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Determination of amino acids by ion-pair liquid chromatography with post-column derivatization using 1,2-naphthoquinone-4-sulfonate☆

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Abstract

A new chromatographic method for the determination of amino acids is proposed. The method is based on the separation of amino acids by means of ion-pair liquid chromatography and post-column derivatization using 1,2-naphthoquinone-4-sulfonate. The analytical column was a Spherisorb ODS 2. Amino acids were separated by an elution gradient with four linear steps based on increasing the concentration of 2-propanol. Two eluents were used to create the gradient profile: eluent A was an aqueous solution of 20 m*M* H_3PO_4 + 20 m*M* $H_2PO_4^-$ + 15 m*M* dodecyl sulfate and eluent B was a mixture of aqueous (25 m*M* H_3PO_4 + 25 m*M* $H_2PO_4^-$ + 18.5 m*M* dodecyl sulfate)–2-propanol (1:1, v/v). The injection volume was 100 μl and the total flow-rate for the mobile phase was 0.8 ml/min. The chromatographic outlet was coupled on-line to the two-channel derivatization system which delivered reagent and buffer solutions. The reaction took place at 65°C in a reaction coil of 4 m × 1.1 mm I.D. The spectrophotometric detection was performed at 305 nm. The separation of common amino acids was done in 90 min, although an additional period of 15 min was required to stabilize the column. The repeatability of the method for lysine is 2.1% and the reproducibility is 2.6%. The detection limit for lysine is 0.09 nmol. The linear range for lysine is up to 32 nmol with a correlation coefficient of 0.999. The method was applied to the determination of amino acids in animal feed and powdered milks. The results of the method are in good agreement with those obtained with the standard amino acid autoanalyzer method.

1. Introduction

The analysis of amino acids is usually done by liquid chromatography. A chemical derivatization is required to improve the detection since most common amino acids are not readily detected by spectroscopy. For this purpose pre- and post-column derivatization methods can be used. Advantages and disadvantages of pre- and post-column labelling have been pointed out [1,2]. Phenylisothiocyanate [3,4], *o*-phthaldialdehyde (OPA) [5,6], dansyl chloride [7], dabsyl chloride [8] and 9-fluorenylmethylchloroformate [9,10] are most popular reagents for pre-column derivatization.

However, only a few post-chromatographic methods are described in the literature. Ninhydrin has been used successfully for post-

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column derivatization of amino acids [11–15], since it was proposed by Spackman et al. [11]. The ninhydrin stream reacts with the chromatographic eluate to give a derivative detected by visible spectroscopy at 570 and 440 nm. Some commercial amino acid analyzers are based on this reaction. In another study [13], ninhydrin has been added to the mobile phase prior the separation. In this case, the labelling reaction is developed by heating the solution emerging from the column in a reaction coil at 140°C. Hence, an additional channel to pump the reagent is not required. 1,2,3-Perinaphthindantrione has been used in the same way [13]. OPA has also been employed in post-column systems for both spectrophotometric and fluorimetric detection of primary amino acids [16–20]. A reducing agent is added to develop the reaction, which takes place at room temperature in a reaction coil coupled on-line to the chromatographic system. However, OPA is not suitable for the analysis of imino acids such as proline or hydroxyproline. Recently, hollow-fibre membrane reactors have been used in the reaction between OPA and amino acids [21–23]. Fluorescamine [24,25], 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole [26] and 4-fluoro-nitrobenzo-2-oxa-1,3-diazole [27] are other fluorogenic labelling agents for post-column derivatization.

Amino acids can be separated by cation-exchange chromatography or by reversed-phase ion-pair chromatography. In cation-exchange chromatography the separation is performed on a sulfonic resin, using a mobile phase based on a lithium or sodium cation elution gradient together with an increasing-pH gradient. In ion-pair chromatography, the ion pairs formed between the acidic form of amino acids and an anionic surfactant are separated on a reversed-phase column. This type of separation should be preferred because reversed-phase columns are cheaper and live longer is higher than sulfonated columns.

In this work, a new chromatographic method for the spectrophotometric determination of amino acids is proposed. The method is based on the separation of amino acids by ion-pair high-performance liquid chromatography and the subsequent derivatization on-line with 1,2-naphthoquinone-4-sulfonate (NQS). The stationary phase is C_{18}-modified silica. The mobile phase is a phosphoric/dihydrogenphosphate buffer solution containing dodecyl sulfate as surfactant and 2-propanol as a solvent. In order to optimize the separation and to decrease the time of analysis an elution gradient of 2-propanol has been used. The outlet of the analytical column is coupled on-line to the derivatization system in which the reaction between amino acids and NQS takes place. The optimum conditions to develop this reaction in flow systems have been previously described [28,29]. NQS reacts with amino acids in basic medium giving a derivative which is spectrophotometrically detected at 305 nm and 480 nm. NQS has advantages over other reagents mentioned above, since it is soluble in water, reacts with primary and secondary amino groups under milder conditions and is quite cheap.

Finally, this chromatographic method has been applied to the determination of amino acids in extracts of commercial powdered milks and animal feed.

2. Experimental

2.1. Reagents

Phosphoric acid (Carlo Erba, analytical grade), sodium dihydrogenphosphate (Carlo Erba, analytical grade), sodium dodecyl sulfate (SDS) (Merck, analytical grade) and 2-propanol (Probus, HPLC grade) were the constituents of the mobile phase.

Amino acids (analytical grade) were supplied by Merck.

Sodium NQS (Aldrich, analytical grade) was used to prepare a $1.2 \cdot 10^{-3}$ *M* solution in 0.1 *M* hydrochloric acid. This solution is stable for at least two weeks.

Buffer stock solution of 0.015 *M* sodium hydrogencarbonate (Scharlau, analytical grade) + 0.185 *M* sodium carbonate (Scharlau, analytical grade) was used to neutralize the

solution emerging from the column and adjust pH for the development of the reaction.

Commercial powdered milk and animal feed samples were obtained from Cooperativa Agropecuaria de Guissona (Lleida, Spain).

2.2. Apparatus

A LKB Bromma 2152 LC controller connected with two LKB Bromma 2150 HPLC pumps was used to pump the eluents and to generate the elution gradient. Samples were injected by means of a Spark Holland Promis automatic injection system. The analytical column was a Spherisorb ODS 2 (150 mm × 4.6 mm I.D., 5 μm of particle size). The column was held at 50°C using an Spark Holland SPH 99 column thermostat. The detector was a Waters 486 tunable absorbance detector with a flow cell of 10 mm path length and 8 μl of dead volume. Data acquisition was performed with a Perkin-Elmer (PE) Nelson 900 Series interface coupled to a microcomputer. Data were stored on floppy disks for further calculations. The equipment for amino acid analysis using the standard method was a Pharmacia LKB autoanalyzer, Model Alpha Plus (Series Two).

2.3. Chromatographic procedure

The experimental set-up used in this study is shown in Fig. 1. Two different mobile phases were prepared: eluent A was an aqueous solution of 20 m*M* phosphoric acid + 20 m*M* sodium dihydrogenphosphate + 15 m*M* SDS, while eluent B was a mixture of aqueous (25 m*M* phosphoric acid + 25 m*M* sodium dihydrogenphosphate + 18.5 m*M* SDS)–2-propanol (4:1, v/v). The elution gradient profile for the chromatographic separation of amino acids was as follows: step 1: time = 0–10 min, % eluent B = 0; step 2: time = 10–85 min, % eluent B = 0–100 (linear); step 3: time = 85–88 min, % eluent B = 100–0 (linear); step 4: time = 88–90 min, % eluent B = 0. The injection volume was 100 μl and the total flow-rate of the mobile phase was set at 0.8 ml/min. The column outlet was coupled on-line to the two-channel derivatization system, in which the NQS reagent and carbonate/hydrogencarbonate solutions were pumped by means of a peristaltic pump (Scharlau HP4) using standard Tygon tubing. Reagent and buffer solutions merged in a mixing chamber. The reaction coil of 4 m × 1.1 mm I.D. was placed in a thermostatic bath (SBS TFB-3) at 65°C. The spectrophotometric detection was performed at 305 nm. The pH of the final reagent solution emerging from the mixing chamber was sufficiently basic to neutralize the chromatographic effluent and provide the proper medium for the development of the reaction (pH 9.7). This final reagent solution cannot be prepared directly as a stock solution because NQS quickly decomposes in basic medium, so it has to be generated on-line with the flow system [28,29].

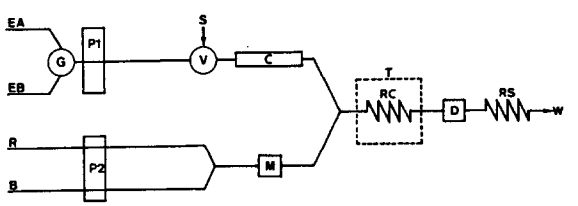

Fig. 1. Flow system scheme. P1 = HPLC pump; P2 = peristaltic pump; G = gradient programmer; V = injection valve; C = analytical column; M = mixing chamber; RC = reaction coil; T = thermostatic bath; D = detector; RS = restrictor; EA = eluent A; EB = eluent B; R = reagent (NQS); B = buffer; S = sample; W = waste.

2.4. Autoanalyzer procedure

The amino acid autoanalyzer is based on the ninhydrin method [11]. Amino acids were separated in a column of 20 cm × 4 mm I.D. packed with a cation-exchange resin Ultropac 7 (8 μm particle size). The column temperature was varied from 20 to 75°C with a Peltier heating/cooling system. The elution is performed by means of a lithium citrate buffer with increasing pH and ionic strength. The chromatographic eluate reacts with ninhydrin in a reaction coil of 0.3 mm I.D. which was contained in a heater jacket at 135°C. The amino acid derivatives were detected spectrophotometrically at 570 and 440

nm using a flow cell of 8 μl volume and 15 mm pathlength.

2.5. Sample treatment

A 2-g amount of feed sample (or 1 g of powdered milk) were subjected to a solid–liquid extraction with 50 ml of 0.01 *M* hydrochloric acid in order to recover free amino acids. The extraction was performed for 60 min in a conical flask by means of magnetic stirring. The extract was centrifuged at 13 000 rpm for 30 min. Solutions obtained in this way were stored in the fridge.

Solutions injected into both HPLC system and amino acid analyzer were prepared from 1000 μl of extract solutions and 100 μl of 10 m*M* norleucine as internal standard, and by filtering through an Ultrafree-MC low-binding cellulose membrane (10 000 NMWL) from Millipore.

3. Results and discussion

3.1. Study of separation conditions

The effect of the concentration of phosphoric acid in the mobile phase on the retention time was studied by varying the concentration of the acid from 1 to 50 m*M*. Concentrations of dihydrogenphosphate and SDS were kept constant at 20 and 15 m*M*, respectively. The retention time of amino acids increased markedly with acidity in the range 1 to 10 m*M*, while from 10 to 50 m*M* the variation was less noticeable. The value chosen for further experiments was 20 m*M*.

Fig. 2 shows the influence of the SDS concentration in the mobile phase on the retention time of alanine. The SDS concentration was varied from 1 to 30 m*M*. The concentrations of phosphoric acid and dihydrogenphosphate were maintained at 20 m*M*. At SDS concentrations higher than 5 m*M*, the retention time of Ala continuously decreases with surfactant concentration. With these conditions, this fact can be attributed to the formation of micelles in the system. The value chosen for the SDS concentration was 15 m*M*.

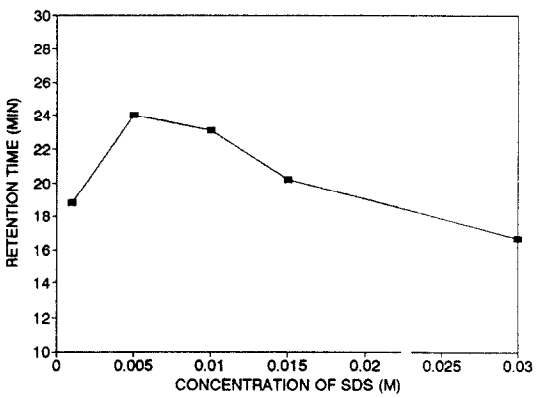

Fig. 2. Effect of the SDS concentration on the isocratic elution of alanine. Amount injected, 100 nmol; reaction coil, 6 m × 1.1 mm I.D.; reaction coil temperature, 65°C; eluent, phosphate buffer (20 m*M* H_3PO_4 + 20 m*M* $H_2PO_4^-$ + SDS)–methanol (4:1, v/v). Other conditions are given in the text.

In these preliminary studies, methanol was added to the mobile phase as solvent to facilitate the elution of amino acids. However, the retention times for several amino acids such as Trp, His, Lys or Arg were too long even at high ratios of methanol/water. In order to reduce the time of analysis methanol was substituted by 2-propanol. Fig. 3 shows the chromatograms for the isocratic elution of several amino acids at three percentages of 2-propanol in the mobile phase. By comparing the common amino acids of this figure (Ala and Pro in Fig. 3a and b, and Nle and Trp in Fig. 3b and c), there is a marked decrease in the retention time with increasing the percentage of 2-propanol. On the basis of these results, different elution gradients were investigated in order to optimize the separation of common amino acids. Fig. 4 shows the chromatogram obtained under the gradient profile chosen. This separation takes 90 min although a further 15 min are required to stabilize the column before the next injection.

3.2. Study of post-column reaction conditions

The most characteristic factors that affect the post-column derivatization of amino acids with NQS were pH, concentration of NQS, tempera-

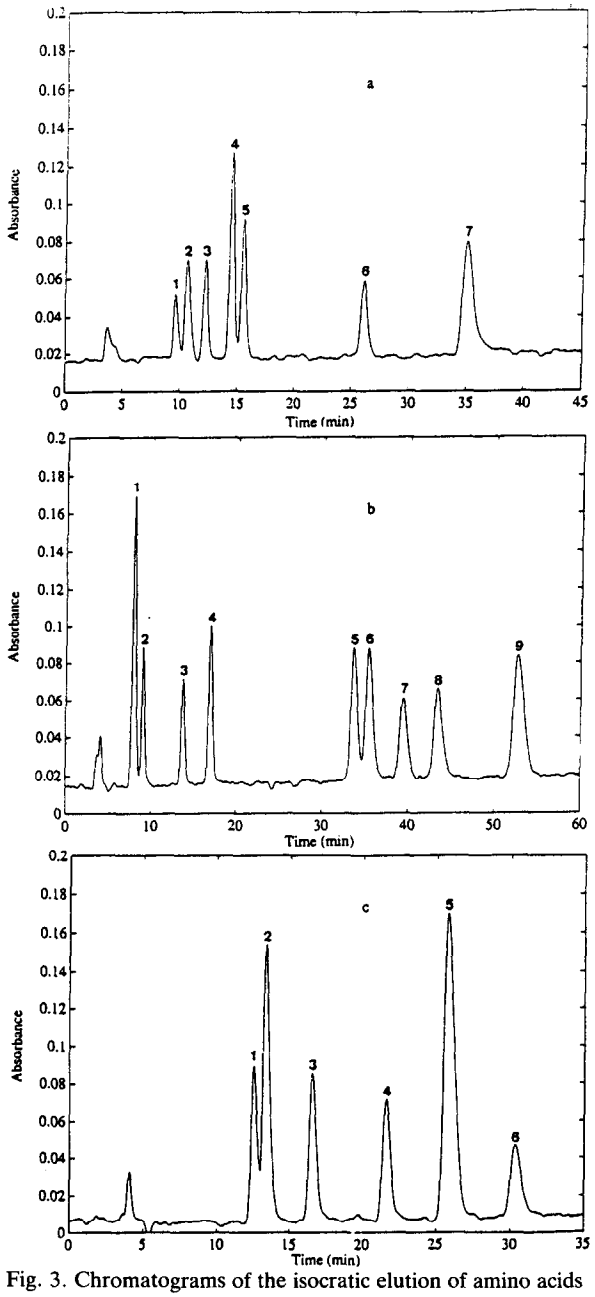

Fig. 3. Chromatograms of the isocratic elution of amino acids at different percentages of 2-propanol in the mobile phase. Injected amounts: Tyr 20 nmol and each other amino acid 40 nmol. Eluent: phosphate buffer (20 m*M* H_3PO_4 + 20 m*M* $H_2PO_4^-$ + 15 m*M* SDS)–2-propanol. Other conditions are given in the text. (a) 0% (v/v) of 2-propanol; peaks: 1 = Asp; 2 = Ser; 3 = Glu; 4 = Gly; 5 = Thr; 6 = Ala; 7 = Pro. (b) 10% (v/v) of 2-propanol; peaks: 1 = Pro; 2 = Asp; 3 = Tyr; 4 = Met; 5 = Ile; 6 = Phe; 7 = Leu; 8 = Nle; 9 = Trp. (c) 20% (v/v) of 2-propanol; peaks: 1 = Nle; 2 = Trp; 3 = His; 4 = Orn; 5 = Lys; 6 = Arg.

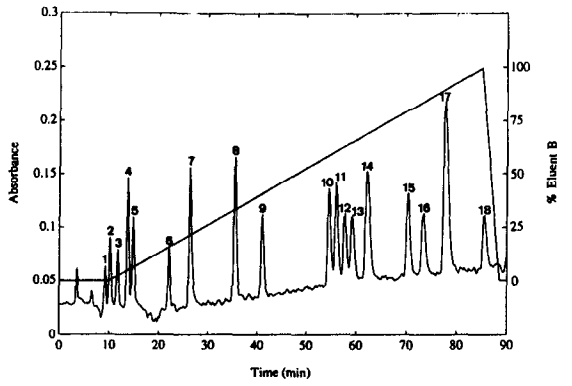

Fig. 4. Chromatogram of amino acids using the elution gradient chosen. Injected amounts: 40 mmol of each amino acid. Peaks: 1 = Asp; 2 = Ser; 3 = Glu; 4 = Gly; 5 = Thr; 6 = Ala; 7 = Pro; 8 = Tyr; 9 = Met; 10 = Ile; 11 = Phe; 12 = Leu; 13 = Nle; 14 = Trp; 15 = His; 16 = Orn; 17 = Lys; 18 = Arg. Line = elution gradient profile.

ture and dimensions of the reaction coil. The experimental conditions for the development of the reaction in flow systems were reported previously [28,29]. In this study, those conditions have been adapted to the new post-column system as follows.

As the separation is performed at pH 2.5 and the reaction between NQS and amino acids occurs at pH 9 to 10, a 0.185 *M* carbonate + 0.015 *M* hydrogencarbonate solution was used as buffer. Under these conditions the pH in the reaction coil was 9.6.

The dimensions of the reaction coil influence both the sensibility and the separation of the method. Reaction coils of 1.1 mm I.D. worked satisfactorily while with the other ones assayed (0.7, 0.5 and 0.35 mm I.D.) the baseline was instable with a high level of fluctuation. This instability was probably due to the incomplete mixing of the chromatographic effluent and the reagent solution. Fig. 5 shows the effect of the length of the reaction coil on the peak height. Maximum absorbance was mostly obtained for a reaction coil of 5 m, although in the range from 4 to 8 m the absorbance was nearly constant. The influence of the length of the reaction coil on the chromatographic resolution of five pairs of close peaks is shown in Fig. 6. From this figure, it can

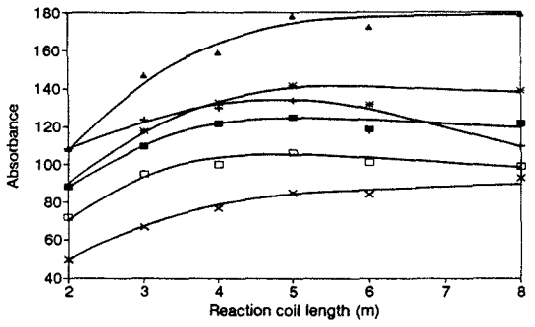

Fig. 5. Influence of the reaction coil on the absorbance at 305 nm. Injected amounts: 40 nmol of each amino acid. Other conditions are given in the text. ■ = Gly; + = Pro; * = Tyr; □ = Trp; × = His; ▲ = Lys.

be seen that peaks become wider and the resolution decreases as coil length increases. Finally, a reaction coil of 4 m × 1.1 mm I.D. was chosen on the basis of a suitable compromise between peak height and peak resolution.

The peak height increases continuously from room temperature to 70°C and remains virtually constant in the range 70–90°C. Thus, although the maximum absorbance was attained in the range indicated, the reaction was developed at 65°C in order to prevent the formation of bubbles in the system.

The effect of SDS and 2-propanol contained in the mobile phase on the post-column reaction was also studied. For this purpose a non-chromatographic system was used, in which solutions with different concentrations of SDS and 2-propanol were pumped to simulate the chromatographic eluate. In the range of concentrations studied [0 to 30 m*M* SDS and 0 to 40% (v/v) of 2-propanol] the absorbance was unaffected. In consequence, the final concentrations chosen for SDS and 2-propanol were those previously selected in the study of separation conditions.

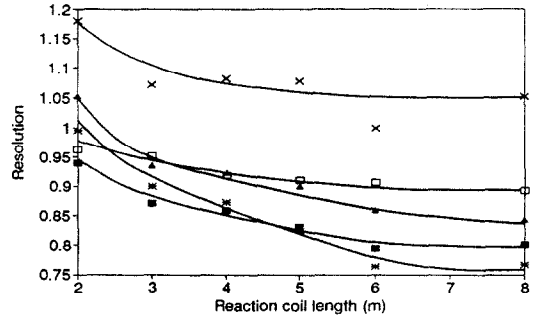

Fig. 6. Influence of the reaction coil on the chromatographic resolution of pairs of close peaks. For conditions see Fig. 5. ■ = Asp–Ser; * = Gly–Thr; □ = Ile–Phe; × = Phe–Nle; ▲ = Nle–Leu.

3.3. Figures of merit

In order to establish the figures of merit for the proposed method a preliminary study was performed to determine which of the following chromatographic parameters provided the best precision: peak area, peak height, peak $\text{area}_{\text{amino acid}}/\text{peak area}_{\text{internal standard}}$ and peak $\text{height}_{\text{amino acid}}/\text{peak height}_{\text{internal standard}}$. The internal standard was $4 \cdot 10^{-4}$ *M* norleucine. Results indicated that the use of Nle as internal standard to perform the calculations did not contribute significatively to the improvement of the results. The lowest R.S.D.s were obtained using the peak height, thus, this parameter was selected for further determination of the figures of merit.

The characteristics of the method at 305 nm, under the optimum conditions described above, are summarized in Table 1. The repeatability of the method was studied by injecting six times consecutively a standard solution of amino acids. The reproducibility on different days was also calculated by injecting the same standard solution for six days. In this case, fresh solutions of reagent, carbonate buffer and eluents A and B were prepared daily. Repeatability and reproducibility were studied both for peak height and retention time. The limit of detection was calculated for a signal-to-noise ratio of 3. In general, for all amino acids tested, the repeatability is better than 4%, the reproducibility better than 5% and the detection limits vary between 0.09 and 0.33 nmol.

3.4. Determination of amino acids in powdered milks and animal feed samples

The proposed method was applied to the determination of the contents of free amino acids

Table 1
Figures of merit for the proposed method at 305 nm under optimum conditions

Amino acid	Retention time		Peak height		Linear range (nmol)	Detection limit (nmol)
	Repeatability (R.S.D., %)	Reproducibility (R.S.D., %)	Repeatability (R.S.D., %)	Reproducibility (R.S.D., %)		
Ser	0.8	1.5	1.7	3.4	Up to 40	0.16
Gly	0.2	1.5	1.9	4.1	Up to 40	0.10
Pro	0.1	0.9	1.6	1.5	Up to 40	0.08
Tyr	0.1	0.8	2.2	3.7	Up to 40	0.11
Met	0.1	1.0	4.1	5.0	Up to 40	0.20
Trp	0.1	0.9	3.8	5.2	Up to 40	0.14
His	0.1	0.5	3.9	4.6	Up to 40	0.26
Lys	0.1	0.6	2.1	2.6	Up to 32	0.09
Arg	0.2	0.4	2.2	4.9	Up to 40	0.33

in feed and powdered milks for animal nourishing. Some essential amino acids such as lysine and methionine are usually added during the elaboration process of this kind of samples in order to correct their lack in proteins used as raw material. Thus, samples were subjected to the solid–liquid extraction previously described, in order to recover the free amino acids.

Fig. 7 shows the chromatograms of the aqueous extracts of a powdered milk and a feed

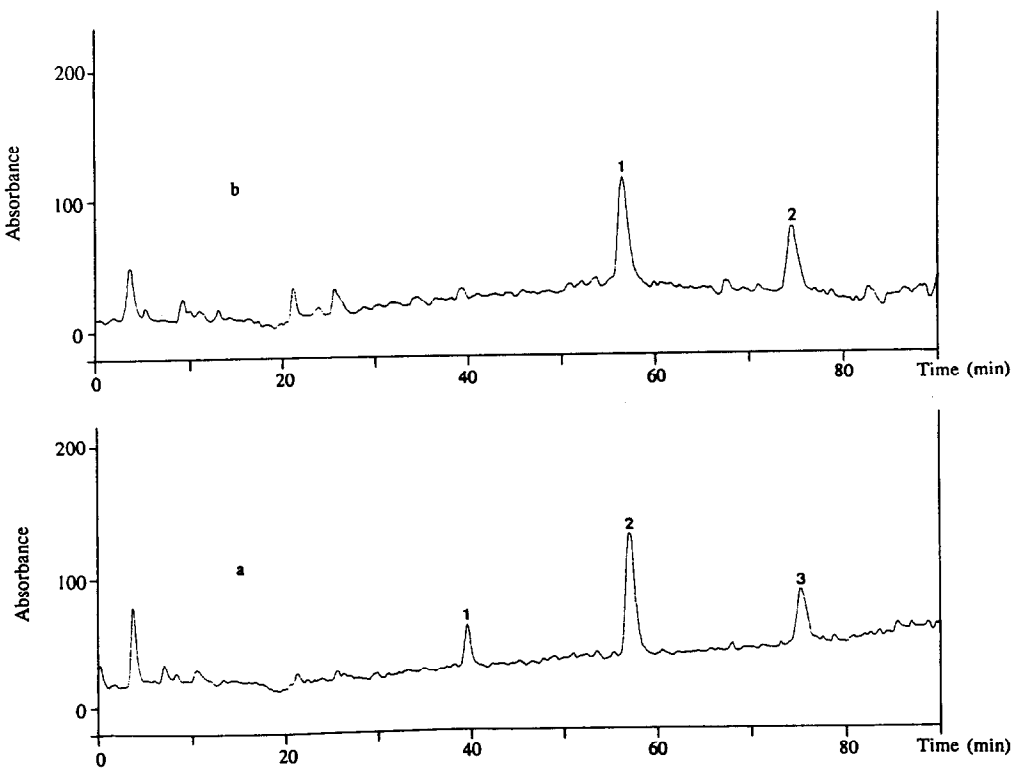

Fig. 7. Chromatograms of extract solutions under optimum conditions. (a) Powdered milk sample; peaks: 1 = Met; 2 = Nle; 3 = Lys. (b) Feed sample; peaks: 1 = Nle; 2 = Lys.

Table 2
Determination of amino acids in feed and powdered milks by the proposed method (NQS method) and the standard method

Sample	Amino acid (g amino acid/100 g sample)			
	Methionine		Lysine	
	NQS method	Standard method	NQS method	Standard method
Milk 1	0.151	0.156	0.056	0.055
Milk 2	0.166	0.168	0.121	0.123
Feed 1	– [a]	– [a]	0.066	0.069
Feed 2	– [a]	– [a]	0.139	0.137

[a]Not present in the sample.

sample, respectively. Although they show that other amino acids (such as proline, arginine and alanine) are present in the samples, the aim of this analysis is focused on the control of the essential amino acids added in the elaboration process, and as a consequence only Lys and Met have been quantified in the samples. Results obtained using the proposed method were compared with those from the standard amino acid analyzer in order to test the accuracy of the method. From Table 2, there is a good agreement between both methods.

4. Conclusions

Sodium NQS is an useful reagent for amino acid analysis using UV–visible spectroscopy. The use of NQS in post-column labelling shows advantages over reagents for pre-column labelling (phenylisothiocyanate, dansyl and dabsyl chlorides and 9-fluorenylmethylchloroformate) because NQS is water soluble and cheaper. In pre-column derivatization methods, the labelling reaction is usually carried out by means of batch procedures. However, the proposed method involves the on-line post-column derivatization, thus the automation is easy, the sample preparation is minimized and problems deal with derivative instability are overcome. OPA and fluorescamine fail in the labelling of secondary amino acids, while NQS reacts with both primary and secondary amino acids. In comparison with the standard method for amino acid determination based on ninhydrin post-column labelling, NQS reacts under milder conditions and dual-wavelength detection is not required in this case. The separation is performed on a reversed-phase column which is cheaper than the cation-exchange columns used in the ninhydrin method. Although the method has been employed in the analysis of feed and powdered milks, it can be easily adapted to other kind of routine samples.

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