Acta Univ. Sapientiae, Alimentaria, **1** (2008) 5–29

Separation and determination of the amino acids by ion exchange column chromatography applying postcolumn derivatization

J. Csapó[1,2]
email: csapo.janos@ke.hu

Cs. Albert[2]
email:
albertcsilla@sapientia.siculorum.ro

K. Lóki[1]
email: loki.katalin@ke.hu

Zs. Csapó-Kiss[1]
email: csapo.janosne@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary;

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. The most perfect method for the determination of the amino acid composition of pure protein, feeds or biological fluids is still the ion exchange column chromatography (IEC). By the help of the lithium buffer system most of the problems on the field of free amino acid analysis of biological fluids can be solved. At IEC most contaminants move rapidly through the post-column system and are discarded before separation of amino acids begins, resulting in better performance. The time of the sample preparation is minimal compared to pre-column methods, and the detection (with ninhydrin or OPA) is chemically specific for amino

Key words and phrases: ion exchange column chromatography, IEC, high performance liquid chromatography, HPLC, amino acids, determination

acids. Nowadays a new off-line sample preparation method was introduced before analysis by anion-exchange chromatography and integrated amperometric detection, which eliminates carbohydrates from amino acid samples. The major problems remained the hydrolysis of the protein, the deproteinization of the biological fluids and the partial decomposition of methionine, cysteine and tryptophan during the sample preparation for analysis. At the moment the traditional IEC with postcolumn ninhydrin derivatization seems to be the best for both pure proteins and feeds and complex mixtures, but in some comparison the HPLC methods were found to be similar to that of the IEC.

1 Introduction

Moore and Stein [17, 18] devoted plenty of time to separation and very precise determination of amino acids in the middle of the 20^{th} century. In 1958, together with Spackman they published the description of the automatic amino acid analyser for quantitative and qualitative determination of amino acid content of the protein based on ion exchange column chromatography (IEC) after postcolumn derivatisation with ninhydrine. For this work in 1972 they have been awarded the Nobel Prize. After they published their method, many researchers tried to improve it, so a lot of ameliorations were elaborated, but the principles of the method were unchanged. Most of the amino acid analyser operates by the traditional principle of Moore and Stein and use ninhydrine or some different postcolumn derivatisation methods [1, 2, 3, 6, 7, 11, 15, 19, 27].

By Parvy et al. [20] in 1990, approximately 94% of the laboratories used an ion-exchanging technique coupled with colorimetric detection after reaction with ninhydrine for determination of the amino acid content of proteins and free amino acids from biological fluids, and only 6% used gas chromatography. Interestingly, no participating laboratory using high performance liquid chromatography (HPLC) with pre-column derivatisation was able to provide usable results, despite several requests to participate and the dispatch of samples. It confirms that the use of HPLC with pre-column derivatization cannot yet be considered to be a routine for determining all the amino acids in biological fluids.

During the recent time the HPLC has become very popular in the field of amino acid analyses, but the determination of the amino acids by means of HPLC brought a number of problems in comparison with the classical Moore and Stein method. These problems explain the small number of HPLC methods in the practice. For HPLC analysis of amino acids perfectly clean samples

are required, otherwise the impurities of the sample destroy the prewash or analytical columns, or the derivatisation of the amino acids is not successful. The IEC method is not so sensitive for the impurities of the sample, and there is no need for precolumn derivatisation of the amino acids.

Since the original publications improvements in the technique were published by several researchers, but these meant no fundamental changes. They intend to improve the sample preparation method, the hydrolysis of the protein, the determination of the sensitive amino acids (methionine, cystine, tryptophan) by different protein hydrolysis methods, and mark the trend to faster analysis and higher sensitivity. The original two columns system described by Moore and Stein [17, 18] has been used for a long time, but after that the single column system has been spread. An accelerated single column lithium buffer system was elaborated for determination of the ninhydrine positive compounds of biological fluids, and others investigated the different postcolumn derivatization method with different agents for improving the sensitivity of the determination [5, 9, 21].

There have been relatively few methodological advancements in the past 15 years, but the technique is still used very wide-spread. In comparing post-column and pre-column methodologies, some advantages of the post-column methods should be noted. Since ion exchange properties dominate when the sample is loaded, most contaminants move rapidly through the post-column system and are discarded before separation of amino acids begins, resulting in a more favourable performance. Sample preparation is minimal compared to pre-column methods. Detection (with ninhydrin or OPA) is chemically specific for amino acids. Considerable literature exists concerning retention times of amino acids and derivatives (over 500 have been catalogued). The accuracy and precision of the data can be maintained at a high level with a reasonable amount of effort [4].

2 Sample preparation

The most correct separation of the samples is the base of the accurate and repeatable analysis of amino acids by automatic IEC. Before the preparation of the samples the protein content or the approximate content of amino acids should be known for the selection of the optimum weighing of the original sample. The sample has to be as pure as possible, because some of the constituents of the sample can assist to destroy the sensitive amino acids. The volume of the sample which can be applied to the ion exchange column vary

for the different instruments. With refinements in instrumentation the tendency has been pointing towards a decrease of the sample volume to 50 μl or less. The preparation of the sample can be divided into two parts depending on the purpose of investigation: releasing the amino acids from protein and peptides by means of hydrolysis, and preparation of samples containing free amino acids when the protein and other disturbing substances are removed. This paper does not deal with the hydrolysis methods (acidic hydrolysis, performic acid oxidation before hydrolysis for the determination of the sulphur containing amino acids, hydrolysis methods for the determination of tryptophan and recent developments in the hydrolysis) of the proteins.

3 Ion exchange chromatography of amino acids

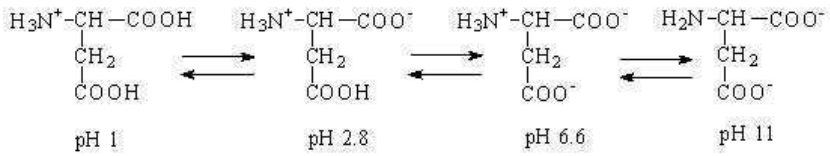
3.1 Introduction

After sample preparation, in most cases meaning hydrolysis of the protein or preparation of the sample for free amino acid analysis, depending on the amino acids present in the sample, sodium or lithium buffers are prepared for separation of the amino acids by IEC. The eluate from the ion exchange column is passed through in a teflon coil placed in a boiling water bath, or other heating apparatus. Before entering, the column effluent is mixed with reduced ninhydrine reagent, which is dissolved in acetate buffer. The ninhydrin reacts with amino acids forming a dye complex. The absorption is determined in a flow photometer, and registered on the chart of a recorder or a computer. The area under the peaks corresponds to the amounts of amino acids present in the sample. The evaluation can be done manually or automatically with an integrator or a computer. The circumstances of the analysis make it possible to quantitate as little as one nanomol amino acid with a high degree of accuracy [1, 2, 17, 18].

At the original two column system for separation all of the protein building amino acids were described first by Spackman et al., and this method was used for manual and automated systems for many years. Nowadays this method is not used, because its problems are related to reliability, accuracy, sensitivity and sample loading system. Nowadays the simple single column system is generally used. By the method of Moore and Stein the amino acids are separated on a cation exchange resin with buffers of carefully defined salt concentration and pH [17, 18]. The ion exchange takes place on resin, consisting of small spherical beads of polystyrene, reacted with divinylbenzene to achieve the required degrees of cross linkage between the two polymerised chains of styrene,

and sulphonated to provide an electrical charge. The chromatographic column is filled with resins of negative charge, and the amino acids are put on the column at a low pH value (pH = 2.2), hence all of them bear a positive charge. In these conditions all of the amino acids will link to the resin, no chromatographic division will occur, and the amino acids are waiting at the beginning of the column for a change in conditions. If the pH and the ionic strength of the elution buffers increase, the isoelectric point of the amino acids will be reached, and the attraction of the ions towards the resin diminishes and so the amino acids will be eluted from the column. The isoelectric point of an amino acid molecule is defined as the pH value, at which the molecule, in the solution, do not dispose any charge. The isoelectric point of amino acids is a function of the pH values of the ionisable groups in the molecule. The conditions of the separation of the amino acids can be modified in a way that the isoelectric points, for all amino acids, are to be reached at various times. For example in the case of aspartic acid *(Figure 1)* the different charges at different pH is the following [6, 15]:

Figure 1: The charges of the aspartic acid at different pH

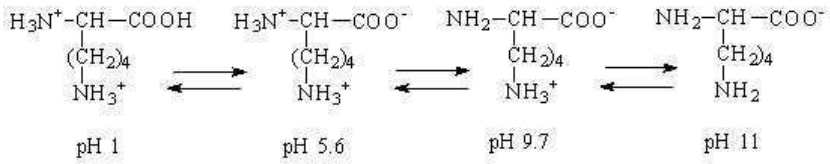


At pH = 1 the molecule has one positive charge, but if the pH value is increasing, larger number of molecules situated in the α-carboxil group will have a negative charge up to the limit of pH = 2.8, when all of them disposes it. This is the isoelectric point of the aspartic acid. The carboxylic group in the side chains less acid than the α-carboxilic acid, and the concentration of the hydrogen ions is sufficient enough to prevent its ionization. If the pH value rises to 6.6, the carboxylic group of the side chain will be ionised, and the molecule will get two negative and one positive charge, and if the pH rise to 11.0, the molecule will dispose only two negative charges.

The lysine has an amino group on its side chain, its isoelectric point is at pH = 9.7. At pH = 1 the lysine possesses two positive, at pH = 5.6 two positive and one negative, at pH = 9.7 one positive and one negative and at pH = 11

one negative charge *(Figure 2)* [6, 15].

Figure 2: The charges of the lysine at different pH



The theoretical treatment of the separation of amino acids supposes that the concentration of the individual amino acids is small, therefore the ratio between the amino acids bound to the resin and free in the solvent have to be regarded as independent of concentration. The process of ion exchange is the following [2]:

$$\mathbf{Matrix–SO_3^- –Na^+ + H_3N^+ –CH(R)COOH =}$$
$$\mathbf{Matrix–SO_3^- –H_3N^+ –CH(R)COOH + Na^+}$$

The distribution coefficient $a_{\text{amino acid}^+}$ for the amino acid is defined as the ratio between free and bound ion in a given section of the column *(Figure 3)*.

Figure 3: The ratio between the free and the bound ion in the column

$$a_{\text{amino acid}^+} = \frac{\left[\mathbf{Matrix - SO_3^- - H_3N^+CH(R)COOH}\right]}{\left[\mathbf{H_3N^+ - CH(R)COOH}\right]}$$

Where $\left[\mathbf{Matrix - SO_3^- - H_3N^+ - CH(R)COOH}\right]$ is the concentration of amino acid bound to the resin,

$\left[\mathbf{H_3N^+ - CH(R)COOH}\right]$ is the concentration of the free amino acids in the buffer.

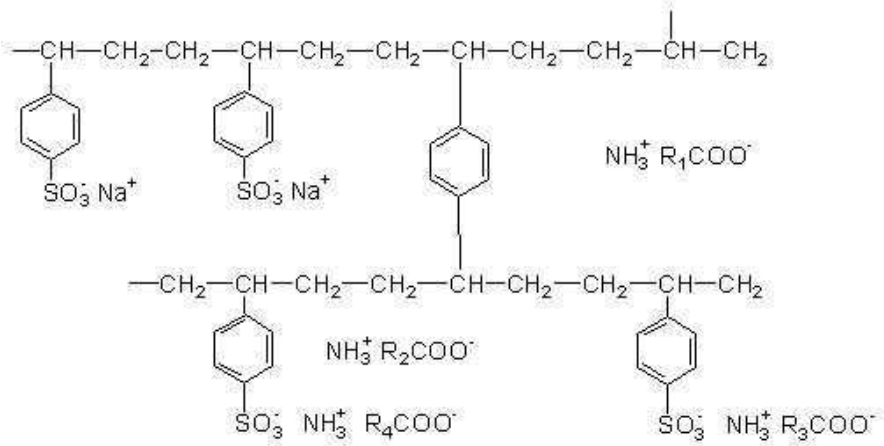
For the ion exchange process the law of mass action can be applied, and from the equilibrium constant (K), the amino acid concentration bound to the

resin, the free amino acid concentration, the counter ion concentration bound to the resin, and the free counter ion concentration we can get information about the elution of the amino acids and the retention time.

3.2 Ion exchange resins

Nowadays spheroidal ball shape ion exchange resins are used [1]. The synthesis is carried out by means of co-polymerization of styrene and divinylbenzene. The share of divinylbenzene applied in the synthesis is approximately 8%. The concentration of divinylbenzene is very important as it forms cross links in the styrene chains leading to the formation of the ball shape, and as depending on the quantity of cross links the resin has more or less favourable properties: terms of rigidity, swelling capacity and porosity. The structure of the resin and the procedure of the ion exchange is the following *(Figure 4):*

Figure 4: The ratio between the free and the bound ion in the column



The cross-linked resin structure is referred to as resin matrix, and if it is sulfonated, then the strongly acid cation exchange resin is obtained. The sections situated inside of the skeleton are called pore and for the charged ions $-SO_3^-$ the term linked ions are used. The ions bearing the opposite charge are referred to as exchangeable ions being assigned to the matrix by means of het-

eropolar links. These are positively charged groups in buffers or amino acids. During the ion exchange the buffer ions bearing opposite charges penetrate to the matrix pores, and exchange places with the ions with opposite charges which are linked there.

The dimension of the particles, the level of sulphonation and cross linking varies in the case of resins used for the amino acid analysis [1, 2, 6, 7, 11, 15, 19]. As the divinylbenzene concentration increase, the cross-linking occurs at shorter intervals and the effective particle size or permeability is reduced, contrary the anchor group is brought closer to each other so that the separating power is increased. The low cross-linking resins with 1–4% divinylbenzene have a higher permeability, their equilibrium is reached more rapidly, and they are capable of handling larger molecules. The capacity of the resins, because of the swollen volume is smaller, the separation power for certain ions is reduced, and the physical stability of the resin is also less. The low cross-linking resins with 8–16% divinylbenzene have small pore size, lesser permeability, but it is sufficient for more minor ions, and the swelling is slight.

Examining the particle size of the resin it is advisable, that the smallest possible particle size is the best. The exchange rate increases with decreasing particle size, since the diffusion path between the active groups become shorter. Short diffusion values improve the sharpness of the separation, and permit to use shorter columns reducing the separation time. Smaller particles have a higher mechanical stability which is to be considered very important, because the resin expands and contrasts in the column through the continuous changes in pH and concentration during the analysis.

The dimension of the separating column is very important as regard to the high resolution separation between the amino acids. The diameter of the columns nowadays is 1–2 mm, but earlier columns with 5–9 mm diameter were widely used. The larger diameter columns are preparative columns. The separating performance depends in addition to the diameter of the ion exchange particles, on a length factor and the column diameter. It is preferable to keep the column as narrow as possible in order to have the largest possible number of the theoretical plate number in the column.

The flow rate of the eluting buffer on the column is very important, as it determines the time of the analysis. If the flow rate through the column is more than the optimal, the fractions leaving the column become unsymmetrical, leading to tailing, in addition the amino acid peaks can overlap. Increasing flow rate leads to a higher back pressure, which is undesirable for safety.

The regeneration of the ion exchange column is indispensable after the sufficient number of amino acid analysis. During the regeneration sodium hy-

droxide or lithium hydroxide is used to wash the impurities from the column and replace the Na^+ or Li^+ ions used during the analysis. Some authors suggest 0.2–1.0 M, but the optimum concentration seems to be 0.4 M for sodium hydroxide and 0.3 for lithium hydroxide. If cation resins contaminated with heavy metals, proteins or other bigger molecules, the resin have to be removed from the column, treated with 1% EDTA in 2 M hydrogen chloride solution for some hours at room temperature, regenerated by boiling the resin in 6 M HCl for half an hour, cooled at room temperature, diluted to 3 M HCl, filtered and washed with 500 cm^3 two times distilled water. Remove the resin from the filter and suspend in 2 M NaOH or LiOH depending fro Na or Li system. Boil the resin for some minute, and dilute to 0.5 M base. This resin is ready to fill in the analytical column [1, 2].

The chromatography activity of the amino acid analysers is still influenced by the column dimensions, eluent flow rate, temperature and the presence of organic solvent in the buffers.

3.3 Buffer systems for separation of the amino acids

Choice of buffer system. Generally protein hydrolysates contain most of all 18 amino acids normally found in proteins, they are easily separated with three sodium buffer system. Physiological fluids contain some of all the 40–50 ninhydrin positive compounds present in different physiological mixtures. For this purpose four or five sodium buffer system is suitable to achieve the satisfactory separation between the ninhydrin positive compounds. The lithium buffer system is suitable for these purposes, but the application of this system is justified rather in the case that simultaneous separation of aspartic acid, asparagine, glutamic acid and glutamine is required. The lithium system is more sensitive to variations than the sodium system. The salts used for making buffers should be at the highest purity. The salts should be dissolved in deionized or carefully distilled water. Not only the ninhydrine positive impurities, but others may cause irregularities in the baseline, for this reason freshly drawn deionized water is preferred. The acidic buffers have a tendency to take up ammonia and other ninhydrin positive compounds, therefore it is advisable to add the HCl as late as possible to the buffers. The source of ammonia is tobacco smoke, cleaning fluids, urine of the laboratory animals and toilets, and vapour of different chemicals. Sometimes thiodiglycol is added to the buffers to prevent oxidation of methionine, which can under certain circumstances influence the baseline shifts. Organic solvents (ethanol, propanol, 2-methoxyethanol) in the case of some resins is also added to the

first buffer to improve the separation between threonine and serine. These peaks become slightly broader as the column ages a further additional organic solvent may be necessary later. It appears that different solvents are more adequate to different resins. Preservatives are added to the buffers to inhibit the growth of microorganisms. Several different chemicals (0.1% phenol, 0.01% pentachlorophenol, 0.01% caprylic acid) can be used for this purpose [1, 2].

Effect on separation by pH, temperature, organic solvents and column flow rate. The pH of the buffer is very critical for the separation of various amino acids. All of the peaks of amino acids emerge earlier and sharper if the pH is too high, and peaks the chromatograph later if the pH is too low. The cystine is the most sensitive for the pH, temperature and the concentration of the ions with an opposite charge of the buffer. Cystine should be eluted and completely separated directly after alanine. With increasing pH and temperature the column accelerates the cystine, thereby shortens its elution time and if the temperature and pH are lower, its elution times become longer, and cystine falls behind. The pH value and temperature must be selected in a way, that cystine can just be positioned between alanine and valine. The pH change has a greater influence on the cystine movement than a change in temperature.

The temperature affects the separation in two different ways: by changing the pH and by altering the affinity of the amino acids to the ion exchange resin. The separation between threonine and serine can be improved by lowering the temperature, but at the same time the backpressure is increased substantially, and it influences the separation of the glutamic acid. Therefore it is important to have a temperature gradient after the separation of the two hydroxy amino acids. Cystine is also sensitive to temperature, but any changes in the retention time caused by the temperature can easily be compensated by the pH. In the system for hydrolysates the increase of the temperature from 50 °C to 70 °C or higher is recommended to decrease the time of analysis, but this rise should not take place before the separation of isoleucine and leucine. The optimum temperature for separation of aspartic acid, hydroxy proline, threonine, serine, asparagine, glutamic acid and glutamine is 37–38 °C with both sodium or lithium buffer systems, as glutamic acid is particularly sensitive even to minor changes of temperature.

The organic solvent added to the first buffer changes the solubility of the different amino acids. It is particularly the extra $-CH_3$ group of threonine as compared to serine that results in melioration in separation. The most frequently used compounds are methanol, ethanol, propanol, isopropanol and

methyl cellosolve. The drawback of these techniques is a slight loss of separation between glycine and alanine and an increased back pressure. It is possible to use as much as 25% of organic solvent, but the normally used concentration is between 2% and 5%. The analysis should be started at a rather low percentage of organic solvent, providing an acceptable separation between threonine and serine, and increases the amounts when the column becomes older, and the peaks slightly broader. The limiting factor should be the separation between glycine and alanine.

A steady buffer flow rate is required for successful and reproducible separations of amino acids by IEC. This can be achieved with a constant pressure or a constant displacement pump. At most of the analysers the pumps are pulse-free and feature an even power output and their utilisation guarantees conformity of the retention times of individual peaks. The pressure limit of the pumps is 1 to 8 MPa, and is controlled by the software. The choice of flow rate is dependent upon the type of resin, the dimensions of the column and the overall design of the instrument, and it varies between models [1, 2, 6, 7, 11, 15, 19].

Preparation of the sodium citrate buffers. Sodium citrate buffers are mainly used for the determination of amino acids in protein hydrolysate *(Table 1).* List of necessary chemicals: citric acid, sodium citrate, sodium chloride, sodium hydroxide, boric acid, thiodiglycol, sodium azide. The table for computation of the quantity of the individuals for the preparation of the sodium citrate buffers is below [1].

Table 1: The composition of the sodium buffers

	Buffers			
	1	**2**	**3**	**4**
M Na	0.2	0.2	0.4	1.12
M citrate	0.066	0.066	0.066	0.066
pH	2.60	3.00	4.25	-
Citric acid (g/dm^3)	30	30	32	-
Sodium citrate (g/dm^3)	19.6	19.6	19.6	19.6
Sodium chloride (g/dm^3)	11.7	11.7	23.4	52.6

Diluting buffer of 0.2 M sodium with pH = 2.2 will be used for the dilution

of both the samples and standards to a required concentration. The *regeneration solution is 0.2 M sodium hydroxyde. The first sodium buffer* (0.20 M Na, pH = 2.95) elutes the following amino acids: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine and cystine. This buffer is designed for the determination of the amino acid content of the hydrolysate, and this buffer is suitable for assaying cysteic acid and methioninesulphone as well. It is also used when it is necessary to determine proline exactly, or when you want to determine the amino acids with the best separation, and the time of the analysis is not a limiting factor. In this buffer smaller ionic strength is used, therefore cystine is eluted after glycine and alanine. With an increased value of pH and increased temperature cystine elute earlier. The separation of threonine and serine as well as glycine and alanine is very good in the case of this buffer. These two groups of peaks behave in the same way as a balancing mechanism, if the separation is improved at one pair, the separation of the other ones become worse. It means that if cysteine is separated well, both of the pairs will be separated at a very good extent.

The second sodium buffer (0.30 M Na, pH = 3.50) elutes the following amino acids: aspartic acid, threonine, serine, glutamic acid, proline, cystine, glycine, alanine and valine. This is a classical buffer designed for the single column system of the determination of the hydrolysate. The cysteine is very sensitive for pH, temperature and concentration of ions with an opposite charge. An increasing pH and temperature accelerates its movement on the column and cystine thereby shortens its elution time. The pH value of the buffer and temperature must be selected in a way that cystine can just be positioned between proline and glycine.

The third sodium buffer (0.40 M Na, pH = 4.25) elutes the following amino acids: methionine, isoleucine, leucine. This buffer is not problematic as all of the amino acids are separated very well. The fourth sodium buffer (1.12 M Na, pH = 7.9) elutes the rest of the amino acids: tyrosine, phenylalanine, histidine, lysine and arginine, and among the amino acids elute ammonia.

Preparation of the lithium citrate buffers. Lithium citrate buffers are used especially for the determination of the free amino acids from physiological samples *(Table 2).* List of necessary chemicals are: citric acid, lithium citrate, lithium chloride, lithium hydroxide, boric acid, thiodiglycol, lithium azide. The table for computation of the quantity of the individuals for the preparation of the lithium citrate buffers is below [1, 22].

Table 2: The composition of the lithium buffers

	Buffers				
	1	**2**	**3**	**4**	**5**
M Li	0.18	0.20	0.35	0.33	1.20
M citrate	0.053	0.060	0.070	0.100	0.220
pH	2.90	3.10	3.35	4.05	4.65
Citric acid (g/dm^3)	27.26	30.07	35.17	38.48	41.65
Lithium citrate (g/dm^3)	14.92	16.92	19.74	28.20	62.04
Lithium chloride (g/dm^3)	7.62	8.47	14.83	13.98	50.87

Diluting buffer of 0.1 M lithium with pH = 2.2 will be used for the dilution of both the samples and standards to a required concentration. The *regeneration solution is 0.3 M lithium hydroxyde. The first lithium buffer* (0.18 M Li, pH = 2.80) elutes the following amino acids: cysteic acid, taurine, phosphoetanolamine, urine, aspartic acid, hydroxyproline, threonine, serine, asparagine, glutamic acid, glutamine. Elution is carried out at the basic temperature of 37 to 40 °C. In terms of pH and temperature the most sensitive ones are asparagine, glutamic acid and glutamine. Glutamic acid is the most responsive and most moveable at a change in pH and temperature, therefore the pH and temperature must be prepared in a way that glutamic acid can just be positioned in the middle between asparagine and glutamine.

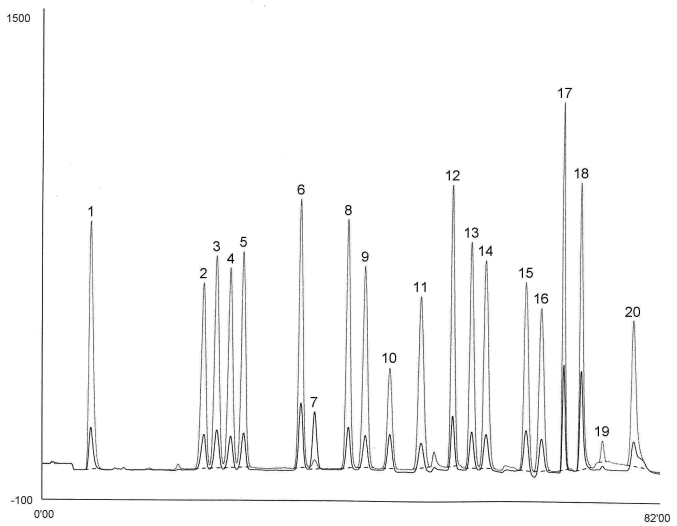
The second lithium buffer (0.20 M Li, pH = 3.05) elutes the following amino acids: α-amino adipic acid, proline, glycine, alanine, citrulline, α-amino butiric acid and valine. Citrullin is very sensible to temperature and pH, its position can be set by the pH of the buffer. *The third lithium buffer* (0.36 M Li, pH = 3.35) elutes the following amino acids: cystine, methionine, cystathionine, isoleucine, leucine. At this buffer only the cystahionine is problematic, which is receptive for both pH and temperature. It is recommended to switching to the higher temperature (60 °C) so that the cystathionine will be positioned in the middle between methionine and isoleucine. In the case of a latter switching of temperature cystathionine is eluted afterwards and it is not sufficiently separated from isoleucine, in opposite case it is eluted with methionine.

The fourth lithium buffer (0.33 M Li, pH = 4.05) elutes the following amino acids: tyrosine, phenylalanine, β-alanine and β-amino butyric acid. This buffer is not accompanied by any problem if the buffer change has been per-

formed in the right place. *The fifth lithium buffer* (1.20 M Li, pH = 4.65) elutes the following amino acids: γ-amino butyric acid, ornithine, lysine, histidine, 1-methyl histidine, 3-methyl histidine and arginine. This buffer is trouble free. The buffer change must be performed after β-amino butyric acid.

Lithium buffers are much more aggressive than Na buffers, that is why it is suitable to rinse approximately once a month with distilled water at the maximum throughput of the pump. Because of Li buffers are more aggressive towards all metals, it is not recommended to leave them for longer times in contact with surfaces of varnishes and metals. At *Figure 5* the chromatogram of the hydrolysate after performic acid oxidation, at *Figure 6* the chromatogram of the free amino acid can be seen.

Figure 5: Determination of the amino acids from hydrolysate after performic acid oxidation of the sample

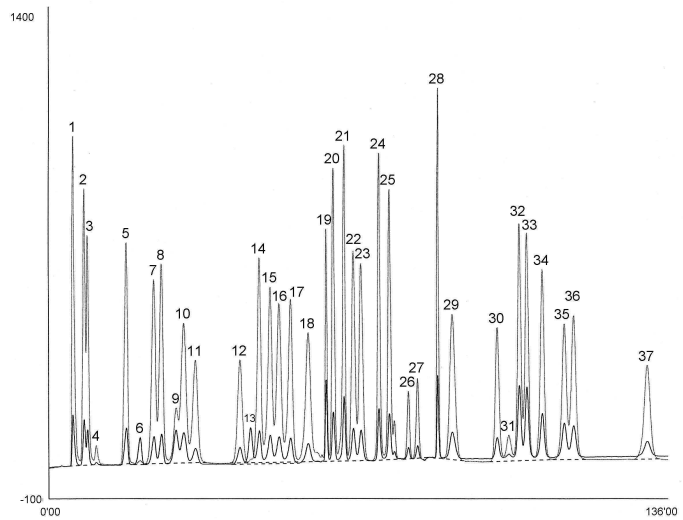


The standard contains 25 nmol of each component except for ammonia. Operating parameters are given below [1]. The amino acids in order of appearance on the chromatogram are: 1. cysteic acid, 2. methionine sulphone, 3. Asp, 4. Thr, 5. Ser, 6. Glu, 7. Pro, 8. Gly, 9. Ala, 10. Cys, 11. Val, 12. Met, 13. Ile, 14. Leu, 15. Tyr, 16. Phe, 17. His, 18. Lys, 19. NH_3, 20. Arg.

Instrument: INGOS AAA400, packing of column: OSTION Lg ANB, column height: 35 × 0.37 cm, buffers: 1: pH 2.7, 0.2 M Na^+; 2: pH 4.25, 0.5 M Na^+; 3: pH 6.9, 1.12 M Na^+; 4: 0.2 M NaOH.
Program:

Time (min)	Temperature (°C)	Buffers
0.00	50.00	1
1.00	50.00	1
29.00	50.00	2
44.00	60.00	3
63.00	74.00	3
66.00	74.00	4
71.00	74.00	1
77.00	60.00	1
82.00	53.00	1
87.00	50.00	1
101.00	50.00	1

Figure 6: Determination of the amino acids from hydrolysate after performic acid oxidation of the sample



The standard contains 25 nmol of each component except for ammonia. Operating parameters are given below [1]. The amino acids and the ninhydrin positive compounds in order of appearance on the chromatogram are: 1. cysteic acid, 2. taurine, 3. phosphoserine, 4. urea, 5. Asp, 6. hydroxyproline, 7. Thr, 8. Ser, 9. Asn, 10. Glu, 11. Gln, 12. α-aminoadipic acid, 13. Pro, 14. Gly, 15. Ala, 16. citrulline, 17. α-aminobutyric acid, 18. Val, 19. Cys, 20. Met, 21. cystathione, 22. Ile, 23. Leu, 24. Tyr, 25. Phe, 26. β-Ala, 27. β-aminoisobutyric acid, 28. γ-aminobutyric acid, 29. chlorophenylalanine, 30. ethanolamine, 31. ammonia, 32. ornithine, 33. Lys, 34. His, 35. 1-methylhistidine, 36. 3-methylhistidine, 37. Arg.

Instrument: INGOS AAA400, packing of column: OSTION Lg FA, column height: 20–22 $\times$ 0.37 cm, buffers: 1: pH 2.8, 0.18 M Li^+; 2: pH 3.1, 0.20 M Li^+; 3: pH 3.35, 0.35 M Li^+; 4: pH 4.05, 0.33 M Li^+; 5: pH 4.65, 1.20 M Li^+; 6: 0.3 M LiOH.

Program:

Time (min)	Temperature (°C)	Buffers
0.00	38.00	2
33.00	38.00	3
45.00	70.00	3
50.00	70.00	4
63.00	70.00	5
95.00	74.00	5
120.00	74.00	6
136.00	53.00	5
139.00	74.00	1
144.00	38.00	1
160.00	38.00	1

3.4 Recent developments in the chromatographic separation

Sample preparation and postcolumn derivatization. For separation of the amino acids after deproteinization or hydrolysis of the sample the column chromatography proved to be the best method. It means high performance liquid chromatography (HPLC) consisting ion exchange column chromatography (IEC) and reversed phase chromatography (RPC) with post- or precolumn derivatization of the amino acids, and gas liquid chromatography (GLC). During IEC the amino acids are separated by sulphonated polystyrene cation exchange resin, mixed with derivatization agent (mainly ninhydrin), passed through a coil and a detector and depending on derivatization agent spec-

trophotometer or fluorometer. During the last two decades the analysis time of IEC was reduced by improvement of the ion exchange resins. The shorter analysis time has been achieved by the use of complex buffer and column temperature systems. During the short time analysis the resolution of the peaks sometimes was not sufficient, and very expensive instruments, ready to use buffers and ninhydrin produced by the manufacturers, had to be used. The detection of the amino acids was mainly based on ninhydrin system but instead of methylcellosolve sulfolane was used as solvent agent of the reduced ninhydrin. This solution buffered with lithium acetate not so toxic, and the stability, the signal to noise ratio, the resolution of the peaks and the baseline is also better than at normal ninhydrin. This reagent does not form precipitates and blockages in the flow lines and in the reaction column, but it is three times as expensive as the normal ninhydrin solution. Other derivatization reagents (fluorescamine, dabsylchloride, 4-fluoro-7-nitro-2,1,3-benzoxadiazole and *o*-phthaldialdehyde) were introduced to improve the sensitivity and the accuracy of the method, but many problems, particularly considering derivatization of proline and hydroxiproline had to be solved. From these reagents only the OPA/mercaptoethanol and the OPA/3-mercaptopropionic acid could be used widely for postcolumn derivatization of the amino acids.

Separation of the free amino acid composition of the biological fluids by lithium buffer system. In the past 25 years, reversed-phase high-performance liquid chromatography (RP-HPLC) has been pervading as a preferred method for the amino acid analysis of protein hydrolysates, but not used widely for physiological samples, because they are so complex that application of RP-HPLC has resulted in poor peak resolution [26]. Analysis of physiological amino acids is traditionally carried out by ion exchange chromatography followed by post-column ninhydrin or *o*-phthaldialdehyde derivatization. Recently with the advances in instrumental design a new generation of amino acid analysers using IE emerged. This system offers the advantage of ease of operation and highly adaptable for analyses of substances than amino acids. Teik et al. [26] in their study described the preparation of lithium citrate buffers and their application in physiological amino acid analysis. Quantitative analysis of results obtained for physiological amino acids was examined in terms of accuracy and precision. The composition of the laboratory-prepared lithium citrate buffers used in obtaining a satisfactory separation of amino acids was the following: lithium eluent 1 contained lithium ion 0.24 M, pH 2.75; the lithium eluent 2 contained lithium ion 0.34 M, pH 3.60, and the lithium regenerant encompassed lithium ion 0.3 M with 0.002 M of EDTA. A complete analysis of

the 44 components in the standard took about 120 min in each case, a somewhat shorter time than reported in the literature for other systems. With these conditions most amino acids were satisfactorily resolved, the exceptions were Trp and HyLys; 1-MeHis and His; and 3-MeHis and Ans. The system reported provided equivalent analytical strengths but also has the advantage of cost saving based on component equipment and laboratory-prepared buffers.

Grunau and Swiader [12] adapted a high-performance liquid chromatographic system to the high-resolution determination of free amino acids. The lithium-based eluent gradients used to allow good separations to be achieved isothermally in 2 h. Although the overall elution pattern correlates strongly with those established automated methods, the differences can be large, and are numerous enough that one type of system cannot serve as a predictor for the other. Relative retention times in the Pickering system were determined for 99 ninhydrin-positive compounds: imino acids, ureides, amino sugars, amino acids and derivatives, with emphasis on those occurring in plants.

Several methods are suitable for the determination of amino acids (AAS) in biological fluids, including gas chromatography, reversed-phase chromatography with pre-column derivatization with various reagents such as *o*-phthalaldehyde (OPA), 9-fluorenylmethyl-chloroformate, phenylisothiocyanate (PITC), dimethylaminonaphthalenesulfonyl chloride, dimethylaminoazobenzenesulfonyl chloride, 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole and ion exchange chromatography with post-column derivatization utilizing OPA or ninhydrin. The latter remains the most widely used because of several technical and practical advantages. The classic ion exchange separation followed by post-column derivatization with ninhydrin has been considerably improved since its initial inception particularly with availability of modern dedicated AA analyzers. However one remaining problem is the relative instability of the ninhydrin reagent, limiting the use of the ninhydrin/acetate buffer mixture to approximately 2 weeks. Probably it also explains why within-run precisions are so poor for an automated technique.

Separation of the amino acids by anion-exchange chromatography. Non-derivatized amino acids and sugars can be separated and detected simultaneously using anion-exchange chromatography in combination with integrated pulsed amperometric detection (IPAD). The simultaneous separation and detection is advantageous for samples containing approximately equimolar levels of amino acids and sugars [16]. If amino acids are to be analyzed in samples containing much higher concentrations of sugars, anion-exchange/IPAD analysis must be preceded by a sugar-eliminating step. Since both classes of

compounds interact with cation and anion exchangers, a combination of the two chromatographic materials appears to be a logical choice for such sample preparation. Therefore Jandik et al. [16] described a new, automated chromatographic procedure eliminating carbohydrates from amino acid samples prior to their analysis by anion-exchange chromatography and integrated amperometric detection. In the first step, a sample was brought onto a short cation-exchange column (trap column) in hydrogen form. Carbohydrates were passing through this column while only amino acids were retained. Subsequently the cation-exchange column, holding the amino acid fraction, was switched in-line with the gradient pump and separator column. The mobile phase used at the beginning of the separation (NaOH; pH 12.7) transferred amino acids from the trap column onto the anion-exchange column and the amino acid separation was completed without any interference by carbohydrates. All common amino acids were recovered following the carbohydrate removal step. The average value of their recovery was 88.1%. The calibration plots were tested between 12.5 and 500 pmol. The mean value of correlation coefficients of calibration plots was calculated as 0.99. The value of relative standard deviations from five replicates was 3.9%.

Ding et al. [10] introduced a new off-life sample preparation that eliminates carbohydrates from amino acid samples containing a high carbohydrate content before analysis by anion-exchange chromatography and integrated amperometric detection. First the sample was introduced into a cation-exchange column in the hydrogen form. Carbohydrates were removed completely using 0.22% formic acid as a transfer fluid, while only amino acids were retained. Amino acids were then extracted from the cation-exchange resin by 10 cm^3 of 1 M ammonia. The collected ammonia was evaporated to dryness and residue redissolved in water containing 20 mg/dm^3 NaN_3 for injection. All amino acids were recovered following the carbohydrate removal step. The average recovery was 97.2%. The relative standard deviation for seven replicates was less than 5.2%.

Hanko et al. [13] used anion-exchange chromatography with integrated pulsed amperometric detection for separation and direct detection of amino acids, carbohydrates, alditols and glycols in the same injection without pre- or post-column derivatization. These separations use a combination of NaOH and NaOH/sodium acetate eluents. They previously published the successful use of this technique, to determine free amino acids in cell and fermentation broth media. They showed that retention of carbohydrates varies with eluent NaOH concentration differently than amino acids, and thus separations can be optimized by varying the initial NaOH concentration and its duration.

Unfortunately some amino acids eluting in the acetate gradient portion of the method were not completely resolved from system-related peaks and from unknown peaks in complex cell culture and fermentation media. They presented changes in method that improve amino acid resolution and system ruggedness.

Comparison of IEC with HPLC at amino acid analysis of physiological fluids. Davey and Ersser [8] compared the high performance liquid chromatography with phenylisothiocyanate derivatisation and a conventional ion exchange method for determination of free amino acid content of physiological fluids. The correlation coefficient for all the amino acids tested was greater than 0.9 except for proline and tryptophan. Various forms of sample preparation were tried for plasma and amniotic fluid; it was finally decided that protein precipitation with acetonitrile was the most suitable. Ultrafiltration was finally decided that protein precipitation while urine was treated the same as a standard mixture. During the ion exchange chromatography of free amino acids in physiological fluids sulphosalicylic acid was used for protein precipitation and norleucine was the internal standard. Amino acids were separated on a heated (42–56 °C) column (350 mm × 3 mm, cation-exchanges resin, 7 μm, 8% DVB) in the Li^+ form using a pH gradient of 2.8–11.5. Post-column reaction was by heating (95 °C) with strongly buffered (pH 6) reduced ninhydrin and the derivatives were detected at 570 and 440 nm. The imprecision compared favourably with standard ion exchange method although each had specific amino acids for which the imprecision was poor. They reported that the HPLC technique is suitable for the same routine clinical analysis purposes as high-resolution ion exchange chromatography. It also offers the advantages of speed of analysis, sensitivity and equipment versatility over the conventional ion exchange methods.

By the opinion of Sarwar and Botting [24] the IEC is still the main method in use. Its use is, however, being replaced by the faster high-performance liquid chromatographic (HPLC) methods of derivatized amino acids. The intra-laboratory variation of the HPLC method was found to be similar to that of IEC. When similar hydrolytic conditions were used in preparing protein hydrolysates, amino acid results obtained with the PITC derivatization method were generally in close agreement with those obtained IEC. There is, however, room for improvement in the HPLC analysis of amino acids in physiological samples.

Schwarz et al. [25] tested whether plasma amino acids can be analyzed using reverse-phase high performance liquid chromatography (HPLC). The reference method for amino acid analysis is ion exchange chromatography (IEC)

with ninhydrin detection because of its ability to resolve in one analysis all clinically important amino acids, its precision and minimal sample preparation. The HPLC method evaluated correlated well with IEC ($0.89 \leq r \leq 1.00$) with linearity up to $2500\,\mu mol/dm^3$. The between and within-run CVs were $<6.0\%$. In addition, this method is able to separate argininosuccinic acid, homocystine and allo-isoleucine, rare but clinically significant amino acids. This HPLC method was comparable to IEC and could represent an alternative for amino acid analysis. The advantages of this method are its ability to separate all amino acids present in plasma in a short time, although two injections per sample are required, and the wide analytic measurement range obtained using a photodiode array detector. The only disadvantages of this method are the column washes needed to maintain column integrity and the fact that it requires two injections per sample in order to achieve separation of all amino acids. This method, however, represents an alternative to ion exchange chromatography for analysis of amino acids in plasma.

Determination of the tryptophan. Hanko and Rohrer [14] presented a new method to rapidly quantify tryptophan (Trp) in proteins, animal feed (Mehaden fishmeal), cell cultures, and fermentation broths. Trp is separated from common amino acids by anion-exchange chromatography in 12 min and directly detected by integrated pulsed amperometry. The estimated lower detection limit for this method is 1 pmol. Alkaline (4 M NaOH) hydrolysates can be directly injected, and therefore they used this method to determine the optimum alkaline hydrolysis conditions for the release of Trp from a model protein, bovine serum albumin (BSA). This method accurately determined the Trp content of BSA and fishmeal. High levels of glucose (2% w/w) do not interfere with the chromatography or decrease recovery of Trp. They used this method to monitor free Trp during an Escherichia coli fermentation.

Ravindran and Bryden [23] developed a chromatographic method for the determination of tryptophan content in food and feed proteins. The method involves separation and quantitation of tryptophan (released from protein by alkaline hydrolysis with NaOH) by isocratic ion exchange chromatography with *o*-phthaldialdehyde derivatisation followed by fluorescence detection. In this procedure chromatographic separation of the tryptophan and α-methyl tryptophan, the internal standard, complete in 15 min, without any interference from other compounds. The precision of the method was 1–4% relative standard deviation. Accuracy was validated by agreement with the value for chicken egg while lysozyme, a sequenced protein, and by quantitative recoveries after spiking with lysozyme. The method allows determination in a

range of feed proteins, containing varied concentrations of tryptophan and is applicable to systems used for routine amino acid analysis by ion exchange chromatography.

4 Detection systems

The colour or fluorescence produced of amino acids varies for different amino acids and it have to be determined for quantification. It can be made by loading a mixture of amino acids containing the same concentration of each amino acid (including the chosen internal standard) and from the areas of the peaks on the recorder trace calculating each response factor in the used way [28]. Sometimes an internal standard, absent from the sample, is used for every analysis carried out. For instance the non-physiological amino acids norleucine or α-amino-β-guanidinobutyric acid may be used. This should be added in a known amount to the sample prior to any sample pre-treatment. If the amount of the internal standard is known, the concentration of the unknown amino acids can be determined using peak area relationship. This paper does not deal with the reaction of the amino acids with ninhydrin, preparation of the ninhydrin reagent and the reaction of the amino acids with other reagents.

5 Controlling of the apparatus and evaluation of the chromatograms

At most of the modern amino acid analysers a software serving helps for controlling the apparatus and subsequent assessment of the results [1]. The evaluation of the results can be done manually or automatically. On a good chromatogram amino acids with the exception of tryptophan give almost symmetrical peaks. For quantitative evaluation the curve with the highest absorption values is used, in most cases the 570 nm curve. Proline and hydroxiproline give their highest absorption at 440 nm, for this reason the suggested evaluation of these two peaks is at 440 nm if it is possible. When two amino acids are not completely separated, an error is introduced. If the separation is better than 65% of the peak height, it is possible to assume that the two peaks are symmetrical and to calculate the width of the peak at a height where the influence of the neighbouring peak is negligible.

During the manual peak evaluation the baseline, total height, net height, half height and the width of the peak at the half height have to be determined, and from these data the basic area of the peak can be calculated by multiplying

the net height with the width. This value represents the area under the peak, which is linear in function to the concentration of the amino acids. If the area is known for a given amount of an amino acid the amount corresponding to any peak size can be determined. If computer program is used for determination of the quantity of amino acids, the peak parameters can be edited directly in the graph or in the peak table. In the graph you can also edit the baseline and the integration marks of the peaks.

6 Acknowledgements

This work has been accomplished with the financial support of OTKA (National Foundation for the Subsidy of Research, Hungary). The number of the contract is T061587. The Sapientia–Hungarian University of Transylvania, Institute of Research Programs, also granted the research. Their financial assistance is gratefully acknowledged.

References

[1] Amino acid analyser AAA 400. User manual (2002) INGOS.

[2] Amino acid analysis. Handbook and application (1982) LKB Biochrom.

[3] Amino acids in animal nutrition. A compendium of recent reviews and reports (2002) Degussa 1–558.

[4] T.T. Andersen, Practical amino acid analysis, *ABRF News,* (1994) 1–5.

[5] R.B. Ashworth, Ion-exchange separation of amino acids with postcolumn orthophthalaldehyde detection, *J. Assoc. Off. Anal. Chem.,* 70 (1987) 248–252.

[6] H.D. Belitz, W. Grosch, *Food chemistry,* (1999) Springer 1–992.

[7] C. Cooper, N. Packer, K. Williams, *Amino acids protocols,* (2001) Humana Press 1–265.

[8] J.F. Davey, R.S. Ersser, Amino acid analysis of physiological fluids by high-performance liquid chromatography with phenylisothiocyanate derivatization and comparison with ion-exchange chromatography, *J. Chromatography,* 528 (1990) 9–23.

[9] R.L. Davies, D.R. Bagient, M.S. Levitt, Y. Mollah, C.J. Rayner, A.B. Frensham, Accuracy and precision in amino acid analysis, *J. Sci. Food Agric.,* 59 (1992) 423–436.

[10] Y. Ding, H. Yu, S. Mou, Off-line elimination of carbohydrates for amino acid analysis of samples with high carbohydrate content by ion-exchange chromatography, *J. Chromatography,* 997 (2003) 155–160.

[11] M. Friedman, Protein nutrition quality of foods and feeds. *Assay methods–biological, biochemical and chemical,* (1975) Marcel Dekker 1–626.

[12] J.A. Grunau, J.M. Swiader, Chromatography of 99 amino acids and other ninhydrin-reactive compounds in the Pickering lithium gradient system, *J. Chromatography,* 594 (1992) 165–171.

[13] V.P. Hanko, A. Heckenberg, J.S. Rohrer, Determination of amino acids in cell culture and fermentation broth media using anion-exchange chromatography with integrated pulsed amperometric detection, *J. Biomolecular Techniques,* 15 (2004) 317–324.

[14] V.P. Hanko, J.S. Rohrer, Direct determination of tryptophan using high-performance anion-exchange chromatography with integrated pulsed amperometric detection, *Analytical Biochemistry,* 308 (2002) 204–209.

[15] D.J. Holm, H. Peck, *Analytical Biochemistry,* (1998) Longman 91–168.

[16] P. Jandik, J. Cheng, D. Jensen, S. Manz, N. Avdalovic, Simplified in-line sample preparation for amino acid analysis in carbohydrate containing samples, *J. Chromatography,* 758 (2001) 189–196.

[17] S. Moore, W H. Stein, Chromatography of amino acids on sulfonated polystyrene resins, *J. Biol. Chem.,* 192 (1951) 663–681.

[18] S. Moore, W.H. Stein, Chromatographie determination of amino acids by the use of automatic recording equipment, *Methods Enzymol.,* 6 (1958) 819–831.

[19] P.J. Moughan, M.W.A. Verstegen, M.I. Visser-Reyneveld, *Feed evaluation. Principles and practice,* Wageningen Pers., 2000.

[20] P. Parvy, J. Bardet, D. Rabier, M. Gasquet, P. Kamoun, Intra- and interlaboratory quality control for assay of amino acids in biological fluids: 14 years of the French experience, *Clin. Chem.,* 39 (1993) 1831–1836.

[21] M.V. Pickering, U.S. Patent 4 274 (1981) 833.

[22] M.V. Pickering, *LC-GC International,* 2 (1989) 25–29.

[23] G. Ravindran, W.L. Bryden, Tryptophan determination in proteins and feedstuffs by ion exchange chromatography, *Food Chemistry,* 89 (2005) 309–314.

[24] G. Sarwar, H.G. Botting, Evaluation of liquid chromatographic analysis of nutritionally important amino acids in food and physiological samples, *J. Chromatography,* 615 (1993) 1–22.

[25] E.L. Schwarz, W.L. Roberts, M. Pasquali, Analysis of plasma amino acids by HPLC with photodiode array and fluorescence detection, *Clinica Chimica Acta,* 354 (2005) 204–209.

[26] Ng.L. Teik, Y.D. Wong, T. Francis, G.H. Andersen, Ion-exchange chromatography for physiological fluid amino acid analysis, *Nutr. Biochem.,* 2 (1991) 671–697.

[27] J.M. Walker, *The protein protocols handbook,* Humana Press 2000. 1–1146.

[28] A.P. Williams, J.E. Cockburn, *Chromatography and Analysis* 1 (1988) 9–11.

Received: August, 2008