Journal of Chromatography, 594 (1992) 173–178
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 857

Determination of glutamine, glutamic acid and pyroglutamic acids using high-performance liquid chromatography on dynamically modified silica

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(First received July 3rd, 1991; revised manuscript received November 7th, 1991)

ABSTRACT

For the determination of glutamine and the products of its degradation, glutamic and pyroglutamic acids, in fermentation broth and other biotechnological samples, a rapid and convenient high-performance liquid chromatographic method is proposed. The method is based on the separation of these compounds on silica dynamically modified with Cu(II) ions. Modification is obtained by the addition of copper sulphate to the mobile phase. The copper complexes of amino acids, including pyroglutamic acid, are easily formed in the column and are detected by UV absorption at 235 nm. If it is necessary to improve the separation, an ion-pairing reagent (sodium dodecyl sulphate) is added to the mobile phase. Using this method, the levels of glutamine and related compounds are determined in fermentation media of industrial strains and other biotechnological samples. The total time of analysis is less then 10 min with a detection limit of 0.1 g/l.

INTRODUCTION

Glutamine is used as a medicine and as a component of tissue culture media. It has been demonstrated that glutamine is unstable and is easily converted into glutamic acid and pyroglutamic (5-oxoproline, 2-pyrrolidone-5-carboxylic) acid.

In our study of glutamine and glutamic acid production by biotechnological methods, a simple and sensitive technique for the determination of glutamine and related products in technological solutions is necessary. The aim of this work was to develop such a method suitable for the simultaneous determination of glutamine, glutamic acid and pyroglutamic acid in biotechnological samples.

Analysis of amino acids is a routine task in biochemistry, medicine, biotechnology and another fields, and different approaches have been proposed. Ion-exchange chromatography with post-column derivatization, pre-column derivatization and reversed-phase chromatography are the most widely used methods [1]. Usually such techniques are developed for the analysis of protein amino acids and are suitable for the determination of glutamine and glutamic acid. However, pyroglutamic acid has a tertiary nitrogen atom which does not participate in the conventional reactions used to form detectable amino acid derivatives.

Several gas chromatographic methods have been proposed for glutamine analysis. These methods are suitable for the determination of pyroglutamic acid but include the complex and time-consuming production of volatile derivatives [2,3].

For the determination of pyroglutamic and glutamic acids in glutamine preparations, it has been proposed to use high-performance liquid chromatographic (HPLC) separation on an ion-exchange column with UV detection at 210 nm. The total time of the analysis is about 20 min [4]. It has also been recommended to determine pyroglutamic acid using reversed-phase chromatography with UV detection at 200 nm. Glutamine and glutamic acid have been determined as *o*-phthalaldehyde derivatives by chromatography, but this technique was very time

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consuming [5]. Neither approach was selective enough for the analysis of fermentation media, which usually contain many organic acid, carbohydrates and other compounds that strongly adsorb in the range 200–210 nm.

Another approach to the separation and detection of amino acids is based on its ability to form a complexes with Cu(II) ions. Such complexes are detectable in the range 230–235 nm. The influence of the chromatographic conditions on such separations has been intensively studied. It was demonstrated that under isocratic conditions all the protein amino acids can be separated on a reversed-phase column using eluents containing ion-pair reagents [6,7]. This approach was recently used for the determination of proline in urine [8]. Modification of this method based on the column switching technique has also been described [9]. The method proposed have for the determination of glutamine and its degradation products in biotechnological samples is based on these studies.

EXPERIMENTAL

Standard samples of glutamine, glutamic acid and pyroglutamic acid were purchased from Sigma, isopropanol (HPLC grade), sulphuric acid and sodium hydroxide (ACS grade) from Aldrich, sodium dodecyl sulphate from Serva and $CuSO_4 \cdot 10H_2O$ (analytical-reagent grade) from Soyuzchimreactiv HPLC-grade water was produced using a Milli-Q system (Millipore).

Stock standard solutions of glutamine, glutamic acid and pyroglutamic acid and samples of fermentation media were stored at −20°C and diluted just before use.

UV spectra of the copper(II) complexes of glutamine, glutamic acid and pyroglutamic acid were obtained using a Spectronic Model 2000 scanning spectrophotometer (Bauseh and Lomb).

All analyses were carried out using an LKB isocratic HPLC system consisting of a Model 2150 HPLC pump and a Model 2151 variable-wavelength monitor operated at 235 nm. Samples were injected using a Model 7410 injector with a 1-μl internal sample loop (Rheodyne). Stainless-steel columns (250 × 4 mm I.D.) packed with unmodified silica (5 μm) were obtained from Labor-MIM. Columns were thermostated at 40°C using a water jacket and Model U1 circulating thermostat. Chromatograms were recorded with a C-R1B computing integrator (Shimadzu) and calculated using the external standard method.

The eluent consisted of 0.01 *M* sodium dodecyl sulphate, 0.001 *M* copper(II) sulphate and up to 10% (v/v) of isopropanol. The flow-rate was 0.5 ml/min. Before analysis, the column was thermostated and equilibrated in the eluent for 1 h. At the end of the working period the column and detector

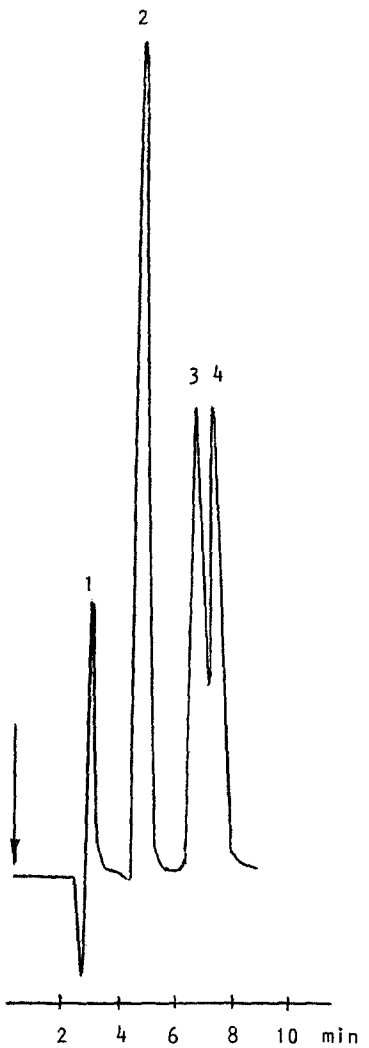


Fig. 1. Typical chromatogram of model mixture. Eluent, 0.001 *M* copper(II) sulphate–2.5% isopropanol; flow-rate, 0.5 ml/min; detection, UV at 235 nm. Peaks: 1 = pyroglutamic acid; 2 = glutamine; 3 = glutamic acid; 4 = proline. Concentrations, 0.5 g/l.

TABLE I

REGRESSION EQUATIONS AND CALIBRATION COEFFICIENTS OF CALIBRATION GRAPHS FOR THE SEPARATION OF GLUTAMINE AND RELATED COMPOUNDS WITH COPPER(II)-CONTAINING MOBILE PHASE

Concentration range, 0.1–100 g/l.

Compound	Regression equation[a]		Correlation coefficient (r^2)
	Slope	Intercept	
Pyroglutamic acid	341.2	−158.6	0.996
Glutamic acid	2325.6	−2545.1	0.998
Glutamine	2683.3	−1278.0	0.998

[a] Peak area = intercept + slope × concentration (g/l).

flow cell were cleaned with 200 ml of 40% aqueous isopropanol.

Fermentation media of glutamine- and glutamic acid-producing strains of *Corinebacterium glutamicum* were used for analysis. A typical sample contained up to 40 g/l of glutamine, 40 g/l of glutamic and 20 g/l of pyroglutamic acid. Cells were separated by centrifugation at 13 000 *g* for 3 min and the supernatant was diluted 50-fold and used for analysis without additional pretreatment.

To study the influence of pH on the stability of glutamine, the pH of the samples of the fermentation broth was adjusted with sodium hydroxide or sulphuric acid. Test-tubes with fermentation media were thermostated in a water-bath at 60°C.

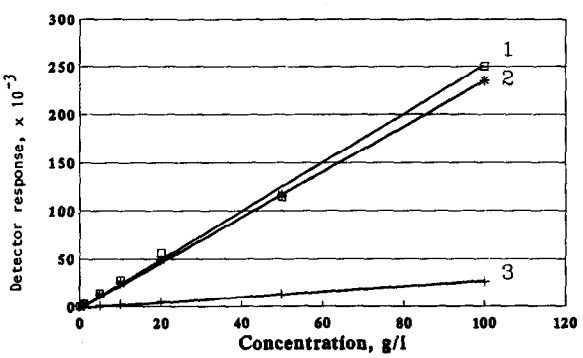


Fig. 2. Linearity of HPLC determination of glutamine, glutamic acid and pyroglutamic acids. Column, 5-μm silica (250 × 4 mm I.D.). Chromatographic conditions as in Fig. 1. The calculated areas are in arbitrary units. Lines: 1 = Glutamic acid; 2 = glutamine; 3 = pyroglutamic acid.

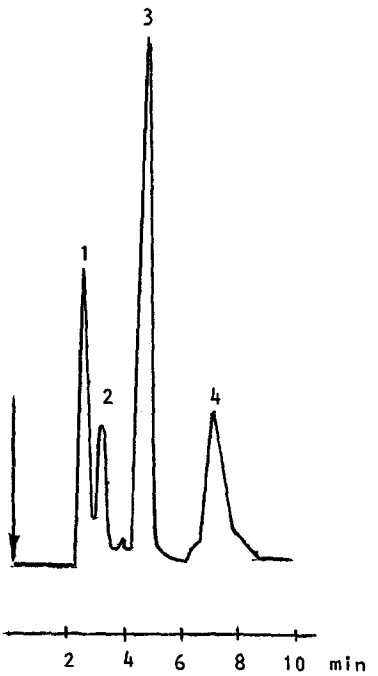


Fig. 3. Typical chromatogram of model mixture. Eluent, 0.001 *M* copper(II) sulphate–2.5% isopropanol–0.01 *M* sodium dodecyl sulphate; flow-rate, 0.5 ml/min; detection, UV at 235 nm. Peaks: 1 = ammonium sulphate; 2 = pyroglutamic acid; 3 = glutamic acid; 4 = glutamine.

RESULTS AND DISCUSSION

Pyroglutamic acid also forms a complex with Cu(II) with an absorbance maximum at 226 nm. Therefore, such a complex can be determined using a conventional UV detector. Considering the ability of some keto acids to absorb at 210–220 nm, we decided to carry out the detection at the highest wavelength, 235 nm. The sensitivity to pyroglutamic acid under these conditions decreases about five-fold, but the selectivity increases. Therefore it is possible to determine glutamine, glutamic acid and pyroglutamic acid simultaneously as their copper complexes.

Analysis was carried out using silica dynamically modified with Cu(II) ions. Owing to the low ability of silica to undergo dispersive interactions, the retention time under such conditions is lower than on reversed-phase sorbents. Under the selected chromatographic conditions it is possible to separate all the studied compounds in model mixtures within 8

TABLE II

REGRESSION EQUATIONS AND CALIBRATION COEFFICIENTS OF CALIBRATION GRAPHS FOR THE SEPARATION OF GLUTAMINE AND RELATED COMPOUNDS WITH COPPER(II)–SODIUM DODECYL SULPHATE-CONTAINING MOBILE PHASE

Concentration range, 0.1–100 g/l.

Compound	Regression equation[a]		Correlation coefficient (r^2)
	Slope	Intercept	
Ammonium sulphate	1016.4	−85.5	0.998
Pyroglutamic acid	260.3	−172.3	0.996
Glutamic acid	2334.7	991.5	0.998
Glutamine	2447.0	1600.1	0.998

[a] Peak area = intercept + slope × concentration (g/l).

min (Fig. 1). The calibration graphs for all compounds are linear in the concentration range 1–100 g/l (Table I, Fig. 2). Hence this approach is suitable for the determination of glutamine and related compounds during separation and purification.

During the analysis of fermentation media it was found that ammonia, which is often present in such samples also forms a complex with Cu(II). This complex elutes too close to the copper complex of pyroglutamic acid. To improve the separation of these compounds we used sodium dodecyl sulphate as an ion-pair reagent. In this instance a nearly baseline separation of the ammonia–pyroglutamic acid pair was achieved (Fig. 3).

Under these chromatographic conditions the cali-

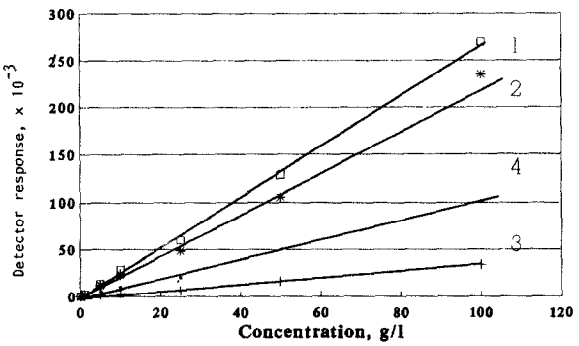
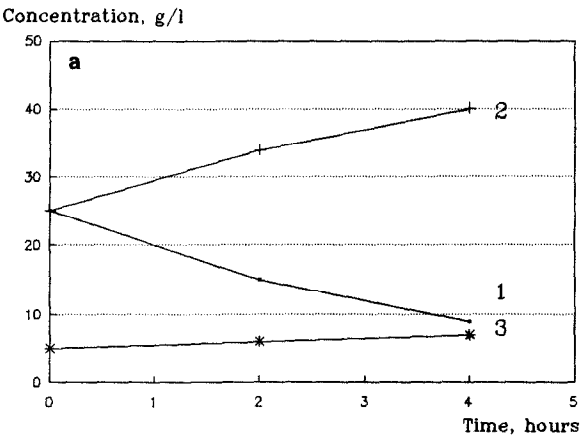
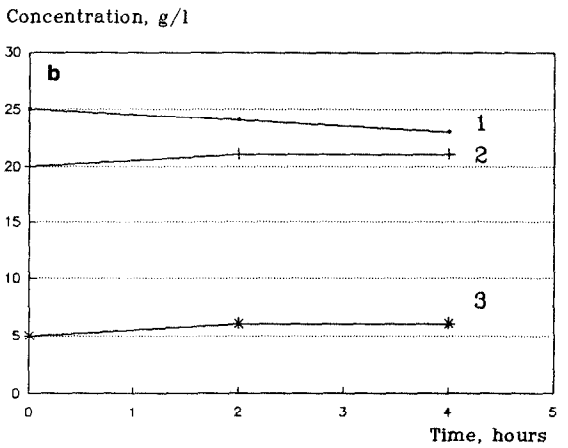


Fig. 4. Linearity of HPLC determination of glutamine, glutamic acid and pyroglutamic acid. Column, 5-μm silica (250 × 4 mm I.D.). Chromatographic conditions as in Fig. 3. The calculated areas are in arbitrary units. Lines: 1 = glutamic acid; 2 = glutamine; 3 = pyroglutamic acid; 4 = ammonium sulphate.





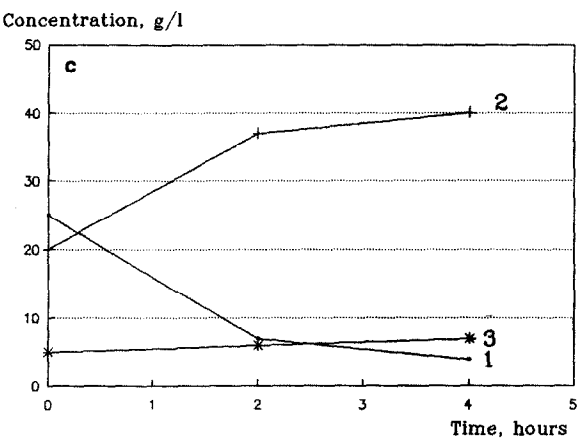


Fig. 5. Degradation of glutamine in fermentation media at 60°C and different pH values: (a) pH 1.5; (b) pH 5.5; (c) pH 10.5. Curves: 1 = glutamine; 2 = pyroglutamic acid; 3 = glutamic acid.

bration graphs were linear within the range 1–100 g/l (Table II, Fig. 4).

Consequently, for the determination of glutamine and glutamic acid it is possible to use the chromatographic conditions as in Fig. 1. The same conditions are suitable for the determination of pyroglutamic acid in model solutions. If it is necessary to determine pyroglutamic acid in samples containing salts of ammonia, *e.g.* in fermentation media, the addition of an ion-pair reagent is recommended.

This technique was used to study the stability of glutamine during its separation from fermentation broth and purification [10]. Previously it was reported that degradation of glutamine to glutamic and pyroglutamic acids in model mixtures may be influenced by temperature, pH, the presence of anions, etc. [5]. Our study demonstrated that at high and low pH values glutamine in model solutions and fermentation media is easly converted into these products (Fig. 5).

At 60°C and pH 1.5 and 10.5, 80% degradation of glutamine occurred in 3 h (Fig. 5a and c, respectively). At the same temperature and at pH 5.5 glutamine is more stable (Fig. 5b).

This technique was also used for the rapid control of glutamine and glutamic acid accumulation in the fermentation media of industrial strains (Figs. 6 and 7). The precision of quantitative analysis was confirmed by techniques such as amino acid analysis and planar chromatography. It was found that owing to the simple sample pretreatment, the possibility of carrying out HPLC analysis under the isocratic conditions and the short time of analysis, the method developed here is suitable for routine appli-

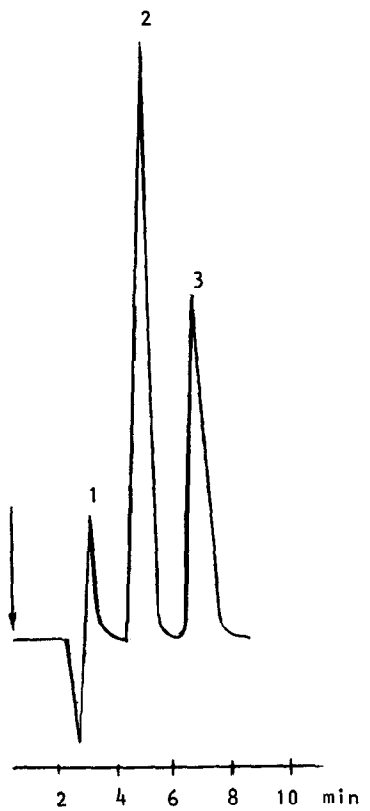


Fig. 6. Typical chromatogram of fermentation media of *Corinebacterium glutamicum*. Dilution, 1:50. Peaks: 1 = pyroglutamic acid; 2 = glutamic acid; 3 = glutamine. Analytical conditions as in Fig. 1.

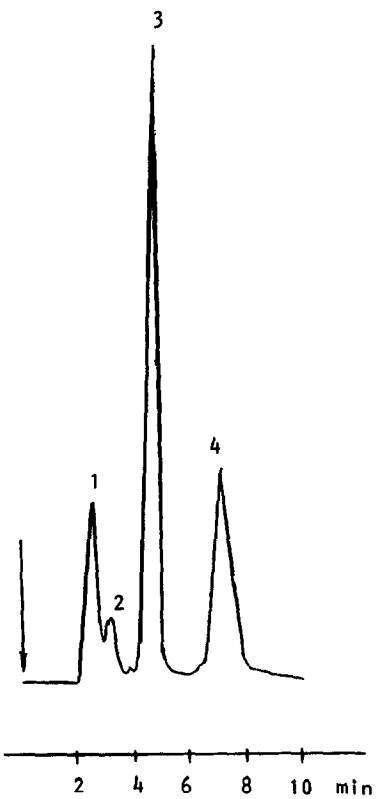


Fig. 7. Typical chromatogram of fermentation media of *Corinebacterium glutamicum*. Dilution, 1:50. Peaks: 1 = ammonium sulphate; 2 = pyroglutamic acid; 3 = glutamic acid; 4 = glutamine. Analytical conditions as in Fig. 2.

cation. The unmodified silica column demonstrates very good stability. After the analysis of more than 500 samples, its chromatographic properties had not changed.

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