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Isocratic high-performance liquid chromatographic method for quantitative determination of lysine, histidine and tyrosine in foods

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Abstract

A method for the quantitative determination of lysine, histidine and tyrosine in foods based on pre-column derivatization with 5-dimethylaminonaphthalene-1-sulfonyl chloride (DnsCl) and reversed-phase liquid chromatography has been developed. Derivatization conditions, including DnsCl concentration, time, temperature, and buffer solution were studied. To establish the reliability of the proposed liquid chromatographic (LC) method, the precision and accuracy of the analyses were evaluated using samples of casein and lysozyme.

1. Introduction

Quantitative determination of the essential amino acids, such as lysine and histidine, and semi-essential amino acids, such as tyrosine, in foods is important from both a technological and a nutritional standpoint.

Lysine is of interest in nutrition, quality control of fortification of wheat-based foods, plant breeding, and work on pure proteins [1–3]. Moreover, it is essential to know how much lysine is present in foods in order to determine available lysine by the difference, pronase digestion and sodium borohydride methods [4–7] and to calculate the percentage of availability. In addition, the formation of lysinoalanine in foods can be detected via analysis for lysine [8,9].

Histidine (His) is an essential amino acid for growing children and for persons suffering from uremic disorders [10,11]. The latest Recommended Dietary Allowances also consider it an essential amino acid for adults. In addition, the histidine content of foods can also be affected by the Maillard reaction [12]. It is important, therefore, that there should be a method for quantifying this amino acid in foods.

Tyrosine (Tyr) is a semi-essential amino acid as a phenylalanine conserver. Under certain circumstances, in which its synthesis from the precursor is disrupted (for instance, in premature babies and persons suffering from liver dysfunction), it can be essential [13]. Moreover, it is one of the main natural substrates for the enzyme polyphenoloxidase, and for this reason enzymatic browning in various fruits and vegetables is considered to be essentially a tyrosine reaction [14]. Consequently, a method of quantifying this amino acid would also be of considerable interest.

The amino acids in foods have commonly been analysed by ion-exchange chromatography (IEC). Properly applied, this technique is un-

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doubtedly highly reliable, but it has the drawbacks of being expensive and time-consuming, particularly if determinations are to be performed for only some, and not all, of the amino acids. In contrast, high-performance liquid chromatography (HPLC) is a faster, less complicated, and less expensive method. Techniques employing pre-column derivatization, which do not require special equipment, are of particular interest.

Pre-column derivatization agents for amino acid analysis include 5-dimethylaminonaphthalene-1-sulfonyl chloride (DnsCl). The reaction of DnsCl with amino acids has been studied by Gros and Labouesse [15], Seiler [16], and Gray [17]. DnsCl reacts with the unprotonated form of the amino group, and thus a pH of 9 or greater is required. However, at high pH this reaction competes with hydrolysis of DnsCl. Neadle and Pollit [18] showed that a second competing reaction takes place between excess DnsCl and dansylated amino acids, leading to the loss of dansylated amino acids and the formation of dansylamide ($DnsNH_2$). Tapuhi et al. [19] proposed using methylamine ($MeNH_2$) or ethylamine ($EtNH_2$) to suppress the dansylation reaction, thus eliminating that source of error.

When quantitative results are required, derivatization conditions must be carefully controlled. The literature contains conflicting data on the applicable conditions. The buffer solutions employed include 500 m*M* borate (pH 9.0) [1,2], 40 m*M* Li_2CO_3 (pH 9.5) [19–21], and 200 m*M* $NaHCO_3$ (pH 9.5) [17]. Certain workers have dissolved the DnsCl in acetone [2,22], others in acetonitrile [19–21]. The concentration of the DnsCl solution used by different researchers has varied, from 5.56 m*M* to 100 m*M*, and different derivatization temperatures and times have also been employed. Moreover, the object of most such work has been to determine the free amino acids in body tissues and fluids and to analyse the terminal NH_2 groups on proteins, and the conditions required for such analyses are quite different from the appropriate conditions for determining the amino acid content of foods.

The initial purpose of the present study was to develop a simple method of determining lysine in foods. Under conditions suitable for determining lysine, histidine and tyrosine were also identified in the chromatogram, and because of the importance of determination of these amino acids in foods, they were included in the study. Thus, the object of the present work was to establish the optimum derivatization conditions with DnsCl for the quantitative determination of lysine, histidine, and tyrosine in foods and to develop a fast, simple, reproducible, and reliable isocratic HPLC method by which to perform the analyses.

2. Experimental

2.1. Chemicals and reagents

L-Amino acids, Dns-amino acids, Dns-amide, DnsCl, methylamine hydrochloride, ethylamine hydrochloride, bovine-milk casein, and chicken egg white lysozyme were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were obtained from Reactivos Scharlau (Barcelona, Spain). Other analytical reagent-grade chemicals were obtained from Merck (Darmstadt, Germany). Water was quartz-distilled and deionized using the Milli-Q system (Millipore, Bedford, MA, USA).

Standard solutions of L-lysine and L-histidine were prepared by dissolving an appropriate amount of amino acid in Milli-Q water. The standard L-tyrosine solution was prepared by dissolving an appropriate amount of amino acid in Milli-Q water–40 m*M* Li_2CO_3 buffer (pH 9.5) at the ratio 1:1. In order to achieve complete solubilization it was necessary to sonicate this last-mentioned solution. Standard solutions of Dns-amino acids were always prepared in methanol. The DnsCl solution was prepared using acetonitrile. An aqueous solution of 2% methylamine was used.

2.2. Preparation of sample protein hydrolysate

A quantity of sample accurately weighed out was poured into a 100-ml Pyrex screw-cap flask and hydrolysed with 6 *M* HCl at 110°C in a nitrogen atmosphere for 24 h. The HCl propor-

tion was 1 ml HCl per mg of protein in the sample [23]. The hydrolysate was filtered through No. 541 Whatman paper and the volume adjusted to 250 ml. An aliquot of this solution was evaporated to dryness in a rotary evaporator at 40°C. Two replications of each protein hydrolysis were performed.

2.3. Pre-column derivatization

Pre-column derivatization of the amino acids was carried out using DnsCl. The dry residue of the protein hydrolysate was reconstituted with Milli-Q water to give a protein concentration of 0.15 to 0.25 mg per ml.

Formation of the dansylated derivatives was accomplished in a 10-ml screw-cap vial protected with aluminium foil, by combining 1 ml protein hydrolysate, 2 ml 40 m*M* Li_2CO_3 (pH 9.5), and 1 ml DnsCl solution (4 mg/ml, 14.83 m*M*), in that order. Two other buffer solutions, 500 m*M* H_3BO_3 (pH 9.0) and 200 m*M* Na_2CO_3 (pH 9.5), were also prepared and tested. The solution was mixed and kept at 60°C for 30 min. Methylamine solution (50 μl) was then added to quench the reaction [19]. The mixture was allowed to stand at room temperature for at least 15 min before HPLC analysis. Each hydrolysate was derivatized and analysed in duplicate.

2.4. Standard solutions

Quantitation was accomplished using external standards for L-lysine–HCl, L-histidine–HCl, and L-tyrosine–HCl. Solutions of each amino acid containing 80 μg of L-lysine, 40 μg of L-histidine, or 65 μg of L-tyrosine per ml were prepared fresh for each experiment. These standard solutions were mixed and diluted with water to a concentration range of from 4–24 μg/ml of L-lysine, 2–12 μg/ml of L-histidine, and 2–14 μg/ml of L-tyrosine. The standards were derivatized in the same way as the sample hydrolysates, except that 3 mg/ml DnsCl (11.12 m*M*) was used.

Calibration curves were obtained for the three amino acids by plotting the peak areas. Correlation coefficients greater than 0.998 were obtained in all cases.

Recovery was tested using casein and lysozyme hydrolysates as follows. A hydrolysate of each protein was divided into two aliquots. One was diluted with Milli-Q water, derivatized, and measured as described above. The standard solution of the three amino acids was added to the other aliquot, which was then derivatized and measured. All determinations were performed in duplicate.

2.5. Chromatography

The HPLC apparatus consisted of two Model 110B pumps and a Model 210A injector from Beckman (Berkeley, CA, USA) equipped with a 20-μl loop and a KNK-029.757 UV–Vis detector (Konik Instruments, Barcelona, Spain). Detection was carried out at 254 nm. Peak areas were determined using an SP-4290 recorder-integrator (Spectra-Physics, San Jose, CA, USA).

Separations were carried out on columns thermostatted at 40°C. Two columns were tested, a 300 × 3.9 mm I.D. column packed with Spherisorb ODS-2 (particle size 10 μm) (Sugerlabor, Madrid, Spain) and a 300 × 3.9 mm I.D. column packed with μBondapak C_{18} (particle size 10 μm) (Waters Assoc., Milford, CT, USA).

The mobile phase was acetonitrile–0.01 *M* phosphate buffer (pH 7.0) (39:61) at a flow-rate of 1.5 ml/min [2].

3. Results and discussion

Fig. 1 presents the chromatograms for standard solutions containing the three amino acids, L-lysine, L-histidine, and L-tyrosine, derivatized as described, on the μBondapak and Spherisorb columns. The reproducibility of the retention time was evaluated for each amino acid by performing six consecutive injections of the standard solution of the three amino acids. The coefficient of variation (C.V.) was less than 1%.

The mobile phase and flow-rate were the same as those given by Peterson and Warthesen [2]. The pH of the mobile phase, 7.0, was similar to

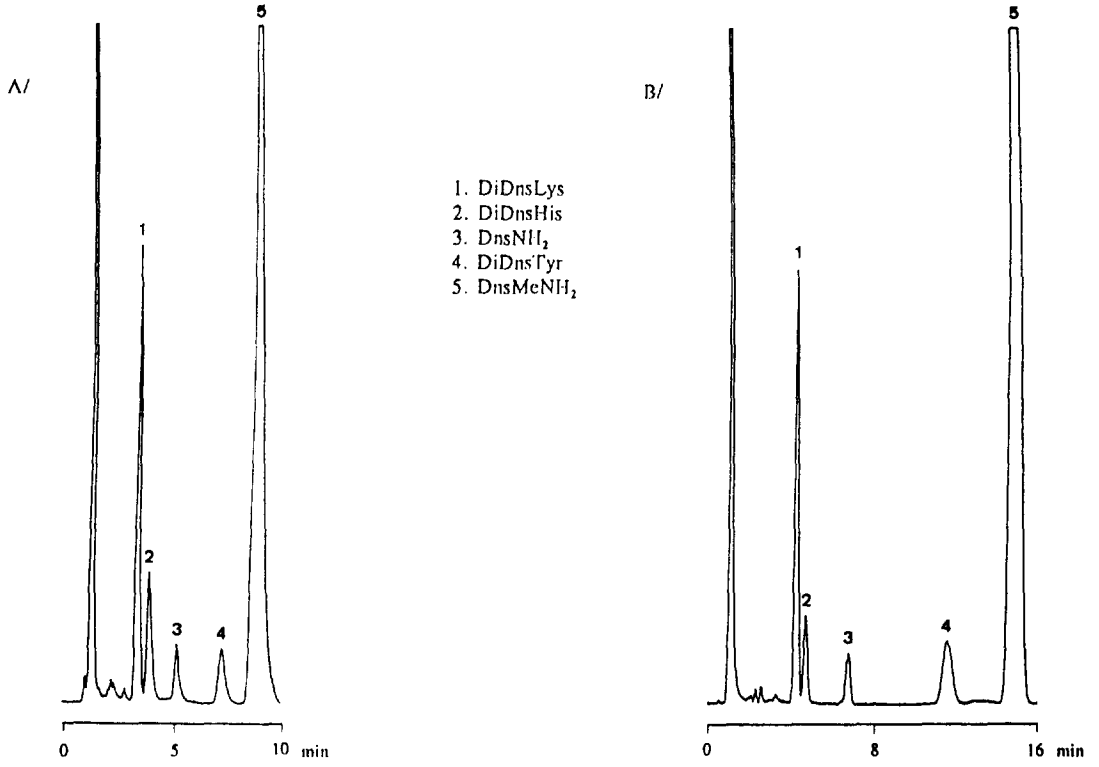

Fig. 1. Chromatogram of a standard solution. (A) μBondapak column, (B) Spherisorb column.

the value reported to be most appropriate for μBondapak columns by Wilkinson [24]. The peak tailing that occurred when the separations were carried out at ambient temperature was reduced by thermostatting the columns to 40°C. Bayer et al. [25] reported the same effect and attributed it to decreased hydrogen bridge formation by polar components. Temperatures of >40°C yielded no advantage. The main drawback of the Spherisorb column was the approximately 6 min longer separation time. The Spherisorb column did, though, have the advantage of reduced peak tailing, and it was also less expensive.

Tapuhi et al. [19] recommended two primary amines, methylamine and ethylamine, as reaction blockers. Methylamine was selected on account of the lower retention time for its dansylated derivative on the μBondapak column, 11 min as opposed to 17 min for the dansylated ethylamine. The aqueous solution of methylamine used could be refrigerated for up to 10 days.

The ratio of DnsCl to buffer was always 1:2 (v/v) as advocated by Tapuhi et al. [19] and Jong et al. [21]. A pH of around 9.0 during derivatization is required to achieve quantitative results. Three buffer solutions were tested for the method: 500 m*M* H_3BO_3 (pH 9.0) [2,25]; 40 m*M* Li_2CO_3 (pH 9.5) [19,21]; and 200 m*M* Na_2CO_3 (pH 9.5) [17]. Using each of the buffer solutions, various aliquots of the standard solution and of the protein hydrolysates were derivatized in the stated experimental conditions; the initial pH and the final pH on quenching the reaction with methylamine were measured. The initial and final pH values for the buffer solutions were 8.5 and 5.0 for H_3BO_3, 9.5 and 8.5 for Li_2CO_3, and 9.5 and 9.0 for Na_2CO_3. The borate buffer was therefore excluded, because it did not provide the necessary pH during derivatization. Of the remaining two buffer solu-

tions, Li_2CO_3 was selected rather than Na_2CO_3 because the Li^+ would further reduce the rate of dansylamide formation [19].

DnsCl is soluble in acetone and acetonitrile. Acetonitrile was selected as acetone is detected by UV, which broadens the chromatogram front so that the trailing edge of the front is near the lysine peak.

Derivatization is normally carried out at ambient temperature over a period of several hours [19,25] or at 40°C for 60 min [1,2,21]. However, Tapuhi et al. [19] obtained good results by carrying out the reaction at a higher temperature for a shorter time. Therefore, six aliquots of each of two standard solutions containing all three amino acids at two different concentrations were taken. Half the aliquots were derivatized at 60°C for 30 min and the other half was derivatized at 40°C for 60 min, and the peak areas for each of the amino acids were compared. The ratios of the peak area at 60°C to the peak area at 40°C for the three amino acids fell in the range 0.9903–1.025, indicating that derivatization was similar under the two sets of conditions. Since derivatization at 60°C for 30 min shortened the analysis time and, at the same time, precluded the variability otherwise in evidence due to changes in ambient temperature at different times of year, that derivatization temperature and time were chosen for the analysis.

Several filtration media were tested with a view to increasing mean column life. To this end, the peak areas for filtered and unfiltered derivatized standard solutions were compared for each of the three amino acids. Cellulose acetate filters are destroyed by acetonitrile, the DnsCl solvent, and were therefore unusable. Nylon filters (0.22 μm) retained around 10–12% of the different amino acids. C_{18} Sep-Pak cartridges (300 mg) (Waters), primed according to the manufacturer's instructions, were then tested. A quantity of 4 ml of sample was passed through the activated cartridge. To ensure complete elution and total recovery of the retained amino acids, 10 ml of mobile phase had to be passed through the cartridge. Since this step extended the overall analysis time and, as pointed out by Hart and White [26], reversed-phase columns are sufficiently inexpensive to be regarded as expendable, it was decided not to filter the solutions.

If the solutions were protected from light, they remained stable, and there were no absorbance losses in the 24 h following derivatization. Nevertheless, the sample should be injected as soon as possible. These findings are in agreement with the results reported by Tapuhi et al. [19] and Jong et al. [21].

Casein and lysozyme hydrolysates were used to evaluate the reliability of the proposed LC method. Fig. 2 presents the chromatograms for samples of casein and lysozyme on the μBondapak and Spherisorb columns, respectively. The protein hydrolysates were always evaporated to dryness before derivatization. As an alternative to evaporation, Peterson and Warthesen [2] neutralized the HCl with 6 *M* NaOH. In preliminary tests carried out at our laboratory, this method resulted in large variation between replicates from a given sample and was therefore not used. This may be attributable to the high saline concentration of the solution after neutralization and the resultant increase in ionic strength, which may exert an effect on the degree of dissociation of the carboxyl groups. Wilkinson [24] reported that this might affect the interaction with the column matrix.

The concentration of the DnsCl solution for derivatizing the samples was 4 mg/ml (14.83 m*M*) for hydrolysate containing between 0.15 and 0.25 mg of protein per ml. The choice of concentration was based on the addition assays, which produced yields of nearly 100%. Table 1 presents the results for recovery of the three amino acids from the casein and lysozyme hydrolysates. Recovery was in all cases nearly 100%, with C.V. values of less than 5.4%. These values indicate that, under the conditions of the assay, the derivatization reaction was quantitative.

Peterson and Warthesen [2] used 150 m*M* DnsCl to determine the lysine content of food samples. In our tests with that concentration, however, a disperse, non-sedimentary, quite voluminous precipitate that could not be redissolved appeared in the derivatized solution. Bayer et al. [25] advised against using large

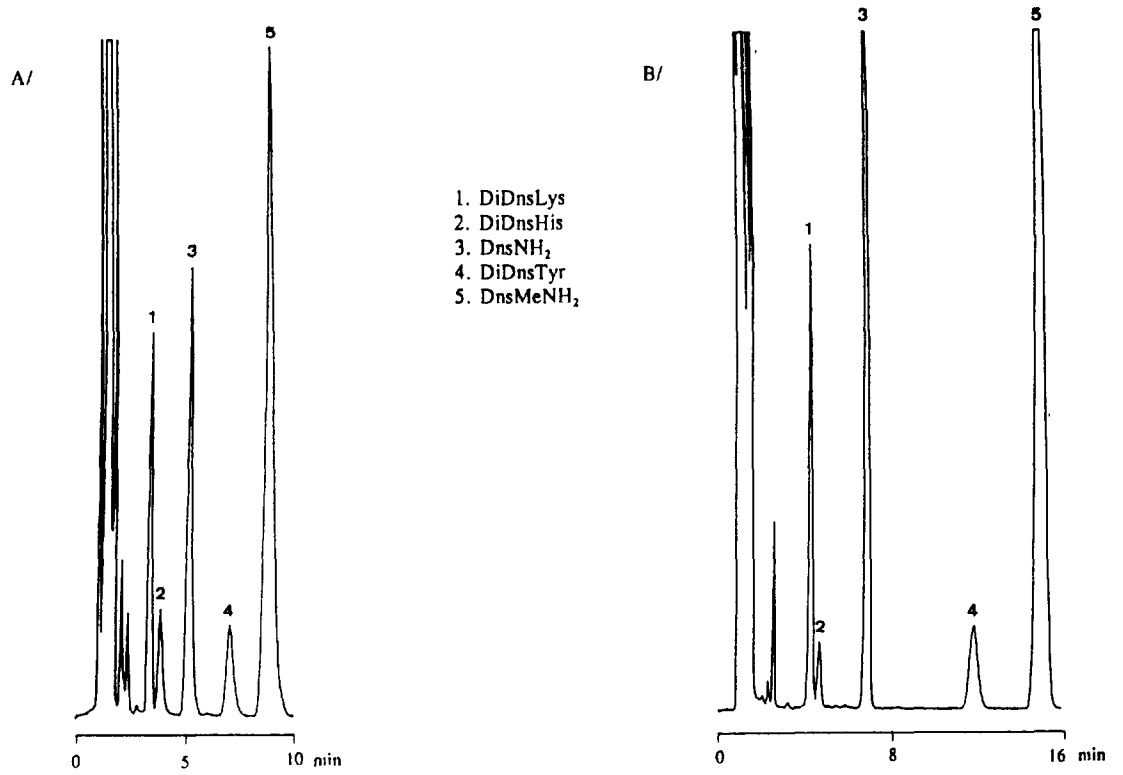

Fig. 2. (A) Chromatogram of a casein sample on the μBondapak column. (B) Chromatogram of a lysozyme sample on the Spherisorb column.

excess amounts of DnsCl, because of conversion to dansylamide and hydrolysis to the sulfonic acid. No other worker has employed such high concentrations of DnsCl.

Precision was evaluated by analysing five separate weighings of the casein and lysozyme hydrolysates. Each was derivatized and injected in duplicate. The variation (expressed as C.V.) did not exceed 2.7% for Lys, 4.6% for His, and 5.7% for Tyr.

Table 1
Recovery of lysine, histidine and tyrosine (mean values of 4 replications)

	Recovery (%)	C.V. (%)
Lysine	100.21	4.18
Histidine	102.56	1.77
Tyrosine	95.06	5.41

To assess the accuracy of the present technique in quantitation of lysine, histidine, and tyrosine, the casein and lysozyme samples were assayed both by the proposed LC method and by conventional IEC. Table 2 presents the results for both procedures along with literature values. Taking the IEC results as reference values, the results for lysine and histidine differed from the IEC values by less than 6.5%. The differences for tyrosine were larger, of the order of 14%, and the IEC values were always higher. The values for other samples, lentil and enteral solution, are also shown in Table 2. Of all events the results for all samples were in good agreement with literature values.

In summary, pre-column derivatization using DnsCl is appropriate for accurate determination of the lysine, histidine, and tyrosine concentration in food hydrolysates. The precision and accuracy of the HPLC method described indicate

Table 2
Lysine, histidine and tyrosine contents (g/16 g N) in the samples

Amino acid	LC[a]	IEC[a]	Literature values
Casein			
Lysine	8.33	8.17	8.29[b]
Histidine	3.91	3.67	2.98[b]
Tyrosine	5.92	6.56	5.94[b]
Lysozyme			
Lysine	5.86	6.12	5.42[c]
Histidine	1.23	1.21	1.08[c]
Tyrosine	3.25	3.79	3.69[c]
Lentil			
Lysine	6.54	–	7.18[b]
Histidine	3.07	–	2.74[b]
Tyrosine	2.92	–	3.26[b]
Enteral solution			
Lysine	10.31	–	10.00[d]
Histidine	2.42	–	2.10[d]
Tyrosine	2.61	–	2.70[d]

[a] Mean of three replications.
[b] FAO [27].
[c] Zumwalt et al. [28].
[d] Submitted by manufacturer.

that it is a reliable alternative to conventional IEC.

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