calculated from determinations based on Amadori compounds.

Direct chemical methods for determining the available lysine are based on the reaction between the free ε-amino group of the lysine in proteins and a chromophoric reagent; the treated protein is then hydrolyzed, and the concentration of the lysine derivative is determined. Compared with indirect methods, such methods afford the advantage of taking into account all alterations affecting the ε-amino group of lysine.

The most widely employed method to date was established by Carpenter (13) using 1-fluoro-2,4-dinitrobenzene (FDNB) for derivatization. The N-ε-dinitrophenyl-lysine (ε-DNP-Lys) that forms is measured spectrophotometrically after acid hydrolysis and organic solvent extraction of the sample. The main problems relating to this spectrophotometric method arise because the ε-DNP-Lys may not be entirely stable during acid hydrolysis and because formation of interfering compounds may result in overestimation of the lysine content (14). Still, most assays have been carried out using the spectrophotometric method, though it is less accurate than current chromatographic methods. Nevertheless, high correlations have been calculated for the lysine values obtained using the spectrophotometric method and rat growth assays (15,16) or determinations of Amadori compounds (12,17).

The use of chromatographic techniques has been proposed to separate ε-DNP-Lys from interfering compounds. Only two high-performance liquid chromatographic (HPLC) methods have been put forward for the quantitation of ε-DNP-Lys (18,19). However, the two methods employ different wavelengths, mobile phase pH, and hydrolysis conditions, and the authors did not explain how to prevent interference from the dinitrophenol precipitate formed. In addition, that earlier work did not include recovery analysis.

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Peterson and Warthesen (18) used a wavelength of 436 nm, whereas Rabasseda et al. (19) used 254 nm. Neither of these is the peak maximum absorption wavelength, and the latter is not even particularly selective. Use of the peak maximum absorption wavelength would enhance the sensitivity of the analytical method.

In the method of Peterson and Warthesen (18), the pH of the mobile phase was 4.0, whereas in the method of Rabasseda et al. (19), the pH was 5.0. Because ∊-DNP-Lys is an ionizable molecule, the pH may exert a strong influence on separation.

Rabasseda et al. (19) used specific conditions of hydrolysis for each sample, considerably increasing the complexity of the analysis. Moreover, they did not evaporate the HCl but instead neutralized it with NaOH, which resulted in an increase in the ionic strength. Wilkinson (20) indicated that this could affect the degree of dissociation of the carboxyl groups and its interaction with the column matrix. On the other hand, Sanz et al. (21) showed that the increase in ionic strength caused large variations between replicates. Peterson and Warthesen (18) neither evaporated nor neutralized the HCl after hydrolysis.

DNP (2,4-dinitrophenol) is the major byproduct of the dinitrophenylation of proteins. In the original spectrophotometric method, its presence was not a problem because it was removed by the ether during the solvent extraction stage. However, chromatographic methods lack any such stage, and the presence of the DNP precipitate may be a source of error.

Consequently, the objective of this study was to develop an isocratic HPLC method for the quantitative estimation of the available lysine in foodstuffs and to evaluate the method's accuracy and precision.

Experimental

Chemicals and reagents

The DNP, 2,4-dinitro-fluorobenzene (FDNB), *N*-∊-2,4-dinitrophenyl-L-lysine, (type III-A) ribonuclease A from bovine pancreas (R-5125), bovine albumin (initial fractionation by cold alcohol precipitation) (A-7638), and chicken egg white lysozyme (L-6876) were obtained from Sigma (St. Louis, MO). HPLC-grade acetonitrile was from Fisher (Morristown, NJ), and the methanol 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 two model 110B pumps and a model 210A injector from Beckman (Berkeley, CA) equipped with a 20-µL loop and a KNK-029.757 ultraviolet–visible (UV–vis) detector (Konik Instruments S.A., Barcelona, Spain). Peak areas were determined using an SP-4290 recorder–integrator (Spectra-Physics, San José, CA).

Total lysine determination

The total lysine content in samples of three pure proteins, albumin, lysozyme, and ribonuclease, was determined by HPLC using the method of Sanz et al. (21). Samples were concisely hydrolyzed with 6M HCl in a nitrogen atmosphere at 110°C for 24 h. The ratio of HCl employed was 1 mL of HCl per milligram of protein in the sample.

Precolumn derivatization of the amino acids was carried out using Dns-Cl. The dry residue of each of the three protein hydrolysates was reconstituted with Milli-Q water to a protein concentration of 0.15–0.25 mg/mL. The dansylated derivatives were formed by combining 1 mL of protein hydrolysate, 2 mL of 40mM Li_2CO_3 (pH 9.5), and 1 mL of Dns-Cl solution (4 mg/mL, 14.83mM) in that order. The solution was mixed and heated at 60°C for 30 min. An amount of 50 µL of methylamine solution was then added to quench the reaction. Each protein hydrolysate was derivatized and analyzed in triplicate. Quantitation was performed using external standards for L-lysine HCl. The standards were derivatized using the same procedure as in the case of the sample hydrolysates, except that 3 mg/mL Dns-Cl (11.12mM) was used. The calibration curve was obtained by plotting the peak areas. Correlation coefficients greater than 0.998 were obtained in all cases.

Separations were carried out on a 300 × 3.9-mm i.d. column packed with Spherisorb ODS-2 (particle size, 10 µm) (Sugerlabor S.A., Madrid, Spain) thermostatted at 40°C. The mobile phase was acetonitrile–0.01M phosphate buffer (pH 7.0) (35:65) at a flow rate of 1.5 mL/min. Detection was carried out at 254 nm.

Available lysine determination

Derivatization of the sample's lysine residues

A quantity of sample accurately weighed out containing about 2.5 mg of total lysine was poured into a 250-mL Pyrex screw-cap flask. Next, 10 mL of 8% $NaHCO_3$ was added, and the suspension was stirred for 10 min. Ethanol (15 mL) and FDNB (0.1 mL) were then added in that order to the reaction mixture, which was stirred at room temperature for 2 h and protected with aluminium foil. Finally, the ethanol was evaporated away completely in a water bath thermostatted at 90°C.

Preparation of sample hydrolysate

After derivatization, the protein was hydrolyzed using 6M HCl. The acid volume was calculated to achieve a ratio of 1 mL of HCl per milligram of protein in the sample (22) and to neutralize the added $NaHCO_3$. Two other concentrations, 8M and 10M, and an additional HCl ratio, 1 mL of acid per 5 mg of protein in the sample, were also tested. The mixture was sonicated for approximately 20 min to remove the CO_2 generated by the neutralization reaction. This procedure is essential to prevent excess pressure from building up during hydrolysis and breaking the flask. Additionally, the flask should have sufficient empty head space as a safety measure. 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, and the screw-cap flask was washed with 125 mL of Milli-Q water. Then 100 mL of acetonitrile was added to achieve complete solubilization of the DNP precipitate that had formed, and the volume was made up to 250 mL with Milli-Q water.

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 (4:1), and the solution was filtered through a nylon filter (0.22-µm) and injected.

Standard solution

Quantitation was performed using an external standard for the ∊-DNP-Lys. The standard solution was prepared by dissolving an appropriate amount of ∊-DNP-Lys in methanol to increase its solubility (19). This standard solution was diluted with Milli-Q water to a concentration ranging from 2–10 µg/mL. The calibration curve was obtained by plotting the peak areas. Correlation coefficients greater than 0.999 were obtained.

Recovery

Lysine itself cannot be used to assess recovery because it forms *N,N'*-di(2,4-DNP)-L-lysine (di-DNP-Lys). ∊-DNP-Lys can be used, but it must be added after the FDNB reaction has been quenched by the acid or it too will be converted to di-DNP-Lys (14). Three assays were performed to verify the recovery level achieved using the method.

In the first assay, pure protein samples (albumin, lysozyme, and ribonuclease) were analyzed using the methods for determining the available and total lysine described above. The obtained values for ∊-DNP-Lys were compared with the total lysine values and with the theoretical values for lysine based on the molecular structure of the proteins.

In the second assay, a standard solution of ∊-DNP-Lys was added to food samples (enteral formula, milk, and lentils) after the FDNB reaction had been quenched by acid. The methanol content in the standard solution used must be minimal to avoid the build-up of excessive pressure in the flask during hydrolysis. The analysis then proceeded in the conditions described above. The same three food samples were assayed without the addition of standard, and recoveries were estimated.

In the third assay, a pure protein, ribonuclease, was used as standard. To this end, a precisely weighed sample of ribonuclease was added to each of the food samples, and the procedure was then completed as described above. Recoveries were estimated by comparison of the results obtained with those for the food samples without the addition of ribonuclease.

Three replications were performed for all determinations. The amount of standard added was calculated to ensure that the final measurement would be within the calibration interval.

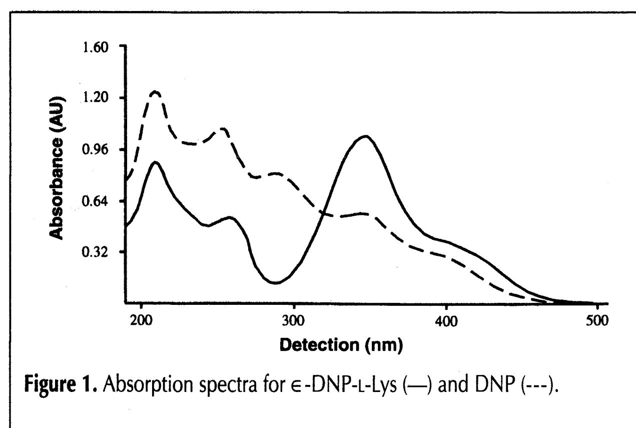

Figure 1. Absorption spectra for ∊-DNP-L-Lys (—) and DNP (---).

Chromatographic method

Separations were carried out on µ-Bondapak C_{18}, 300 × 3.9-mm i.d. columns (particle size, 10 µm) (Waters, Milford, CT) thermostatted at 50°C. The mobile phase was acetonitrile–0.01M acetate buffer (pH 5) (22:78) at a flow rate of 2 mL/min. Detection was carried out at 360 nm.

Results and Discussion

Chromatographic conditions

Figure 1 depicts the absorption spectra for ∊-DNP-Lys and DNP between 200 and 500 nm. The spectrum for ∊-DNP-Lys had three absorption peaks (at 211, 258, and 346 nm) and a shoulder at 400 nm, whereas the spectrum for DNP had peaks at 210, 253, and 288 nm and low absorption levels from 350 nm.

To select the detection wavelength, regression lines for the calibrations were plotted for ∊-DNP-Lys at 254, 360, and 435 nm. The slope of the line at 360 nm was 2.3 times greater than the slope of the line at 254 nm and 2.5 times greater than the slope of the line at 435 nm; therefore 360 nm was the wavelength that showed the highest sensitivity. Furthermore, because absorption of DNP, the principal byproduct of the reaction, was very low at that wavelength, 360 nm was selected as the absorption wavelength.

The chromatographic conditions for separation were studied using a mixture of standard solutions of ∊-DNP-Lys and DNP. Because the chromatographic column was the same as that used by Peterson and Warthesen (18), the mobile phase they used was tested: acetonitrile–0.01M acetate buffer (pH 4.0) (20:80). However, with that mobile phase, it was impossible to separate the ∊-DNP-Lys and DNP peaks, even when the ratio of the two phase components was varied slightly. Given that the effect of pH on the separation may be strong because ∊-DNP-Lys is an ionizable molecule and DNP is not, the pH of the buffer was changed to 5.0, the value used by Rabasseda et al. (19). That change enabled the two peaks to be separated. Resolution was further improved by increasing the proportion of acetonitrile in the mobile phase to 22. Thus, testing showed that the best chromatographic separations were obtained using a mobile phase comprising acetonitrile–0.01M acetate buffer (pH 5.0) (22:78) at a flow rate of 2 mL/min and a column temperature of 50°C, which effectively reduced peak tailing.

Figure 2 presents a chromatogram for a standard solution containing DNP and ∊-DNP-Lys with retention times of 2.8 and 6.5 min, respectively. Reproducibility of the retention time for ∊-DNP-Lys was assessed by performing nine consecutive injections of the standard solution. The coefficient of variation (CV) was less than 1%.

Several different filtration media were tested to increase mean column life, and the peak areas for filtered and unfiltered standard solutions of ∊-DNP-Lys were compared. Acetonitrile dissolved away cellulose filters, which were therefore unusable. Nylon filters (0.22 µm) did not retain this component in that there were no significant differences between the peak areas for the filtered and unfiltered solutions. C_{18} Sep-Pak cartridges (300 mg) (Waters) primed according to the manufac-

turer's instructions were also tested. A 4-mL amount of solution was passed through the activated cartridge, and 15 mL of mobile phase was used to elute; test results showed that using larger amounts of mobile phase did not cause more component to elute. However, the results also showed that approximately 6% of the ∊-DNP-Lys was retained by the cartridge. Consequently, nylon filters were selected.

Derivatization and hydrolysis conditions of the sample

The amount of FDNB used in derivatization of the lysine residues in the sample depends on the sample's total lysine content. For that reason, a ratio of 0.1 mL of FDNB per 2.5 mg of lysine, the ratio reported by Booth (14) and used by most workers, was employed. Ethanol should be added before the FDNB to denature the sample proteins because dinitrophenylation is easier when proteins have been denatured (23).

Carpenter (13), Roach et al. (24), and Nordheim and Coon (25) used a small volume of 8M HCl to hydrolyze the proteins after derivatization. However, Rao et al. (26) found that losses of ∊-DNP-Lys were greater when the hydrolysis was carried out in the conditions used by Carpenter than when 6M HCl was used. Furthermore, Matheson (23) tested the effect of sample concentration during hydrolysis on recovery of ∊-DNP-Lys by varying the volume of 5.6M acid used in the hydrolysis and concluded that higher yields of ∊-DNP-Lys were obtained when the hydrolyzed sample was dilute than when it was concentrated. This was also reported by Rao et al. (26), El-Nockrashy (27), and Blom et al. (28).

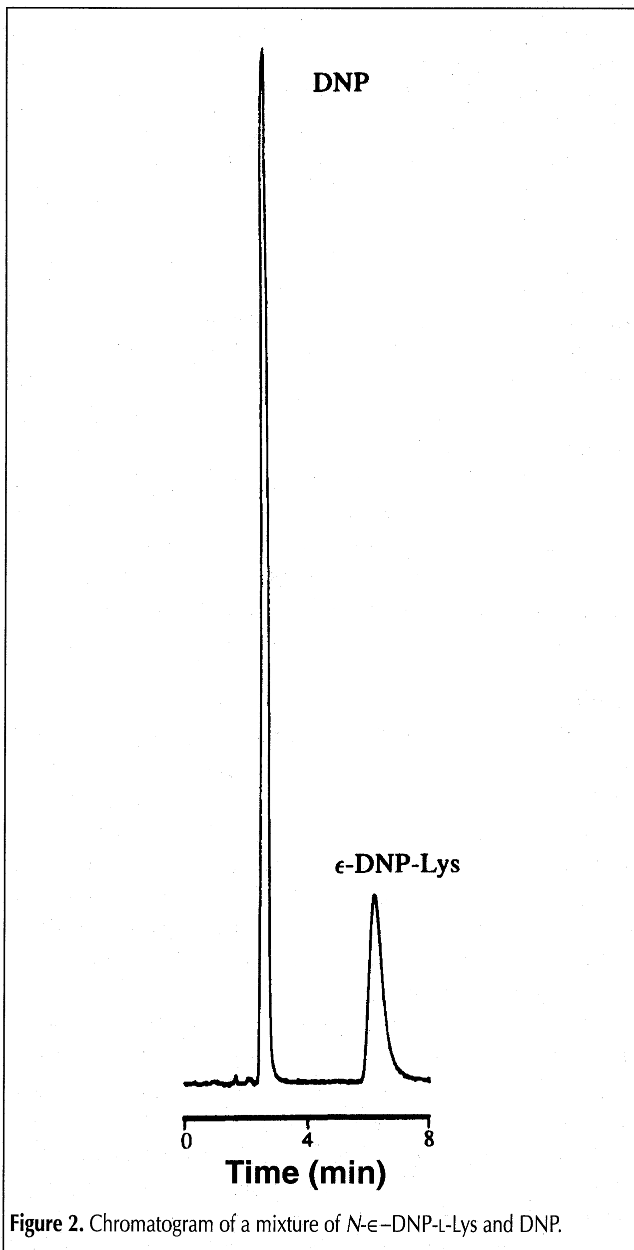

Figure 2. Chromatogram of a mixture of *N*-∊-DNP-L-Lys and DNP.

Because of these discrepancies, the influence of sample concentration and the ratio of HCl used in the hydrolysis was tested. A sample of milk and another sample of an enteral formula were hydrolyzed using three different concentrations of HCl (6M, 8M, and 10M) and two different ratios of acid at each concentration (1 mg and 5 mg of protein in the sample per milliliter of acid). The results obtained appear in Table I. At the ratio of 1 mg of protein per milliliter of HCl (dilute hydrolysis), the yields obtained for ∊-DNP-Lys were similar for both 6M and 8M HCl, whereas the yield decreased when 10M HCl was used, possibly due to partial breakdown of the ∊-DNP-Lys during hydrolysis. At the ratio of 5 mg of protein per milliliter of acid (concentrated hydrolysis), the yields of ∊-DNP-Lys were lower, perhaps because protein hydrolysis was incomplete in those conditions. In view of these results, which were consistent with the results reported by Rao et al. (26) and Matheson (23), we decided to perform the hydrolysis using 6M HCl. The requisite volume of acid was calculated to achieve a ratio of 1 mg of protein per milliliter of acid after neutralization of the $NaHCO_3$. That ratio has been used by most researchers in analyzing amino acids (22).

A DNP precipitate forms during dinitrophenylation of the protein. Carpenter (13) cooled the solution before filtration after hydrolysis to minimize precipitation of DNP in the filtrates. Booth (14) proposed hot filtration and washing of residues to avoid ∊-DNP-Lys adsorption on incompletely washed cold residues. Both hot and cold filtration were tested in this study. In both cases, a suspension formed after filtration of the hydrolysate through no. 52 Whatman paper, making topping up and precise measurement of a representative aliquot

Table I. ∊-DNP-Lys Content (g/100 g protein) in Food Samples Hydrolyzed with Different Concentrations and Ratios of HCl*

	Ratio of acid	
Acid concentration	1 mg protein/mL acid	5 mg protein/mL acid
	Enteral formula	
6M	6.37 (1.04)	4.74 (2.03)
8M	6.47 (0.24)	6.15 (1.51)
10M	5.81 (0.64)	5.87 (0.11)
	Milk	
6M	7.47 (2.08)	5.40 (0.97)
8M	7.44 (6.90)	6.84 (7.26)
10M	7.12 (6.42)	6.93 (1.68)

* Mean values of three replications; CV is given in parentheses.

Table II. ∊-DNP-Lys and Total Lysine Content (g/100 g protein) in Pure Protein Samples*

		Experimental Lys		Theoretical Lys		Recovery (%)	
Pure protein	∊-DNP-Lys	Unadjusted value	Adjusted value	Unadjusted value	Adjusted value	Experimental Lys	Theoretical Lys
Albumin	11.34 ± 0.1	11.16 ± 0.5	-	-	-	101.61	-
Lysozyme	4.93 ± 0.2	5.86 ± 0.1	4.88	6.12	5.10	101.02	96.67
Ribonuclease	9.97 ± 0.3	10.45 ± 0.1	9.41	10.68	9.61	105.95	103.74

* Mean values of three replications.

difficult. Because the peak areas for the ∊-DNP-Lys were unaffected by the filtration temperature and hot filtration substantially raised the amount of DNP in the chromatogram, we decided to perform the filtration after the hydrolysate had cooled to room temperature, wash the residues with a large amount of water, and add the minimum amount of acetonitrile sufficient to achieve complete solubilization of the precipitate.

Recovery

Three pure proteins (albumin, lysozyme, and ribonuclease) were used to assess the reliability of the proposed method. Three samples of each pure protein were assayed by the proposed method and by the total lysine method as described above. Because the proteins used were pure proteins, all the lysine present in the samples was available lysine; hence comparison of the values obtained using the two methods should provide an indication of the accuracy of the proposed method. Table II presents the results of both procedures along with the theoretical values for lysine based on the molecular structure (29) of lysozyme and ribonuclease. In both these proteins, each protein molecule has an *N*-terminal lysine residue that was not determined in the proposed available lysine method because it is converted into di-DNP-Lys; therefore, the theoretical and experimental total Lys values were adjusted to remove the contribution by *N*-terminal lysine using the following equations:

$$\text{\% adjusted theoretical Lys} = \frac{\text{number of non-}N\text{-terminal Lys residues per molecule} \times \text{MW Lys}}{\text{MW protein}} \times 100$$

$$\text{\% adjusted experimental Lys} = \frac{\text{\% experimental Lys in protein} \times \text{number of non-}N\text{-terminal residues per molecule}}{\text{number of total Lys residues per molecule}}$$

The theoretical values for the albumin could not be calculated because the molecular structure of that protein was unavailable.

Table II indicates that the results for available lysine by the proposed method showed good agreement with the adjusted experimental and theoretical values of total lysine for all the protein samples. Kakade and Liener (30) reported that one *N*-terminal lysine residue per molecule produces an error in the available lysine determination that can be regarded as experimental error. However, Table II shows that this was not the case and that the error may be high. The initial assay of the pure proteins indicates that the derivatization reaction is quantitative and that ∊-DNP-Lys is stable during hydrolysis in the absence of carbohydrates, thus confirming that ∊-DNP-Lys is one of the most stable DNP-amino acids (31).

After verifying that the analytical method allowed complete recovery of the ∊-DNP-Lys when the sample was devoid of carbohydrates, the next step was to consider recovery in the presence of carbohydrates. Recovery was tested using three different types of foods with differing carbohydrate compositions: an enteral formula, milk, and lentils. Sanger (32), Matheson (23), and Booth (14) reported that the stability of free ∊-DNP-Lys and protein-bound (endogenous) ∊-DNP-Lys may be quite different, so the stability of both types of ∊-DNP-Lys was analyzed. For the former, a standard solution of ∊-DNP-Lys was added to the food samples after derivatization and acidification. For the latter, a pure protein, ribonuclease, was added to the food samples at the beginning of the analysis prior to derivatization and hydrolysis.

Table III presents the results for the recoveries of free and endogenous ∊-DNP-Lys. Recovery of free ∊-DNP-Lys was greater than 95% in all three types of food sample, though it differed for each food. Recovery was lowest for lentils, followed by milk and then the enteral formula. The differences may be attributable to the different types of primary carbohydrates in the respective foods, namely, monosaccharides, disaccharides (lactose), and polysaccharides (maltodextrines). That order is the same as for decreasing carbohydrate reactivity in the Maillard reaction, which could explain the different recovery values for the three food samples.

Recovery of endogenous ∊-DNP-Lys (Table III) was close to 100% in all three food samples, and no relationship could be established between the recovery value and the carbohydrate composition of the samples, hence protein-bound ∊-DNP-Lys appears to be stable during hydrolysis. Booth (14) tested the recovery with ∊-DNP-Lys and with previously dinitrophenylated proteins using the spectrophotometric method and found different stabilities in each case. As a possible cause of the differing

Table III. Recovery of Free and Endogenous ∊-DNP-Lys in Food Samples*

	Free ∊-DNP-Lys		Endogenous ∊-DNP-Lys	
Food sample	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Enteral formula	99.31	0.32	97.99	2.13
Milk	97.58	2.83	103.78	2.07
Lentils	95.02	1.30	102.60	0.30

* Mean values of three replications.

stabilities of free and endogenous ε-DNP-Lys, Booth postulated that breakdown products of the carbohydrates arising early in hydrolysis may react readily with the added ε-DNP-Lys, resulting in reduction of the nitro groups on the molecule, whereas endogenous ε-DNP-Lys released gradually from DNP-protein early in hydrolysis may be less susceptible to reduction. Consequently, because recovery of the free form is not comparable to the real situation in foodstuffs during hydrolysis, a correction factor calculated based on that recovery value would not be correct.

Figure 3 presents the chromatograms for the samples of milk and lentils. The chromatograms had only three peaks, one for DNP, one for ε-DNP-Lys, and an intermediate peak between the two that has not yet been identified. In comparison, the chromatogram obtained by Rabasseda et al. (19) had many more peaks as a result of the lower selectivity of the wavelength they used (254 nm).

The repeatability results of the assays for the enteral formula, milk, and lentil samples are set out in Table IV. Four replications of each analysis were performed. The maximum CV was around 3%.

Table IV. ε-DNP-Lys Content (g/100 g protein) in Food Samples*

Food sample	ε-DNP-Lys Mean	ε-DNP-Lys CV (%)
Enteral formula	6.46	2.42
Milk	6.89	3.19
Lentils	1.28	1.41

* Mean value of four replications.

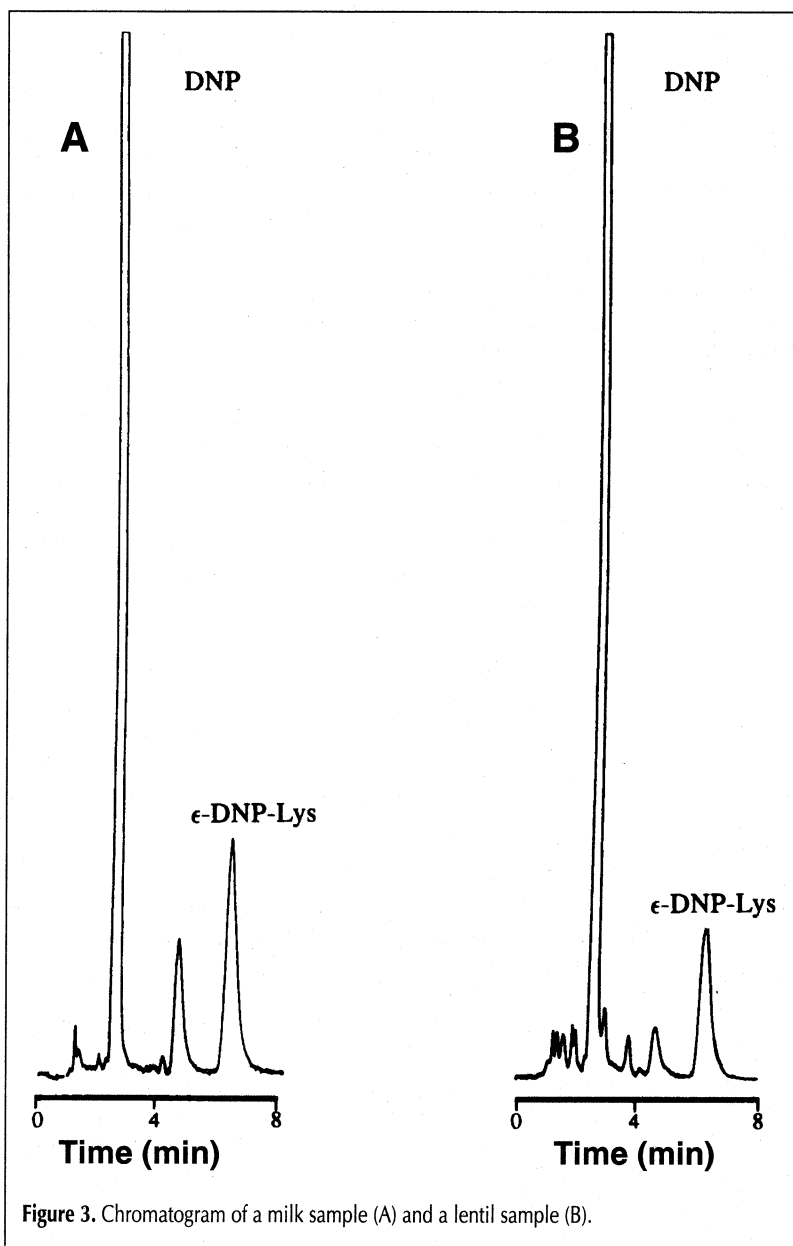

Figure 3. Chromatogram of a milk sample (A) and a lentil sample (B).

Conclusion

The derivatization of lysine using FDNB is an excellent method for the accurate and precise determination of the available lysine in foodstuffs.

FDNB is a very potent irritant with toxic effects that may cause skin sensitization. Use of nitrile gloves offers the best protection for the skin during laboratory work. Thompson and Edmonds (33) reviewed the toxic effect of FDNB and the precautions to be taken. The authors recommend reading that paper before undertaking any laboratory work using FDNB.

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