ELSEVIER

Journal of Chromatography A, 740 (1996) 21–30

JOURNAL OF CHROMATOGRAPHY A

Chromatographic determination of amino acids by pre-column derivatization using 1,2-naphthoquinone-4-sulfonate as reagent

J. Saurina, S. Hernández-Cassou*

Departament de Química Analítica, Universitat de Barcelona, Diagonal 647, 08028 Barcelona, Spain

Received 16 November 1995; revised 24 January 1996; accepted 24 January 1996

Abstract

A new method based on the pre-column derivatization of amino acids with 1,2-naphthoquinone-4-sulfonate followed by the chromatographic separation, on a C_{18} column, of the derivatives formed is proposed. The reaction is developed at pH 10 and the reaction mixture is thermostated at 65°C for 5 min. An elution gradient with seven steps based on increasing the percentage of acetonitrile separates common amino acids present in protein hydrolysates. For an injection volume of 50 μl, the linear range of the method for glycine was up to 20 nmol, its precision expressed as R.S.D. was 2.6% and the detection limit was 60 pmol. The method has been applied to the determination of amino acids in hydrolysates of feed samples. Results obtained by the method proposed show satisfactory agreement with those given by the standard method.

Keywords: Derivatization, LC; Amino acids; Naphthoquinone sulfonate

1. Introduction

The determination of amino acids is usually carried out by HPLC with spectroscopic detection. However, direct detection provides low sensitivity for most amino acids, thus a chemical derivatization procedure is often required to increase the response. The characteristics, advantages and disadvantages of the two alternatives for derivatization (pre- and post-column techniques) have been widely discussed [1].

Most of the reagents reported in the literature for amino acid analysis are based on the reactivity of the amino group. Ninhydrin was used for the development of some amino acid analyzers [2,3]. It is applied in post-column labellings, and requires drastic reaction conditions. Dual wavelength detection is often used, since their primary and secondary amino acid derivatives absorb at different wavelenghts. Phenylisothiocyanate (PITC) [4,5] and other isothiocyanates [6] have been used in amino acid derivatization before the separation step. The addition of the amino function on the isothiocyanate is followed by a rearrangement of the chemical structure with the participation of the carboxyl group to give a thiohydantoin, which can be detected by spectrophotometry or fluorimetry. Dansyl [7] and dabsyl [8] chlorides have been also applied to amino acid derivatization in pre-column methods under drastic reaction conditions of temperature and time. These compounds react with amino groups forming fluorescent sulfonamines. *o*-Phthaldialdehyde (OPA) allows both pre- [9,10] and post-column [11,12] formation of the corresponding amino acid derivatives. The reaction must be developed in the presence of thiols, such as 2-mercaptoethanol, or mercaptopropionic acid. OPA derivatives are unstable in

*Corresponding author

0021-9673/96/$15.00
PII S0021-9673(96)00100-8

solution, thus, these derivatives have to be injected immediatly after the derivatization. OPA is suitable only for primary amino groups, while for secondary amino acids an additional oxidation step using hypochlorite is required. Moreover, the excess of reagent does not cause interfering fluorescence. Alternatively, the secondary amino acids can be derivated with another reagent, such as PITC, dansyl-Cl or 9-fluorenylmethylchloroformate (FMOC) [13]. Fluorescamine [14] is another fluorogenic reagent utilized in the post-column labelling of amino acids. It reacts quickly with primary amino acids, whereas longer reaction times and higher temperatures are required for secondary amino acids. 4-Chloro- [15] and 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole [16] produce fluorescent derivatives of both primary and secondary amino acids under mild reaction conditions. The use of FMOC [17,18] for pre-chromatographic derivatization of amino acids is possible. In contrast with previous reagents (except ninhydrin), 2,4,6-trinitrobenzenesulfonic acid is only useful for spectrophotometric detection [19].

In this study a new chromatographic method based on the pre-column derivatization of amino acids with 1,2-naphthoquinone-4-sulfonate (NQS), followed by the separation of the corresponding derivatives, is developed. The derivatization is performed in batch by mixing reagent, buffer and amino acid solutions. Conditions for the quantitative formation of the amino acid derivatives, such as temperature, time and pH, have been studied. The reaction is developed completely at pH 10 in a water bath at 65°C for 5 min. The amino acid derivatives are separated in a C_{18} modified silica column by generating an elution gradient based on increasing the percentage of acetonitrile. The method is applied to the determination of amino acids in protein hydrolyzates of feed samples.

NQS has several important characteristics. It is soluble in water at any pH and is able to react with both primary and secondary amino groups. Both types of derivative can be detected spectrophotometrically at the same wavelength, which avoids the need for multiple wavelength detection. Electrochemical detection is also available [20]. The reaction is quantitatively developed in a few min. The cost of this derivatization is low. This reagent has also been applied to amino acid determination by developing the reaction in flow systems, in particular, on-line post-column derivatization [21], flow injection analysis [22] and continuous flow analysis [23].

2. Experimental

2.1. Reagents and solutions

Chromatographic eluents were prepared from sodium acetate (Probus, analytical grade), hydrochloric acid 25% (w/w) (Merck, analytical grade) and acetonitrile (Carlo Erba, HPLC grade).

The reagent solution was composed of $3 \cdot 10^{-2}$ *M* sodium 1,2-naphthoquinone-4-sulfonate (Aldrich, analytical grade) in 0.1 *M* hydrochloric acid. The buffer solution was 0.05 *M* boric acid plus 0.09 *M* sodium hydroxide (both from Merck, analytical grade).

All amino acids were supplied by Merck and were of analytical grade.

The amino acid hydrolysates were prepared using feed samples obtained from Cooperativa Agropecuaria de Guissona (Lleida, Spain).

2.2. Apparatus

Eluents were pumped using an LKB Bromma 2152 LC controller connected to two LKB Bromma 2150 HPLC pumps. This system was also used for the generation of the elution gradient. The sample was injected by a Spark Holland Promis automatic injection system. Two different columns were utilized; the studies of reaction conditions, stability of amino acid derivatives and isocratic separation experiments were performed using a Spherisorb ODS 2 column (150×4.6 mm I.D., 5 μm particle size). Gradient elution studies, figures of merit and feed sample analysis were carried out using a Spherisorb ODS 2 column (3 μm particle size). In all cases, the column was thermostated at 50°C by a Spark Holland SPH 99 column thermostat. The spectrophotometric detector was a Waters 486 tunable absorbance detector, with a flow cell (10 mm path length) and 8 μl of dead volume. Data acquisition and treatment was performed with a Perkin-Elmer (PE) Nelson 900 interface coupled to a personal computer. A Phar-

macia LKB autoanalyzer, Model Alpha Plus (Series Two) was used for the analysis of amino acids according to the standard method.

2.3. Pre-column derivatization procedure

The derivatization reaction of amino acids was carried out in a vial (batch procedure) by mixing 150 μl of each of the following solutions: (A) reagent solution, $3 \cdot 10^{-2}$ *M* NQS in 0.1 *M* HCl; (B) buffer solution, 0.05 *M* sodium borate plus 0.09 *M* NaOH; (C) amino acid solution. The pH of this mixture was 10.0. The reaction was developed for 5 min by placing the vial in a thermostatic bath (SBS-TFB 3) set at 65°C. Under these conditions, the formation of derivatives was quantitative. After that, 60 μl of 0.25 *M* HCl was added to the solution containing the derivatives, providing a final pH of about 4.5. The solution obtained in this way was injected into the chromatographic system.

2.4. Chromatographic procedure

A 50-μl aliquot of the final solution containing the amino acid derivatives was injected onto the column. Their separation was performed by using two different eluents: Eluent A was an aqueous solution of 0.05 *M* acetic acid and 0.05 *M* sodium acetate (pH 4.75). Eluent B was prepared from eluent A plus acetonitrile (1:1, v/v). The elution gradient used was as follows: step 1: time=0–15 min, % eluent B=0; step 2: time=15–20 min, % eluent B=0–6 (linear); step 3: time=20–30 min, % eluent B=6; step 4: time=30–31 min, % eluent B=6–14 (linear); step 5: time=31–45 min, % eluent B=14–17 (linear); step 6: time=45–46 min, % eluent B=17–27 (linear); step 7: time=46–60 min, % eluent B=27–29 (linear). The total flow-rate was kept constant at 0.8 ml/min during the separation. Spectrophotometric detection was performed at 305 nm. After eluting all derivatives, the following procedure was applied to wash and prepare the column for the next injection: step 8: time=60–62 min, % eluent B=29–100 (linear); step 9 (washing period): time=62–70 min, % eluent B=100; step 10: time 70–72 min, % eluent B=100–0 (linear); step 11 (conditioning of the column): time 72–75 min, % eluent B=0.

2.5. Standard procedure

The Pharmacia LKB amino acid autoanalyser is based on the post-column derivatization with ninhydrin, as proposed by Spackman et al. [2]. Amino acids were injected onto a cationic-exchange column (200 ×4 mm I.D., packed with Ultropac 7 resin with a particle size of 8 μm). The separation was performed by using an elution gradient based on increasing pH and ionic strength with several lithium citrate buffers, together with increasing the temperature from 20 to 75°C. The outlet of the analytical column was coupled on-line to the derivatization system, where amino acids react with the ninhydrin solution in a PTFE reaction coil of 0.3 mm I.D., maintained at 135°C. The amino acid derivatives were detected spectrophotometrically at 570 and 440 nm in a flow cell with a path length of 15 mm and with 8 μl of dead volume.

2.6. Sample treatment

Feed samples were subjected to the following treatment to hydrolyze the proteins present: 0.5 g of feed sample plus 2 ml of 6 *M* hydrochloric acid (containing 1% phenol) were placed in a 10-ml glass-stoppered vial and hydrolysed in an aluminium block kept at 105°C for 24 h. The content of the vial was neutralized with a 0.1 *M* sodium hydroxide solution, diluted to a volume of 100 ml and finally filtered through a nylon membrane of 0.22 μm pore size. Hydrolyzate solutions obtained in this way were derivatized with NQS following the proposed procedure.

Solutions to be injected into the amino acid autoanalyzer were prepared by mixing 975 μl of hydrolyzate solution plus 25 μl of 0.01 *M* norleucine solution, which was used as the internal standard. Before the injection, this solution was filtered through an Ultrafree MC low-binding cellulose membrane (10 000 NMWL) from Millipore.

3. Results and discussion

3.1. Study of pre-column reaction conditions

A solution containing Glu, Gly, His, Lys, Pro, Arg and Tyr was used to establish the derivatization

conditions. These seven amino acids were selected to cover a wide range of possibilities (e.g. primary and secondary amino acids, amino acids with basic, acidic or neutral character). The concentration of each amino acid was $8 \cdot 10^{-4}$ *M*.

These initial studies were performed by injecting 200 μl of sample solution into the system. The chromatographic separation of derivatives was achieved with an Spherisorb ODS-2 column (150 $\times$4.6 mm I.D. and 5 μm particle size). The mobile phase was an aqueous solution of 0.05 *M* acetic acid and 0.05 *M* sodium acetate (pH 4.75) with 5% (v/v) acetonitrile.

3.1.1. Effect of NQS concentration

Reagent solutions with NQS concentrations ranging from $2.5 \cdot 10^{-3}$ *M* to $5 \cdot 10^{-2}$ *M* were prepared. In all of these NQS solutions the concentration of hydrochloric acid was 0.1 *M*. The amino acid derivatization was carried out at pH 9.5 using a buffer solution of 0.05 *M* sodium borate plus 0.083 *M* sodium hydroxide. The reaction was developed at room temperature for 30 min. Fig. 1 shows the influence of the NQS concentration on the chromatographic peak height of several derivatives, expressed in absorbance units. Amino acids such as Pro and Tyr achieved their maximum absorbance at NQS concentrations between 0.015 and 0.05 *M*. Others such as Gly and Glu required NQS concentrations higher than 0.03 and 0.04 *M*, respectively. Because Lys has two amino groups that are able to react with NQS, two different Lys derivatives can be obtained: the mono- and the di-derivative, depending on whether one or two amino groups have reacted. The values of peak height for Lys in Fig. 1 correspond to the mono-derivative. At low NQS concentrations, the mono-derivative predominated (thus the peak height increased to 0.01 *M*), whereas when the NQS concentration increased, the remaining free amino group of Lys reacted to give the di-derivative (thus the mono-derivative peak height decreased, for concentrations higher than 0.01 *M*).

3.1.2. Effect of pH on the derivatization reaction

The influence of pH on the reaction was studied in the range 8.0 to 12.5 by using different buffer solutions of sodium borate and sodium hydroxide.

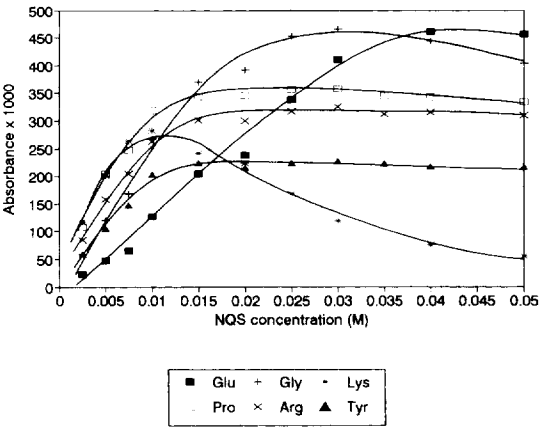

Fig. 1. Effect of the NQS concentration on the peak heights of amino acid derivatives. Peak height is expressed as absorbance units at 305 nm. Derivatization conditions: buffer solution, 0.05 *M* $NaBO_2$ and 0.083 *M* NaOH, pH 9.5; amino acid concentration, $8 \cdot 10^{-4}$ *M*; volume of reagent, buffer and amino acid solutions to prepare the batch solution, 500 μl of each; reaction time, 30 min; reaction temperature, 25°C. Acidification of 1500 μl of the batch solution was performed with 100 μl of 0.25 *M* HCl. Chromatographic conditions: Injection volume, 200 μl; mobile phase, 0.05 *M* acetic acid and 0.05 *M* sodium acetate with acetonitrile (95:5, v/v); column particle size, 5 μm.

The study, with NQS concentrations of 0.02, 0.03 and 0.05 *M*, was always carried out in 0.1 *M* hydrochloric acid. Results in Fig. 2a for a NQS concentration of 0.05 *M* show that the reaction was completely developed in a pH range from 9 to 12.5. However, in contrast to the other amino acids, Glu (see Fig. 2b) was only derivatized quantitatively under more extreme conditions, giving the maximum absorbance value of 0.59. At a NQS concentration of 0.02 *M*, its absorbance increased continuously with pH (there was no plateau zone). At a NQS concentration of 0.03 *M*, Glu is only completly derivatized at pH 12.5 while at 0.05 *M*, the maximum absorbance is attained at a pH value higher than 10. The optimum pH finally chosen was 10.0.

3.1.3. Kinetic study

The effects of temperature and reaction time were simultaneously studied because these variables are interrelated. The temperature was varied from 22 to 80°C while the reaction time was varied from 5 to 60

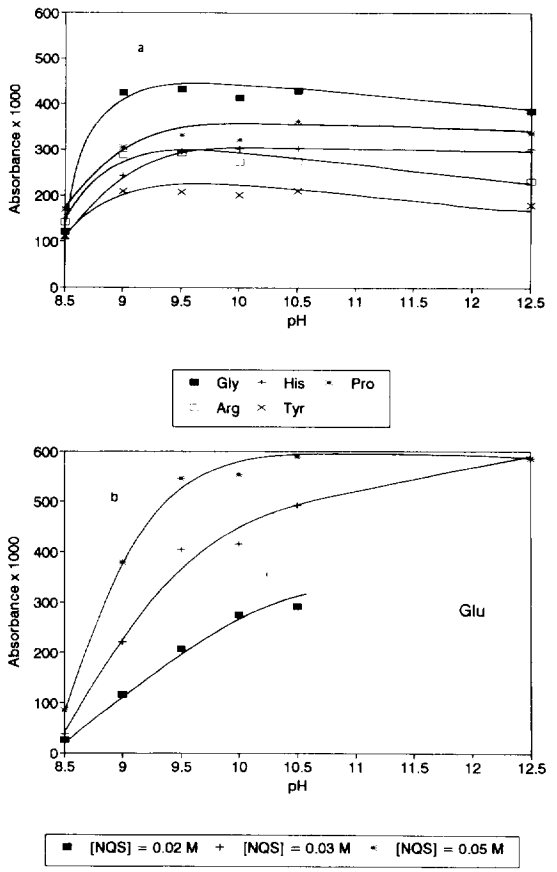

Fig. 2. Effect of pH on the peak heights of different amino acid derivatives. (a) Absorbance curves at NQS concentration of 0.05 *M*. (b) Peak heights of the Glu derivative at three NQS concentrations. See Fig. 1 for other experimental conditions.

min. For every amino acid derivative, a set of 25 experimental points was obtained. These points can be represented as a response surface as shown in Fig. 3 for Glu, Gly and Tyr derivatives. These three amino acids were selected since their reaction kinetics are slow, fast or very fast, respectively. The optimal experimental conditions selected were those leading to the total development of the reaction for all amino acids in a minimum time, which was 5 min at 65°C. Higher temperatures are not advisable because, as the surface for Gly shows, the peak becomes lower as the derivative decomposes. Considering these three previous studies, the final NQS concentration chosen was 0.03 *M*.

3.2. Study of separation conditions

The effect of the pH of the eluent on the retention time of several amino acid derivatives was the first factor studied. Seven different mobile phases composed of aqueous buffer solution and acetonitrile (95:5, v/v) were prepared. These buffer solutions contained the appropriate concentrations of acetic acid and sodium acetate to give pH values in the range of 3.5 to 6.0. Fig. 4 shows that, in general, derivatives obtained from amino acids with basic character differed from the rest. Thus, His and Arg derivatives showed an increase in retention time when the pH was increased. Conversely, for the other derivatives such as Pro and Gly, a decrease in retention time with pH was observed. This effect is more marked for amino acids with acidic character like Glu. Finally, a pH of 4.75 was chosen for the eluent.

The influence of the amount of acetonitrile in the mobile phase on the isocratic elution of the amino acid derivatives was investigated using a C_{18} column of 5 μm particle size. For all derivatives considered, the retention time decreased with increasing percentage of acetonitrile (Fig. 5). Moreover, depending on this amount of acetonitrile, the order of elution of some closely eluted derivatives (e.g. Ile and Lys or Gly and Glu) changed, which was taken into account in the establishment of the optimum separation conditions. This study was the basis for the optimization of an elution gradient that was able to separate complex amino acid mixtures, such as those generated in the hydrolysis of proteins. However, in order to improve the resolution of some close peaks, the elution gradient was established using a C_{18} column of 3 μm particle size. Fig. 6 shows the chromatogram of a standard amino acid mixture obtained under the gradient profile chosen under optimum conditions. The separation took 60 min, although an additional period of 15 min was required to wash the system and to prepare the column for the next injection.

3.3. Study of the stability of the amino acid derivatives

As mentioned above, the derivatization reaction was developed in basic medium, although hydrochlo-

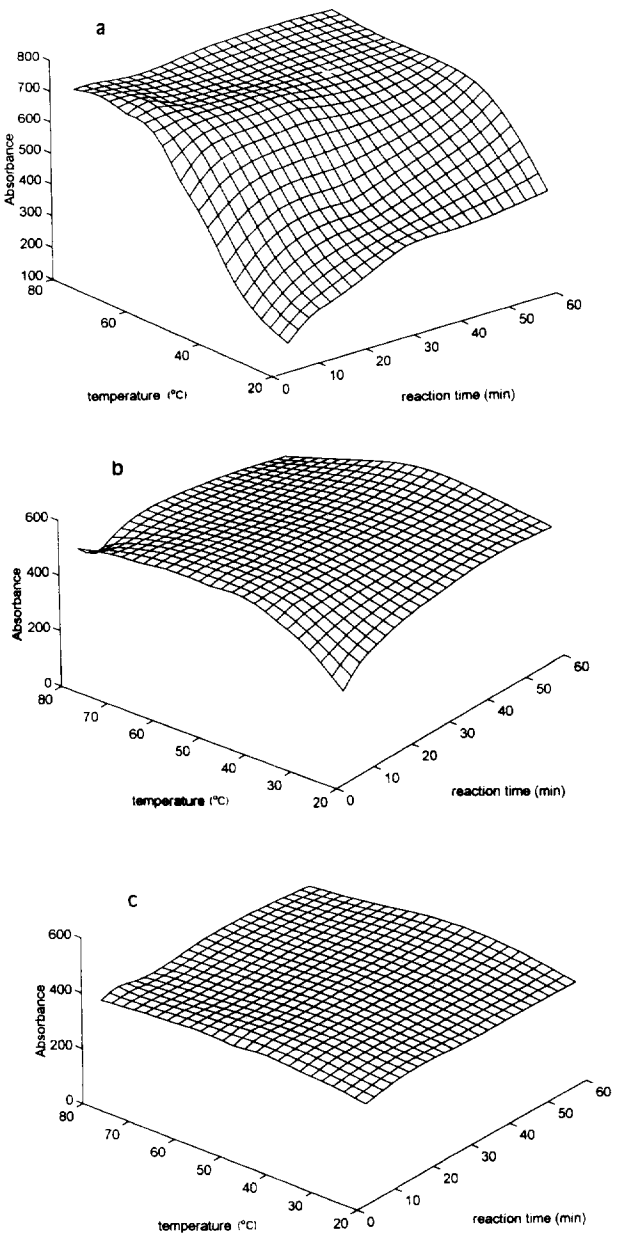

Fig. 3. Effect of temperature and reaction time on the peak heights of amino acid derivatives: (a) Glu, (b) Gly and (c) Tyr. Derivatization conditions: Reagent solution, 0.03 *M* NQS in 0.1 *M* HCl; buffer solution, 0.05 *M* $NaBO_2$ and 0.09 *M* NaOH (reaction pH 10.0); See Fig. 1 for other experimental conditions.

ric acid was added before injection onto the chromatographic column. For this reason, the stability of the amino acid derivatives was studied for several hours (see Fig. 7), by delaying the injection of the derivative solutions after the addition of 60 μl of 0.25 *M* HCl (final pH of 4.5). A general decrease in the peak heights with time was observed after 30 min for most amino acid derivatives, indicating progres-

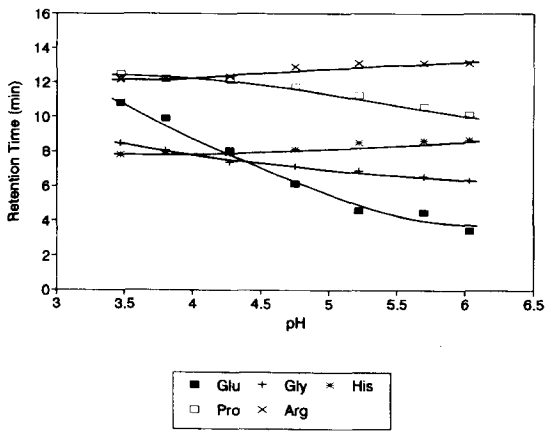

Fig. 4. Effect of the pH of the mobile phase on the isocratic elution of amino acid derivatives. Derivatization conditions: Reagent solution, 0.03 *M* NQS in 0.1 *M* HCl; buffer solution, 0.05 *M* $NaBO_2$ and 0.09 *M* NaOH (reaction pH=10.0); amino acid concentration, $8 \cdot 10^{-4}$ *M*; volume of reagent, buffer and amino acid solutions used to prepare the batch solution, 150 μl of each; reaction time, 5 min; reaction temperature, 60°C. Acidification of 450 μl of batch solution was carried out using 60 μl of 0.25 *M* HCl. Chromatographic conditions: injection volume, 200 μl; mobile phase, acetic acid and sodium acetate with acetonitrile (95:5, v/v); column particle size, 5 μm.

sive decomposition. This decomposition was especially marked for the Pro derivative, since its peak disappeared in 3 h. When Pro is to be determined, the sample solution should be injected inmediately after the addition of hydrochloric acid.

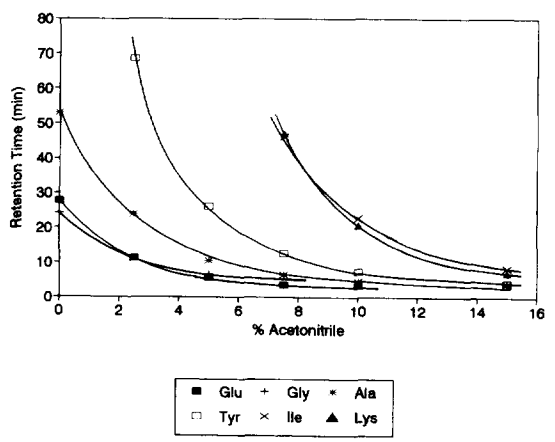

Fig. 5. Effect of percentage of acetonitrile (v/v) of the mobile phase on the isocratic elution of amino acid derivatives. Mobile phase, 0.05 *M* acetic acid and 0.05 *M* sodium acetate with acetonitrile. See Fig. 4 for other experimental conditions.

3.4. Separation of amino acid standards

Table 1 shows the characteristics of the method for seventeen amino acids under the optimized conditions selected. For determination of the linear range of the method, six standard solutions of $4 \cdot 10^{-5}$, $8 \cdot 10^{-5}$, $1.6 \cdot 10^{-4}$, $2 \cdot 10^{-4}$, $3.2 \cdot 10^{-4}$ and $4 \cdot 10^{-4}$ *M* of every amino acid were utilized. The repeatability of peak area and retention time on the same day was calculated as the relative standard deviation (R.S.D.) of seven consecutive injections of a standard solution with a concentration of $2 \cdot 10^{-4}$ *M* of every amino acid. The reproducibility of peak areas and retention times on different days was the R.S.D. of six injections of a standard solution containing a $2 \cdot 10^{-4}$ *M* concentration of every amino acid performed on six different days. The limit of detection was calculated for a signal-to-noise ratio of 3. Taking Gly as an example, the linear range was up to 20 nmol, the repeatability was 2.6%, the reproducibility was 3.6% and the detection limit was 0.06 nmol. For other amino acids (see Table 1), the linear range varied between 16 to 20 nmol, the repeatability between 2.0 and 4.6%, the reproducibility between 3.1 and 7.7%, and the detection limit between 0.04 and 0.1 nmol. With respect to the repeatability and reproducibility of the retention time, the relative standard deviation varied between 0.03 and 0.10 and between 0.1 and 0.4, respectively.

3.5. Determination of amino acids in feed samples

The proposed method was applied to the determination of amino acids in feed samples after the hydrolysis of the proteins present. The hydrolysis procedure is described in Section 2. Fig. 8 shows the chromatogram of amino acid derivatives obtained for a hydrolyzate solution. Results obtained in the analysis of four feed samples using the proposed method were compared with those obtained with the standard method using the amino acid analyzer (Table 2). This table indicates that there is good concordance between the two methods, since the total percentage of error in the quantification of amino acids by the proposed method with respect to

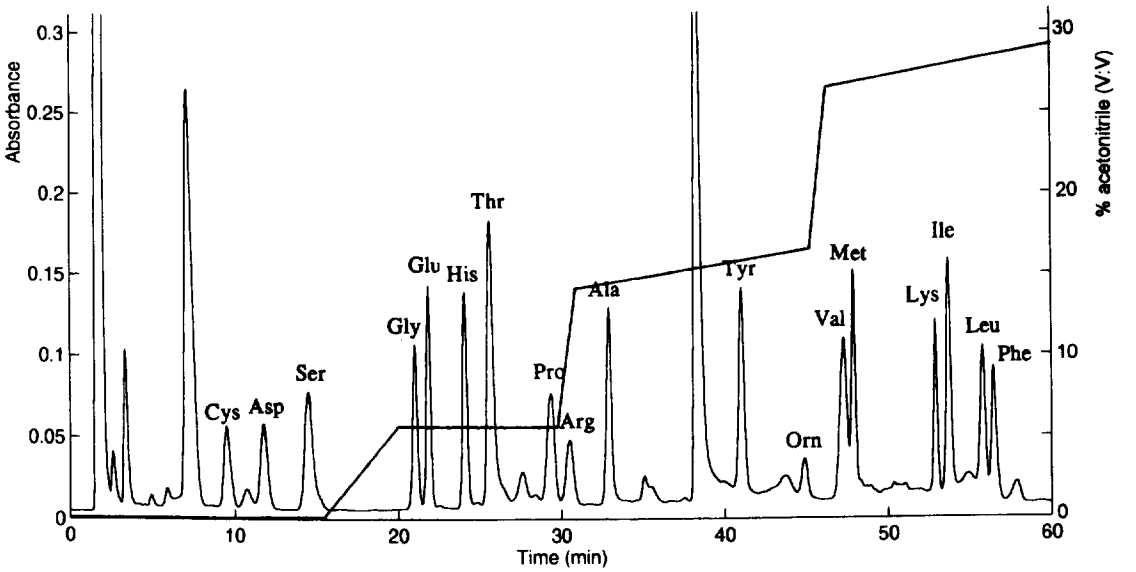

Fig. 6. Chromatogram of amino acid derivatives for a standard amino acid solution, under the optimized derivatization conditions and using the elution gradient chosen. Amino acid concentration used was 10^{-4} *M*. See Fig. 4 for other reaction conditions. Chromatographic conditions: injection volume, 25 μl; mobile phase A, 0.05 *M* acetic acid and 0.05 *M* sodium acetate; mobile phase B, mobile phase A and acetonitrile (1:1, v/v); column particle size, 3 μm. The continuous line indicates the elution gradient profile.

the standard was 4.16. For each sample, the quantification error was always lower than 5%.

4. Conclusions

The present study shows the utility of NQS in the pre-column derivatization of amino acids. The free solubility of NQS in water is an important advantage over other labelling agents, since the reaction may be carried out in aqueous solution. Conditions for quantitative reaction are faster and milder than with other reagents, such as dansyl and dabsyl chloride and isothiocyanates. In contrast to *o*-phthaldialdehyde, NQS is able to react with imino acids such as proline and hydroxyproline under the same reaction conditions as those of the primary amino acids. Reagents such as FMOC require removal of the excess reagent after the derivatization by liquid–liquid extraction or by chemical reaction. However, these treatments are not necessary with NQS, since its excess does not interfere in the chromatographic separation proposed.

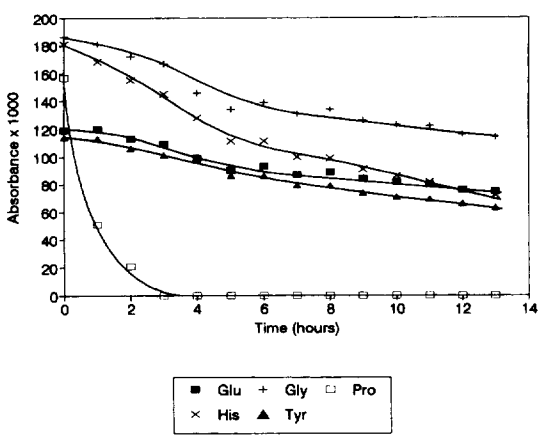

Fig. 7. Influence of time on the stability of amino acid derivatives in an aqueous solution at pH 4.5. See Fig. 6 for other reaction conditions.

In comparison with the post-column NQS method for the determination of amino acids [21], the detection limit of the present pre-column derivatization method is about three times lower. The repeatability and reproducibility in the responses are similar, while repeatability and reproducibility with respect to the retention time are better in the present method. Finally, since NQS is a general reagent for amino groups, this method can also be applied to other analytes, such as biogenic amines and pharmaceuticals.

Table 1
Figures of merit of the proposed method for several amino acids under the optimum conditions

Amino acid	Retention time repeatability [R.S.D. (%)]	Retention time reproducibility [R.S.D. (%)]	Peak area repeatability [R.S.D. (%)]	Peak area reproducibility [R.S.D. (%)]	Linear range (nmol)	Detection limit (nmol)
Cys	0.07	0.3	2.1	3.4	20	0.08
Asp	0.10	0.3	2.0	3.1	20	0.09
Ser	0.08	0.4	2.2	3.2	20	0.05
Gly	0.03	0.3	2.6	3.8	20	0.06
Glu	0.03	0.3	2.4	3.7	20	0.05
His	0.04	0.1	3.8	5.2	20	0.05
Thr	0.04	0.1	4.7	7.7	20	0.10
Pro	0.08	0.3	2.5	4.3	20	0.05
Arg	0.05	0.3	3.7	3.2	20	0.10
Ala	0.10	0.2	2.2	5.7	20	0.05
Tyr	0.07	0.2	3.2	3.6	20	0.04
Val	0.04	0.2	2.1	5.3	16	0.04
Met	0.04	0.3	2.1	5.2	20	0.06
Lys	0.06	0.2	4.4	3.7	20	0.08
Ile	0.05	0.2	2.6	3.8	20	0.04
Leu	0.04	0.2	2.5	3.7	16	0.04
Phe	0.05	0.2	2.9	3.5	16	0.06

Acknowledgments

The authors thank Dr I. Casals and co-workers from "Serveis Científico-Tècnics de la Universitat de Barcelona" for their help in the chromatographic study, and the Cooperativa Agropecuaria de Guissona for providing the samples. J. Saurina also thanks the Ministerio de Educación y Ciencia for an

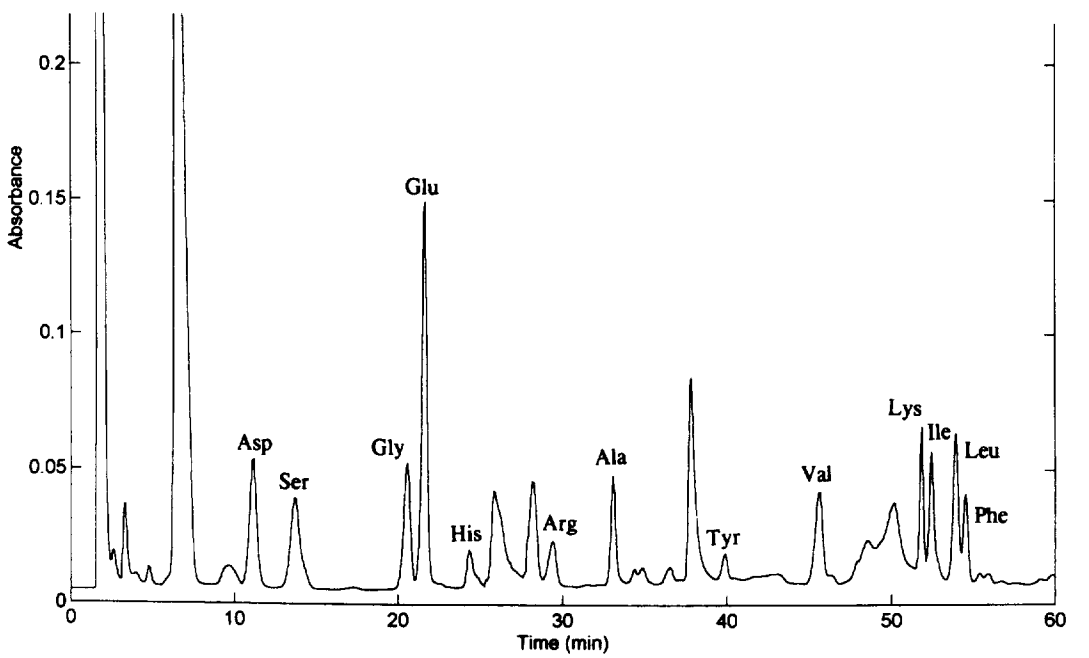

Fig. 8. Chromatogram of amino acid derivatives obtained from a protein hydrolyzate solution of a feed sample using the proposed method. See Fig. 6 for other reaction conditions.

Table 2
Determination of amino acids in hydrolysates of proteins of four different feed samples using the proposed method (NQS method) and the standard method

Amino acid	Sample A		Sample B		Sample C		Sample D	
	NQS method	Standard method	NQS method	Standard method	NQS method	Standard method	NQS method	Standard method
Asp	1.55	1.56	2.29	2.35	3.04	2.95	3.19	3.35
Ser	0.38	0.37	0.45	0.42	0.82	0.82	0.51	0.52
Gly	0.65	0.66	0.80	0.74	0.96	0.85	0.51	0.52
Glu	2.22	2.25	2.63	2.62	3.48	3.54	3.21	3.18
His	0.27	0.25	0.34	0.31	0.42	0.38	0.39	0.39
Arg	0.43	0.43	0.69	0.67	0.72	0.66	0.81	0.80
Ala	0.57	0.55	0.69	0.63	0.85	0.83	0.82	0.77
Tyr	0.06	0.07	0.09	0.12	0.07	0.06	0.10	0.15
Val	0.62	0.49	0.62	0.58	0.71	0.68	0.63	0.62
Lys	0.35	0.37	0.65	0.66	0.83	0.80	0.90	0.85
Ile	0.38	0.35	0.49	0.46	0.71	0.68	0.63	0.62
Leu	0.72	0.75	1.08	0.95	1.38	1.35	1.15	1.12
Phe	0.37	0.41	0.55	0.56	0.83	0.78	0.73	0.72

Amounts are expressed as g of amino acid in 100 g of feed sample.

FPI grant. This work has been partially financed by DGICYT project PB93-0744.

References

[1] E. Heftmann (Editor), Chromatography — Fundamentals and Applications of Chromatography and Related Differential Migration Methods, Part B: Applications, Elsevier, Amsterdam, 5th ed., 1992.
[2] D.H. Spackman, W.H. Stein and S. Moore, Anal. Chem., 30 (1958) 1190.
[3] J.A. Grunau and J.M. Swiader, J. Chromatogr., 594 (1992) 165.
[4] V. Fierabracci, P. Masiello, M. Novelli and E. Bergamini, J. Chromatogr., 570 (1991) 285.
[5] M. Puig-Deu, S. Buxaderas, J. Chromatogr. A, 685 (1994) 21.
[6] J.V. Scheller, J.B. Shear, M.A. Zare and R.H. Scheller, Anal. Chem., 63 (1991) 496.
[7] M. Simmaco, D. DeBiase, D. Barra and F. Rossa, J. Chromatogr., 504 (1990) 129.
[8] E.H.J.M. Jansen, R.H. van der Berg, R. Both-Miedema and L. Doorn, J. Chromatogr., 553 (1991) 123.
[9] H. Brückner, M. Langer, M. Lüpke, T. Westhauser and H. Godel, J. Chromatogr. A, 697 (1995) 229.
[10] R. Schuster, J. Chromatogr., 431 (1988) 271.
[11] A. Fiorino, G. Frigo and E. Cucchetti, J. Chromatogr., 476 (1988) 83.
[12] R.B. Ashworth, J. Assoc. Off. Anal. Chem., 70 (1987) 248.
[13] T. Bartók, G. Szalai, Zs. Lörincz, G. Börcsok and F. Sági, J. Liq. Chromatogr., 17 (1994) 4391.
[14] M.C. Miedel, J.D. Hulmes and Y.C.E. Pan, J. Biochem. Biophys. Methods, 18 (1989) 37.
[15] M. Ahnoff, I. Grundevik, A. Arfwidsson, J. Fonsellus and B.A. Persson, Anal. Chem., 53 (1981) 485.
[16] Y. Watanabe and K. Imai, Anal. Chem., 55 (1983) 1786.
[17] P.A. Haynes, D. Sheumack, J. Kibby and J.W. Redmond, J. Chromatogr., 540 (1991) 177.
[18] H. Brückner and M. Lüpke, J. Chromatogr. A, 697 (1995) 295.
[19] J. Molnár-Perl and G. Vitány, Analusis, 16 (1988) 173.
[20] Y. Nakahara, A. Ishigami and Y. Takeda, J. Chromatogr., 489 (1989) 371.
[21] J. Saurina and S. Hernández-Cassou, J. Chromatogr. A, 676 (1994) 311.
[22] J. Saurina and S. Hernández-Cassou, Anal. Chim. Acta, 281 (1993) 593.
[23] J. Saurina, S. Hernández-Cassou and R. Tauler, Anal. Chem., 67 (1995) 3722.