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Short communication

Determination of available lysine in infant milk formulae by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatography (HPLC) method to determine available lysine is proposed. Available lysine was measured by an optimised fluorodinitrobenzene method on the basis of the reactivity of the free ε-amino group of the lysine. The classical acid hydrolysis has been improved and shortened from the usual time of 12 h to 2 h 30 min using an oil bath. Optimal resolution and quantitation of N^{ε}-dinitrophenyllysine was obtained with a Nova-Pak C_{18} column using an isocratic elution with 35% methanol and 65% 0.01 *M* sodium acetate buffer (pH 4.5) and a flow-rate of 1 ml/min. Satisfactory results were obtained for the reliability of the method in terms of linearity from 0.1 to 5.0 mg/l of lysine-free base, precision (R.S.D. values between 4.3% and 7.8%), recovery (91.5%) and sensitivity (detection limit of 0.02 mg/l). The proposed method has also been checked for lack of interferences from other dinitrophenyl-amino acids. © 1997 Elsevier Science B.V.

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1. Introduction

Lysine is an essential amino acid usually used as an indicator of the potential biological value of food protein [1–4]. The ε-amino group of lysine is susceptible to chemical reactions which can render an amino acid nutritionally unavailable. During heat treatment, in some food, Maillard reactions occur between reducing carbohydrates and lysine and/or other amino acids and can lead to serious losses of nutritive value. Maillard reactions also occur under mild or moderate conditions such as food storage [3,5]. Knowledge of the amount of unmodified (available) and modified (blocked) lysine facilitates evaluation of the effects of treatment on protein quality. The stability of the available lysine is one of the most important nutritional factors [1] in food processed or stored in the presence of reducing sugars, such as infant milk formulae. Thus, the ε-amino group of the protein-bound lysine can react with lactose to form the 'Amadori product' (lactuloselysine), which is not susceptible to attack by proteolytic enzymes during digestion [6].

Chemical estimation of lysine in foods can be achieved by the measurement of total lysine or available lysine. Total lysine is generally determined after acid hydrolysis treatment, but it does not always reflect the amount of nutritionally available lysine. The value of total lysine should be close to the value of available lysine only in foods in which no Maillard browning has occurred. Most of the chemical methods used to determine available lysine depend on the reaction of a derivative reagent with a free ε-amino group of protein-bound lysine. Thus,

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only available lysine yields the derivate-lysine compound, whereas bound lysine, in which the ε-amino group is already blocked (unavailable lysine), cannot react.

The derivatization reaction most extensively used to determine available lysine was established by Sagner [7] using 1-fluoro-2,4-dinitrobenzene (FDNB). The derivative formed is N^{ε}-dinitrophenyl-lysine (N^{ε}-DNP-Lys), which can be measured spectrophotometrically after acid hydrolysis and extraction [8]. This classical direct method provides similar results to those obtained from both biological evaluation and enzymatic digestion in vitro [9]. Booth [10] and Hurrell and Finot [11] also concluded that the FDNB technique was the most reliable method for the determination of available lysine. One shortcoming of this method is that the N^{ε}-DNP-Lys is not completely stable during acidic hydrolysis and so recoveries are low [12]. However, reports on the stability of N^{ε}-DNP-Lys during hydrolysis are contradictory [2,7]. Furthermore, the derivatization reaction is not specific for lysine [7,10] and the carbohydrates present in food can lead to the formation of interfering compounds that cause a false estimation of lysine content [13–15].

Attempts have been made to separate N^{ε}-DNP-Lys from interfering amino acids and other compounds formed during hydrolysis by paper chromatography [16], liquid–liquid partition chromatography [13] and ion-exchange chromatography [17,18]. High-performance liquid chromatography (HPLC) is a rapid and efficient method for the separation of N^{ε}-DNP-Lys. Many of the sources of error inherent to the procedures described above are eliminated through the use of HPLC [14,19].

Few HPLC methods for the direct determination of available lysine are reported in foods. In these methods, the complex and prolonged sample treatment of Carpenter [8] modified by Booth [10] is usually employed [14,20]. Particularly for infant milk formulae, only one method has been reported, in which the sample treatment took more than 72 h [21].

The aim of this work was to optimise a method for the determination of available lysine in two types of infant formula: powdered and liquid infant milk. This last one has recently been introduced in the European market. Special emphasis was given to reducing the duration of the hydrolysis step in the sample preparation, based on the Rabasseda et al. [19] sample treatment to determine available lysine in soybean and fish.

2. Experimental

2.1. Chemicals

Methanol HPLC grade was obtained from SDS (Barcelona, Spain), ultrapure water was obtained from a Milli Q-System of Millipore (Bedford, MA, USA). Sodium acetate was from Merk (Barcelona, Spain). The derivative reagent FDNB was obtained from Fluka (Madrid, Spain). Standards N^{ε}-dinitrophenyl-L-lysine hydrochloride and N^{α}-acetyl-L-lysine were both from Sigma (Madrid, Spain).

2.2. Chromatography

The HPLC system (Waters Chromatography, Milford, MA, USA) consisted of a Waters 600E System Controller Pump, a Waters 715 Autosampler, a Waters 486 spectrophotometric detector. Data acquisition was accomplished by a System Millenium 2.10 version (Waters).

The separation was performed on a Nova Pak C_{18} column 150×3.9-mm, 4-μm particle size (Waters) with a matching guard cartridge. Optimum resolution was obtained using an isocratic elution at a flow-rate of 1 ml/min, with a mobile phase composed of 35% methanol and 65% 0.01 *M* sodium acetate solution adjusted to pH 4.5 (with glacial acetic acid), filtered through a 0.45-μm filter and degassed before use. Chromatographic analysis was carried out at room temperature. When steady baseline was recorded, 20 μl of standard solutions or prepared sample were injected. Ultraviolet detection was performed at 362 nm.

2.3. Derivative reagent and standard solutions

2.3.1. Derivative reagent

A 3% solution of FDNB in ethanol was made up fresh daily. FDNB is a powerful skin irritant and extreme care should be exercised in handling this compound [8,22].

2.3.2. Standards

N^{ε}-DNP-Lys. Stock solution: a solution of 500 mg/l expressed as lysine-free base was prepared in a methanol–water mixture (1:4). Working solutions of 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1, 2, 3, 4 and 5 mg/l were prepared by dilution of the stock solution. All the standard solutions was passed through 0.45-μm filter, stored in a refrigerator and protected from light. N^{α}-Acetyllysine: a solution of 1000 mg/l expressed as lysine-free base in methanol–water (1:4) was used for the recovery study. This solution was stored in a refrigerator and protected from light.

2.4. Sample preparation

Approximately 2 g of liquid infant milk or 0.25 g of powdered infant milk sample were accurately weighted and placed in a Tekator® digestion flask. Then, 10 ml of 8% (w/v) sodium bicarbonate solution was added and the contents were thoroughly mixed by gentle swirling. 15 ml of 3% FDNB solution were added to the digestion flasks, which were then stoppered and shaken for 2 h on a magnetic plate at room temperature. Ethanol was evaporated in a water bath at 95°C for 15 min, until no further effervescence was produced, even when shaking. Then, 40 ml of 8 *M* HCl was added and the samples were refluxed for 2 h 30 min at 160°C in an oil bath. Flasks were connected to a vacuum system to prevent loss of HCl. The dark brown reaction mixtures were filtered, while still hot, through a Whatman paper number 541 and collected into a 250-ml volumetric flask. The digestion flasks and the residues were washed thoroughly with water until the total filtrate had a volume of 250 ml. The filtrate was strongly agitated to prevent the formation of a precipitate of dinitrophenol, which can adsorb the N^{ε}-DNP-Lys. A 15-ml aliquot was transferred into a small beaker and adjusted to pH 5.0 with 2 *M* NaOH. Then, it was transfered to a 50-ml volumetric flask and made up to a volume with 10 ml of methanol and distilled water. Before the injection, each sample was filtered through 0.45 μm.

2.5. Calculations

The available lysine content was obtained directly by interpolation from calibration curves. Concentration of available lysine in milk, expressed as g lysine/100 g of protein, was calculated for powdered milk (C_{pm}) and liquid milk (C_{lm}) with the following formulae:

$$C_{pm} = (C \cdot 8.33 \cdot 10^{-2})/(\%_{protein} \cdot W)$$

$$C_{lm} = (C \cdot \delta \cdot 8.33 \cdot 10^{-2})/(\%_{protein} \cdot W)$$

where C = concentration from the calibration curves, $\%_{protein}$ = milk protein content, W = sample weight and δ = milk density.

2.6. Statistical analysis

All statistical analyses were performed using the statistics package SPSS for Windows 6.0.1 (SPSS Inc., Chicago IL).

3. Results and discussion

3.1. Optimal conditions for acid hydrolysis

An acid hydrolysis step is needed to determine any DNP-amino acids from DNP-protein after the Sagner reaction [7]. Therefore, it is necessary to measure the stability of DNP-derivatives during this step. Optimal conditions for acid hydrolysis would imply minimum losses of N^{ε}-DNP-Lys. Previous methods for the determination of available lysine generally used reflux times ranging from 12 to 24 h during the acid hydrolysis step. Only Rabasseda et al. [19] reported a hydrolysis time of 4 h in closed vessels in an autoclave used to determine available lysine in soybean and fish samples.

Table 1
Available lysine content as a function of the acid hydrolysis time

Hydrolysis time	Available lysine content (g lys per 100 g protein) mean (standard deviation)	R.S.D. values %
2 h	4.29 (0.86)[a]	20.08
2 h 15 min	4.63 (0.42)[a]	9.09
2 h 30 min	5.84 (0.29)[a]	5.05
2 h 45 min	4.68 (0.22)[a]	4.73

[a] Values bearing common superscript letters were not statistically different at a $p<0.05$ (Multiple Range test, LSD test).

Hydrolysis was carried out in a Tekator digestion flask connected to a vacuum system and inmersed in an oil bath, which allowed a constant temperature of 160°C. Thus, the time of hydrolysis was drastically reduced. Various hydrolysis conditions were studied to find the optimal combination of time/temperature (Table 1). The best results were achieved when the time applied was 2 h 30 min ($p<0.05$). Longer or shorter hydrolysis times increased the loss of lysine.

3.2. Optimal LC conditions

Several mobile phases were tested to achieve the optimum resolution of N^{ε}-DNP-Lys from interferences. The best resolution was obtained using a mobile phase of methanol–0.01 *M* sodium acetate solution (35:65) adjusted to pH 4.5. Potential interference from dinitrophenol, FDNB and other compounds was avoided. By using the mobile phases described above, the chromatograms obtained were relatively simple and identification was sure (Fig. 1). Identification was performed on the basis of retention time by comparison with standard solution. The average retention time of N^{ε}-DNP-Lys was 6.02 min ($n=10$) and the relative standard deviation (R.S.D.) was 2.24%.

N^{ε}-DNP-Lys was eluted in 6 min in isocratic conditions. However, a routine gradient program was designed to clean the chromatographic column. Cleaning was necessary to eliminate the excess FDNB and other brown compounds such as dinitrophenol (a decomposition product from FDNB), aminonitrophenol, humin and dinitroethoxybenzene [8,10,14,19], which are compounds formed during the hydrolysis step. Therefore, just after the elution of N^{ε}-DNP-Lys, an increase of the proportion of methanol up to 95%, and maintained for 4 min, was applied. Afterwards, the proportion was returned to 35% and requilibrated in 10 min. In this way, the interference to subsequent injections was avoided and the life of the column was increased.

3.3. Method validation

Reliability of this method was tested for linearity, precision, recovery and sensitivity. In addition, lack of interference was also verified.

3.3.1. Linearity

Detector response was linear between 0.1 and 5.0 mg/l expressed as lysine-free base. Least-squares analysis produced a correlation coefficient of $r=0.9985$ ($p<0.001$) with a coefficient of determination (r^2) of 99.71%. The linearity of the LC method was verified by analysis of variance of regression ($F_{\exp(1,40)}=14125.7$ and $p<0.0001$).

3.3.2. Precision

Eight determinations of a milk sample were performed on the same day using the same reagents and apparatus. R.S.D. values were 5.4% for powdered milk and 7.8% for liquid milk. Therefore, the %R.S.D. values of the method proposed were satisfactory according to the Kolthoff criterion [23].

The variability of the chromatographic system was also tested, after 8 injections of the same extract from a milk sample. The variability due to the chromatographic system was low, since it represented a relative standard deviation of 1.10%.

3.3.3. Recovery

N^{α}-Acetyllysine was the standard used to calculate the recovery of method, since, as Sagner [7] reported, this compound has the N^{α}-amino group blocked, as lysine in proteins. The use of free N^{ε}-DNP-lysine does not reflect what happens when DNP-protein is hydrolysed because free N^{ε}-DNP-Lys is less stable to acid hydrolysis than DNP-Lys-protein [10], especially in the presence of large amounts of carbohydrate [13], as occurs in milk. Free lysine cannot be used either, because it gives N,N-*bis*-DNP-Lys [7], which cannot be estimated in the conditions established to determine N^{ε}-DNP-lysine [13].

Two millilitres of 1000 mg/l N^{α}-acetyllysine standard solution was added in both types of infant formula, powdered and liquid milk. Eight samples of each type of milk were analysed and the recovery results are shown in Table 2. By using the Students *t*-test, we verified that the accuracy of the method does not depend on the type of sample ($p>0.05$).

3.3.4. Sensitivity

The detection limit (DL) and the determination limit (DtL) were calculated by applying the Long and Winefordner criterion [24]. The blank used was

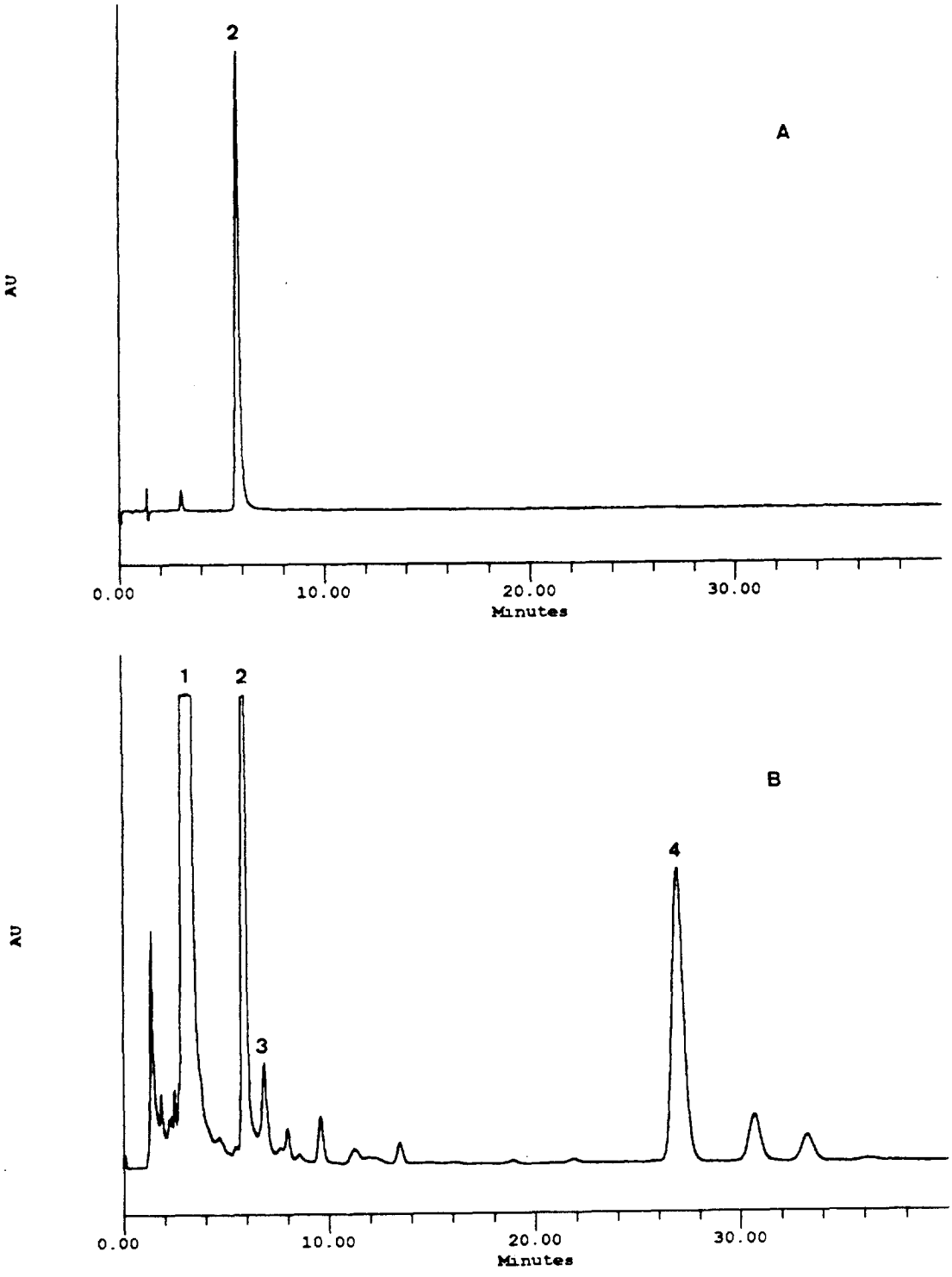

Fig. 1. Typical liquid chromatograms of N^{ε}-DNP-lysine: (A) standard solution of 2 μg/ml, (B) infant milk formula. Peak identities: (1) dinitrophenol; (2) N^{ε}-DNP-lysine; (3) FDNB and (4) dinitroetoxibenzene.

Table 2
Recovery of the method to determine available lysine in infant milk formula

	Initial content (g Lys per 100 g protein)	Content after addition[b] (g Lys per 100 g protein)	Recovery (%)
Powdered milk	5.97 (0.32)[a]	10.42 (0.54)	91.40 (4.39)
Liquid milk	5.02 (0.40)	8.81 (0.30)	92.01 (3.31)

[a] Mean (standard deviation).
[b] The standard addition, in both cases, was 2 ml of 1000 μg/ml of N^{α}-acetyllysine, which corresponds to 5.1 g lys per 100 g protein and 4.5 g lys per 100 g protein in powdered and liquid infant milk respectively.

2 ml of water–methanol (4:1) which was treated under the same conditions described for milk samples. From the results of 8 determinations, the DL was 0.02 mg/l and the DtL was 0.06 mg/l, both expressed as lysine-free base.

The sensitivity of the method was also checked by the analysis of very low concentration standard solutions [23], to identify the lowest concentration that could be detected by the chromatographic system. This procedure showed that 0.005 mg/l of lysine-free base was the minimum concentration of a standard solution detectable and 0.05 mg/l was the minimum concentration which yields an acceptable chromatographic response variability (less than 10% of R.S.D.). The difference in the DL values seems to be related to the baseline interference in the sample extracts.

3.3.5. Lack of interferences

The method was tested for the potential interference from other DNP-amino acids, which can be detected at the same wavelengths as N^{ε}-DNP-Lys.

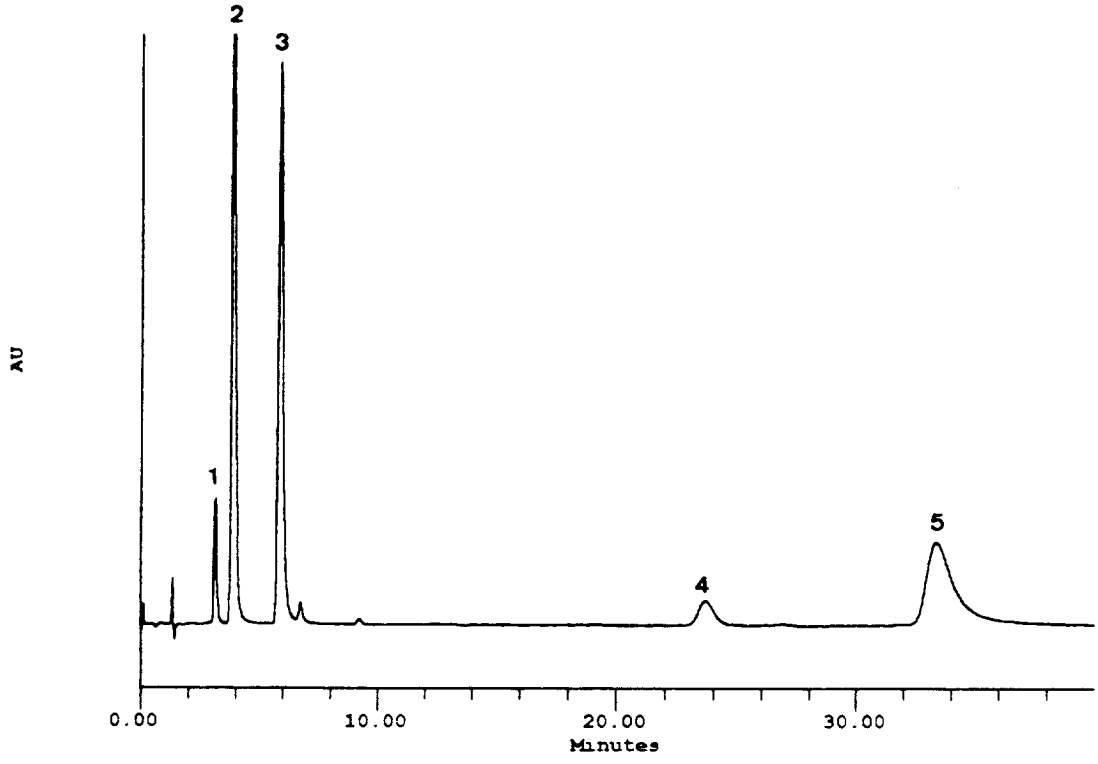

Fig. 2. Typical liquid chromatogram of a solution of DNP-amino acids: (1) N,S-di(DNP)-cysteine; (2) N-DNP-arginine; (3) N^{ε}-DNP-lysine; (4) N,N′-di(DNP)-histidine and (5) N,N-di(DNP)-cystine.

By injecting standard solutions of N-DNP-arginine, N,N′-di(DNP)-histidine, N,N-di(DNP)-cystine and N,S-di(DNP)-cysteine. No interference was observed in N^{ϵ}-DNP-Lys resolution (Fig. 2).

4. Conclusions

The proposed HPLC method to determine available lysine in infant milk formulae (liquid and powdered) yielded satisfactory results in terms of linearity, precision, recovery and sensitivity. The main advantage of the method described is that sample treatment is easier and faster than in other analytical procedures. In addition, the N^{ϵ}-DNP-Lys can be well resolved without interference from other DNP-amino acids.

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