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

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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

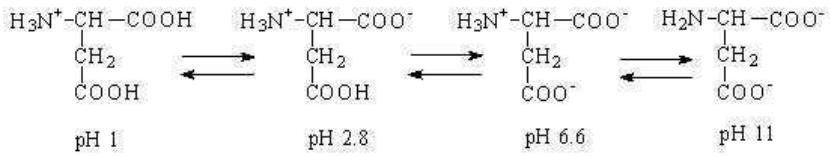
3.1 Introduction

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

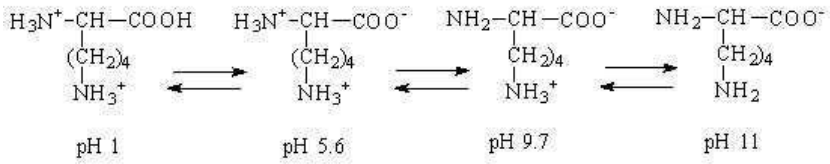


At pH = 1 the molecule has one positive charge, but if the pH value is increasing, larger number of molecules situated in the α-carboxil group will have a negative charge up to the limit of pH = 2.8, when all of them disposes it. This is the isoelectric point of the aspartic acid. The carboxylic group 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.

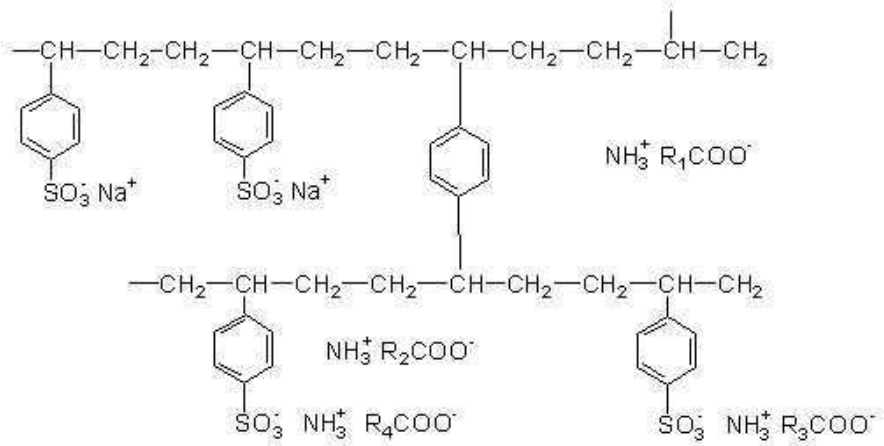
For the ion exchange process the law of mass action can be applied, and from the equilibrium constant (K), the amino acid concentration bound to the

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.

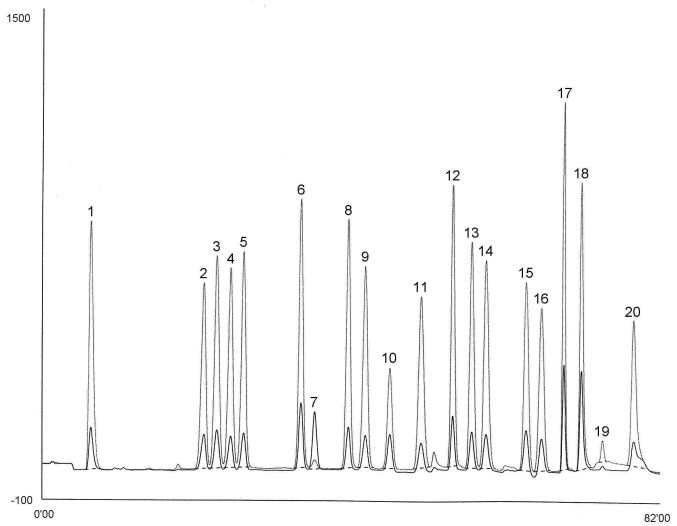
The second lithium buffer (0.20 M Li, pH = 3.05) elutes the following amino acids: α-amino adipic acid, proline, glycine, alanine, citrulline, α-amino butiric acid and valine. Citrullin is very sensible to temperature and pH, its position can be set by the pH of the buffer. *The third lithium buffer* (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

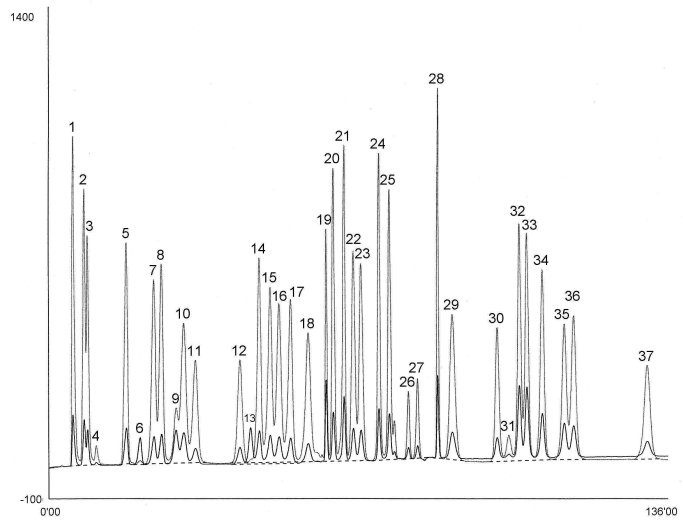


The standard contains 25 nmol of each component except for ammonia. Operating parameters are given below [1]. The amino acids in order of appearance on the chromatogram are: 1. cysteic acid, 2. methionine sulphone, 3. Asp, 4. Thr, 5. Ser, 6. Glu, 7. Pro, 8. Gly, 9. Ala, 10. Cys, 11. Val, 12. Met, 13. 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.

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Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization, in order to determine the enantiomers of D- and L-amino acids

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Abstract. Racemization of free amino acids is considerably lower than that of amino acids bound in peptide. In the same experimental conditions, the rate of racemization of free amino acids is only 20–80% of that of peptide bound amino acids. When using to traditional protein hydrolysis, racemization was 1.2–1.6 times as high as that obtained at high temperatures (160–180 °C), under conditions ensuring total hydrolysis of the protein. This lower degree of racemization may be explained by the fact that, at high temperatures, the protein hydrolyses more rapidly into

Key words and phrases: sample preparation; protein hydrolysis; racemization; amino acids, tryptophan, determination of D-amino acids

free amino acids and the racemization of free amino acids is considerably slower that of amino acids bound in polypeptides. When hydrolysis is conducted at lower temperatures for longer times, the amino acids bound in the peptide chain are exposed for a longer time to the effects actually causing racemization. As a result, we may say that any factor which speeds up hydrolysis, will lower the degree of racemization.

Racemization was higher for proteins in milk powder than for pure proteins. This may be explained by catalysis of racemization by the heavy metals present. After 48 hours at 110 °C and in presence of 4 M barium hydroxide, all amino acids (whether free or bound in peptide) totally racemized. Therefore the racemization of tryptophan cannot be determined using barium hydroxide promoted protein hydrolysis. High temperature hydrolysis (at 160 °C for 45 to 60 minutes, at 170 °C for 30–45 minutes and 180 °C for 30 minutes) are recommended for those who would like to hydrolyse the protein for short times and to determine the degree of racemization occurring in the polypeptide chain, but do not wish to use enzyme hydrolysis.

1 Introduction

The role of optical activity in living organisms has long been known. The large group of biologically active molecules – such as the amino acids – are all optically active. Thus in order to know their roles in living organisms, we should be able to separate and determine their enantiomers. Recently, considerable effort has been devoted as to separation and quantitation of amino acid enantiomers. Among these is the archaeometric application whereby one can establish the age of archaeological relics based on racemization of amino acids, specifically the epimerization of isoleucine [15, 43, 52]. Another example of recent work is the study of the composition of extraterrestrial materials [12]. Recent research demonstrated that, due to technological intervention or the alteration of microbiological status, foods may contain sizeable amounts of D-amino acids [3, 8, 17, 23, 24, 25, 27, 30, 31, 32, 34, 36, 40, 42, 45]. Several articles dealt with the D-amino acid content of milk [4, 5, 6, 28, 16]. From these reports it became obvious that the appearance of D-amino acids is not solely due to technological interventions (heat treatment, heat preservation), but may also result from alteration of microbiological status.

When attempting to quantify amino acid enantiomers, it is not sufficient to separate these from each other. One also has to pay attention to the separation of these from the other amino acids and their derivatives. The amino acid derivative on which we decide to depend should be detectable

with good sensitivity. Lately, pre-column derivative formation has been used with fluorescent reagent, followed by Reversed Phase Chromatography (RPC) of the derivatives. Using these methods, the detection limits for the amino acids of interest are extremely low. On the other hand, the flexibility of this analytical method provides outstanding advantages [21, 38, 51]. Thus, automatic methods have been developed for the simultaneous determination of optically inactive *o*-phthalaldehyde (OPA)/mercapto-ethanol (ME) and α-amino acids [49], and of 9-fluorenyl-methyl chloroformate (FMOC-Cl) in the presence of α-amino and imino acids [2, 13]. The reaction of optically active (chiral) amino acids with chiral reagents yields dia-stereoisomer compounds. In theory, one should be able to separate these using a non-chiral column. If the chiral reagent is another amino acid, then the separation and determination of the diastereomer dipeptide may be achieved using ion-exchange column chromatography [12, 18, 19, 33].

Following derivative formation with chiral reagents, the enantiomers of protein building block amino acids may be separated in a single run using RPC. Since the chromatographic separation takes 50–70 minutes, it is of paramount importance that the analytical method be adaptable to full automation. Another prerequisite is that the derivative formation should be simple, proceeding in a short time at room temperature. The reaction between the optically active thiols, the OPA and the amino acids to be determined has been used to separate and quantify amino acid enantiomers [1, 7]. The use of chiral 1-(9-fluorenyl) ethyl chloroformate (FLEC) for the separation of enantiomers has the advantage of being able to form derivatives, not only with the α-amino acids, but also with the imino-acids [22].

It is very important to know whether or not racemization occurs during protein hydrolysis. If so, the results of the determination will be influenced adversely. Various studies reported that the degree of racemization during hydrolysis of peptide is dependent on protein type and amino acid background. It was found [26, 35, 41, 50] that amino acids bound in peptide racemize faster than free amino acids.

Several reports appeared in the literature [9, 29, 46, 54] dealing with the use of microwave technology in protein hydrolysis. Some authors reported excellent results [11, 14] using high temperatures and short times for the hydrolysis process. It appears that, during microwave promoted hydrolysis, significant racemization occurs, because microwaves have been purposely used [10] to trigger racemization of amino acids. Reports have been published describing the increase of D-enantiomers in foods [39] under the influence of microwave treatment. Racemization is no cause for concern if one does not wish to de-

termine the enantiomers of amino acids. However, if our aim is the separation and determination of amino acid enantiomers, the protein hydrolysis procedure selected should be such that the accompanying racemization is as small as possible. This is necessary since, in the case of significant racemization, we are unable to distinguish between the amino acid enantiomers initially present in the sample and those that appear during the hydrolysis process. Several methods have been developed [20, 47, 48] which restrict racemization occurring during hydrolysis. However, these proved to be lengthy and tedious. As a consequence, objective was to develop a protein hydrolysis method having the lowest possible degree of racemization, by using high temperatures for a short time duration.

2 Material and methods

Hydrolysis and processing of the hydrolysate. Pyrex reusable hydrolysis tubes having 8 mm I.D. (Pierce Chemical Company, Rockford, IL, USA) were used for hydrolysis of proteins or for treating free amino acids. Each tube can contain up to 8 ml of hydrolysing agent without making contact with the PTFE (polytetrafluoroethylene) sealing cup. 1 ml of 6 M hydrochloric acid (HCl) was added to each tube for preparation of protein and peptide hydrolysate. Each tube had two PTFE sealing caps to get complete leak-free operation during heating at 160, 170 or 180 °C.

Either 1 mg peptide, protein, or free amino acids or 20 mg fat-free milk powder was weighed into Pyrex tubes previously washed with hydrochloric acid and deionised water. One ml 6 M HCl was added to each sample (HCl was obtained from Pierce Chemical Company, Rockford, IL, USA) and nitrogen was bubbled for five minutes through the hydrolysing agent by glass capillary. After bubbling with nitrogen, the Pyrex tubes were immediately closed, and put into the heating oven at 160, 170 and 180 °C for 15, 30, 45 or 60 minutes. One sample of each examined material was hydrolysed at 110 °C for 24 h, according to the method of More & Stein [44], with 6 M HCL. Another sample was hydrolysed at 110 °C for 48 h using 4 M barium-hydroxide for determination of tryptophan from samples of food and feed. After hydrolysis, the tubes were cooled at room temperature and HCl was evaporated by lyophilization and the residue of the sample was dissolved in 0.01 M HCl. After the barium-hydroxide hydrolysis, pH of the hydrolysate was set to neutral with 1 M HCl, and the barium was removed from the hydrolysate in the form of barium-sulphate. During neutralisation, the temperature was held below 30 °C with the help of

a sodium chloride - ice mixture. Next all of the hydrolysates were filtered and stored at $-25\,^{\circ}\mathrm{C}$ until the analysis of D- and L-amino acid enantiomers by HPLC.

Materials tested. The following materials were used for testing the racemization during hydrolysis: Bovine ribonuclease, lysozyme, citochrom C, mare's milk powder, and individual free amino acids as follows: L-aspartic acid, L-glutamic acid, L-threonine, L-alanine, L-valine, L-phenylalanine, L-histidine and L-tryptophan. The protein content of milk powder (22.7%) was determined using a Kjel-Foss 16200 (Foss Electric, Denmark) rapid nitrogen analyser. The protein content was calculated from nitrogen % using a conversion factor of 6.38. Peptides and proteins were hydrolysed at varying temperature-time combinations. The free amino acids samples were subjected to the same temperature-time treatments. HPLC was used for determination of L-aspartic acid, L-glutamic acid, L-threonine, L-alanine, L-valine, L-phenylalanine, L-histidine and L-tryptophan content of the samples.

High performance liquid chromatography (HPLC) for separation and determination the D- and L-amino acids. *Instruments:* The chromatographic system was assembled from ISCO 100 DM syringe pumps (Isco Inc. Lincoln, Nebraska, USA) and a Rheodyne (Berkeley, California, USA) injector equipped with a $20\,\mu$l loop. The separation process was monitored and chromatograms stored on an ISCO Chem Research (Isco Inc. Lincoln, Nebraska, USA) system. The derivative formation and sample injection were performed manually. The excitation and observation wavelengths were 325 and 420 nm, respectively.

Reagents: Acetonitrile and methanol were purchased from Rathburn Ltd (Walkeburn, England). The AA standards, the *o*-phthalaldehyde and the TATG were obtained from Sigma Chemical Co., Inc. (St. Louis, MO). The buffers used for elution were prepared from mono- and disodium phosphate. The pH was adjusted with 4 M sodium hydroxide.

Synthesis of derivatives: The reaction was carried out in a $120\,\mu$l microvial which was placed in another vial (volume, 1.8 ml) that had TeflonR coating, internal cover plate, and a screw cap. The sample (free AA or protein hydrolysate evaporated by lyophilization), dissolved in $90\,\mu$l borate buffer (0.4 M; pH 9.5), was mixed with $15\,\mu$l of reagent (8 mg of *o*-phthalaldehyde (OPA) and 44 mg of 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (TATG) dissolved in 1 ml of methanol). The mixture was then homogenized by bubbling through

approximately 100 μl of nitrogen and left standing for 6 min. Then, 25 μl of the reaction mixture were injected into the analytical column. After injection, the system was rinsed three times with approximately 100 μl of a 70:30 acetone–water (v/v) solution. Synthesis of derivatives was performed manually and mixing of reagent solution was made with the aid of an IKA Vibro Fix instrument (Janke and Kunkel, IKA-WERK, Breisgau, Germany).

Separation and quantitation of the enantiomers: Separation of the enantiomers was made according to the method of Einarson et al. [6], using a reversed-phase analytical column packed with Kromasil octyl C-8 (250 × 5.6 mm internal diameter; 5 μm particle size, EKA Nobel AB, Bohus, Sweden). To increase the lifetime of the column, a safety column was fitted between the sample injector and the analytical column (RP-8, Newguard, 25 × 3.2 mm internal diameter, 7 μm particle size, EKA Nobel AB, Bohus, Sweden), and a cleaning column (C18, 36 × 4.5 mm internal diameter, 20 μm particle size, Rsil, EKA Nobel AB, Bohus, Sweden) was installed between the pump and the sample injector. In order to separate the enantiomers, the two component gradient system had the following composition: A = 40% methanol in phosphate buffer (9.5 mM, pH = 7.05) and B = acetonitrile. The flow rate was 1 ml/min, and the elution of the gradient as a function of time is shown below.

Table 1: Eluent composition applied for the separation of OPA-TATG derivatives of amino acid enantiomers

Time (min)	**A**[1]	**B**[2]
	%	
0	95	5
10	95	5
35	83	17
55	72	28
56	67	33
74	67	33
75	62	38

[1] 40% methanol in phosphate buffer (9.5 mM, pH = 7.05)
[2] Acetonitrile

3 Results

D-amino acid composition of bovine ribonuclease as related to time and temperature. Bovine ribonuclease was hydrolysed by 6 M HCl at 110 °C for 24 h and at 160, 170 and 180 °C for 15, 30, 45 and 60 minutes. The D-amino acid compositions of ribonuclease after hydrolysis at 110 °C for 24 h and at elevated temperatures for shorter times are in *Tables 2, 3* and *4.* The data in *Table 2* showed that both traditional hydrolysis (6 M HCl, 24 h, 110 °C) and high temperature–short duration hydrolysis, tryptophan almost completely decomposed.

Table 2: D-amino acid content of bovine ribonuclease hydrolysed by 6 M HCl at 160 °C for different times

Amino acid	6 M HCl 110 °C, 24 h	6 M HCl, 160 °C for			
		15 min	30 min	45 min	60 min
Asp	6.73	1.73	2.78	3.11	3.34
Glu	4.58	1.58	2.59	2.61	2.84
Thr	3.64	1.47	1.70	1.97	2.12
Ala	2.95	1.41	1.58	1.60	1.73
Val	2.34	1.22	1.29	1.51	1.54
Phe	3.28	2.13	2.47	2.93	3.21
His	1.96	0.92	1.41	1.52	1.64
Trp*					

The values refer to the percent of racemization expressed as the ratio (D/D+L)×100. Each values is the mean of triplicate determinations. Hydrolysis conditions: 6 M HCl 110 °C for 24 h and 160 °C for different times using Pyrex No. 9826 tubes.
*Almost totally decomposed during 6 M HCl hydrolysis at 160 °C for 15–90 min.

Table 3: D-amino acid content of bovine ribonuclease hydrolysed by 6 M HCl at 170 °C for different times

Amino acid	6 M HCl 110 °C, 24 h	6 M HCl, 170 °C for 15 min	30 min	45 min	60 min
Asp	6.73	2.23	3.02	3.62	3.89
Glu	4.58	1.97	2.74	2.93	3.28
Thr	3.64	1.99	2.16	2.54	2.84
Ala	2.95	1.69	1.99	2.22	2.54
Val	2.34	1.61	1.90	2.03	2.17
Phe	3.28	2.30	2.83	3.01	3.10
His	1.96	1.22	1.59	1.63	1.81

Data expressed as in *Table 2.*

Table 4: D-amino acid content of bovine ribonuclease hydrolysed by 6 M HCl at 180 °C for different times

Amino acid	6 M HCl 110 °C, 24 h	6 M HCl, 180 °C for 15 min	30 min	45 min
Asp	6.73	2.69	4.28	6.19
Glu	4.58	2.94	3.42	4.61
Thr	3.64	2.45	3.06	3.39
Ala	2.95	2.37	2.89	3.31
Val	2.34	1.99	2.43	2.70
Phe	3.28	2.97	3.12	3.78
His	1.96	1.77	2.09	2.53

Data expressed as in *Table 2.*

As a result, we shall not report this amino acid in the following tables. It is clear that, among the examined amino acids, the highest degree of racemization [D/(D+L)×100] is recorded for aspartic acid, in both traditional and short duration hydrolysis. This is followed in decreasing order by glutamic acid, threonine, phenyl-alanine, alanine, valine and histidine. At 160 °C racemization degree increases as hydrolysis time increases. In the case of every amino acid tested, the lowest racemization was recorded at 15 minutes hydrolysis times. Increasing the hydrolysis time from 15 to 60 minutes, racemization

increased from 1.73% to 3.34% in the case of aspartic acid, 1.58% to 2.84% for glutamic acid, 1.47% to 2.12% for threonine, 1.41% to 1.73% for alanine, 1.22% to 1.54% for valine, 2.13% to 3.21% for phenyl-alanine and 0.92% to 1.64% for histidine.

In earlier studies [14], it was reported that, protein hydrolyses performed at 160 °C for 15–45 minutes were insufficient for complete hydrolyses of proteins, and especially for breakage of the bond adjacent to Val, Ile and Leu. Therefore, for hydrolysis made at 160 °C, only the 60 minute times have practical importance. If we compare the racemization obtained after 60 minutes hydrolysis with results of the traditional method, it is found that the racemization degree for the traditional method, on the average, is 1.5 times as high as that of brief hydrolysis performed at 160 °C.

We reach similar conclusions if we analyse the data featured in *Tables 3* and *4*. When performing the hydrolysis at 170 °C, the hydrolysis reaction practically concludes after 45 minutes and after 60 minutes, even the very stubborn bonds adjacent to Val are broken. At 180 °C, 30–45 minutes are sufficient for complete hydrolysis. Therefore, when comparing results obtained during traditional hydrolysis, it is advisable to make comparisons with data obtained at 170 °C for 45 minutes and 180 °C for 30 minutes. Hydrolysis made at 160 °C for 60–90 minutes yields racemization similar to hydrolysis performed at 170 °C for 45 minutes. Hydrolysis performed at 180 °C for 45 minutes yields a racemization ca. 1.5 times as high as that of hydrolysis carried out at a lower temperature which results in total breakage of bonds. Obviously, both increasing temperatures (from 160 to 180 °C) and increasing time (from 15 to 60 minutes), produced a higher degree of racemization. However, at all three temperatures, continuation of hydrolysis until total hydrolysis of the peptide bond (e.g. at 180 °C for 30 minutes), produced a degree of racemization which represented only ca. 50–70% of that observed in the case of traditional hydrolysis.

Data in *Tables 2, 3* and *4* were subjected to analysis of variance with temperatures, times and amino acids representing main effects. All main effects plus interactions of temperature–time and temperature–amino acids were highly significant ($P<0.001$) sources of variance affecting degree of racemization. Increases of temperature and time of hydrolysis caused increases in racemization. The degree of racemization, when averaged over all time–temperature treatments, varied from 1.16% for His to 2.52% for Asp.

Degree of racemization values (average for all amino acids and for Asp) were fitted to a curvelinear and interactive function of time and temperature. The model explained 94 to 96% of the variation in the dependent variable. The function for Asp indicated that 3.6% racemization would occur at 60, 42 and

28 minutes, respectively, for 160, 170 and 180 °C temperatures. Corresponding times for average racemization were 70, 35 and 20 minutes to produce 2.33% racemization.

Influence of the hydrolysis method on the D-amino acid content of lysozyme, cytochrome C and milk powder. After the experiments with ribonuclease, we hydrolysed lysozyme, cytochrome C and milk powder using the traditional method. The results thus obtained were compared with the data obtained at 160 °C for 60 minutes, at 170 °C for 45 and 60 minutes and finally at 180 °C for 30 minutes. The degree of racemization was compared among the various hydrolysis conditions. The selection of the these time–temperature combinations was based on the time–temperature combinations required to produce total amino acid hydrolysis. The data in *Tables 5* and *6*, show that the degrees of racemization for lysozyme and cytochrome C were virtually identical to that obtained for ribonuclease at the same time and temperature.

Table 5: D-amino acid content of lysozyme (A), cytochrome (B) and milk powder (C) hydrolysed by 6 M HCl at different temperatures for different times

Amino acid	6 M HCl					
	110 °C for 24 h			160 °C for 60 min		
	A	B	C	A	B	C
Asp	6.62	7.01	7.89	3.27	3.42	4.15
Glu	4.58	4.61	5.93	2.79	2.84	3.61
Thr	3.62	3.74	4.38	2.29	2.31	3.14
Ala	2.99	3.21	4.02	1.69	1.65	2.13
Val	2.11	2.24	2.53	1.69	1.84	2.33
Phe	3.31	3.42	3.64	3.19	3.37	3.57
His	1.83	1.89	2.38	1.64	1.67	2.01

Data expressed as in *Table 2.*

In the case of milk powder, for each temperature–time combination, we obtained degree of racemization 15–25% higher than that of the three pure proteins. The higher racemization may be partially explained by the mineral matter content of milk powder. It is known that, with the exception of nickel, heavy metals catalyse the racemization of amino acids.

Table 6: D-amino acid content of lysozyme (A), cytochrome (B) and milk powder (C) hydrolysed by 6 M HCl at different temperatures for different times

Amino acid	6 M HCl					
	170 °C for 45 min			180 °C for 30 min		
	A	B	C	A	B	C
Asp	3.29	3.57	4.42	3.84	3.99	4.67
Glu	2.81	2.89	3.74	3.51	3.63	3.92
Thr	2.11	2.23	3.04	2.87	3.14	3.42
Ala	1.72	1.77	2.11	2.81	2.89	3.04
Val	1.71	1.82	2.27	2.54	2.57	2.82
Phe	2.89	3.11	3.60	2.97	2.83	3.20
His	1.52	1.60	1.99	1.79	1.93	2.11

Data expressed as in *Table 2.*

Racemization of free amino acids during hydrolysis. Racemization of free amino acids has been reported to be lower than that of amino acids bound in peptide. In order to test the hypothesis that there is difference between racemization of free amino acids and those bound in peptide, we have treated each free amino acid with 6 M HCl for various times, and at various temperatures. The results of this investigation are shown in *Table 7.*

When samples were treated for the same length of time, racemization increased with increased treatment temperature. Also, racemization increased with increased treatment times. The high temperature treatments yielded 20–55% less racemization than was observed for traditional treatment of 110 °C for 24 h. Even in the case of the sample treated at 180 °C for 30 minutes, the racemization, except of valine was only 70–90% of that seen for traditional treatment.

If we compare the racemization of free *(Table 7)* and peptide bound *(Tables 2, 3, 4, 5, 6)* amino acids, we find that the percentage racemization of peptide bound amino acids is 4 to 6 times as great as that of free amino acids at 110 °C for 24 h. When both free amino acids and peptide bound amino acids were subjected to the same high temperature, short time hydrolysis, the above ratio ranged from 5 to 7.

Table 7: Racemization of free amino acids treated by protein hydrolysis with different temperatures for different times

Amino acid	6 M HCl 100 °C for 24 h	6 M HCl					
		160 °C for		170 °C for		180 °C for	
		45 min	60 min	30 min	45 min	15 min	30 min
Asp	1.42	0.79	0.93	0.73	1.04	0.94	1.12
Glu	1.07	0.54	0.82	0.68	0.89	0.91	1.03
Thr	0.83	0.31	0.38	0.32	0.41	0.42	0.61
Ala	0.69	0.22	0.29	0.27	0.35	0.37	0.52
Val	0.54	0.19	0.25	0.17	0.24	0.37	0.57
Phe	0.72	0.21	0.27	0.20	0.31	0.32	0.47
His	0.47	0.11	0.18	0.10	0.19	0.21	0.33

Data expressed as in *Table 2.*

Racemization when using barium hydroxide promoted hydrolysis. It was observed earlier in this report that, during hydrolysis with 6 M HCl, tryptophan decomposed almost completely. If we wish to determine the degree of racemization for tryptophan, we must resort to a hydrolysis method which does not cause decomposition of Trp. Since we have been using the barium-hydroxide hydrolysis for the determination of Trp content of proteins, we decided to examine racemization associated with this process. We hydrolysed pure proteins (ribonuclease, lysozyme, cytochrome C), milk powder and free amino acids with 4 M barium hydroxide. We found that, in all materials examined (including free amino acids), all amino acids were fully racemized. In other words we were able to demonstrate the presence of 50% L- and 50% D-enantiomers. Because of this, barium hydroxide based hydrolysis may not be used to measure racemization of the tryptophan contained in proteins.

Conclusions and recommendations. Racemization of free amino acids was considerably lower than that of amino acids bound in peptide. In the same experimental conditions, the degree of racemization of free amino acids was only 20 to 80% that of peptide bound amino acids.

Traditional protein hydrolysis produced racemization is 1.5 times as high as that obtained at high temperatures (160–180 °C), under conditions ensuring total hydrolysis of the protein. This lower degree of racemization may be

explained by the fact that, at high temperatures, the protein hydrolyses more rapidly into free amino acids. Racemization of free amino acids is considerably less that of amino acids bound in polypeptides. Therefore, high temperature hydrolysis promotes conversion to the free state in which amino acids are less subject to racemization. When hydrolysis is conducted at lower temperatures for longer times, the amino acids bound in the peptide chain are exposed for a longer time to the effects actually causing racemization. As a result, we may say that factors which speed up hydrolysis, will lower the degree of racemization.

In the case of milk powder, racemization was higher than in the case of pure proteins. This may be explained by catalysis of racemization associated with the heavy metals present. After 48 hours at 110 °C and in presence of 4 M barium hydroxide, all amino acids (whether free or bound in peptide) totally racemized. Therefore the racemization of tryptophan cannot be determined using barium hydroxide promoted protein hydrolysis.

We recommend that protein samples be hydrolysed at high temperature for a short time (160 °C for 60 minutes and 170 °C for 45 minutes) for all those who would like to determine the degree of racemization occurring in the polypeptide chain, but do not wish to use enzyme hydrolysis.

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Mercaptoethanesulfonic acid as the reductive thiol-containing reagent employed for the derivatization of amino acids with o-phthaldialdehyde analysis

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Abstract. Mercaptoethanesulfonic acid (MES-OH) can be applied for hydrolyzing proteins. The aim of our research was to examine if it can be used also as a derivatization reagent for fluorescens detection of amino acids together with OPA (*o*-phthaldialdehyde) instead of ME (mercaptoethanol) owning to its thiol-group. Corn, soybean and meatmeal samples were hydrolized with hydrochloric acid or MES-OH, derivatized with ninhydrin or OPA/ME or OPA/MES-OH and analysed with IEC (ion exchange chromatography) or RP-HPLC (reversed phase high performance liquid chromatography). There were no significant differences among the amino acid composition results of samples irrespective of choice of

Key words and phrases: Column liquid chromatography, reversed phase chromatography, hydrolysis of proteins, derivatization for fluorescence detection, mercaptoethanesulfonic acid

the hydrolysis or derivatization methods. MES-OH can be applied not only for hydrolysis but also for derivatization of amino acids. In case of samples with high protein content (>50%), due to the dilution after hydrolysis the MES-OH concentration could be insufficient for derivative formation, in these cases an extra MES-OH addition is required prior to derivatization.

1 Introduction

Hydrolysis of peptides and proteins with mercaptoethanesulfonic acid (MES-OH) has often been applied prior to determination of amino acid content of food and feed samples (with the exception of cysteine), and tryptophan content of samples with low amount of carbohydrates [4]. Side-chains of amino acids which are susceptible to oxidation during hydrolysis can be protected with the use of MES-OH due to its thiol group with reducing properties [2]. With the use of MES-OH oxidized forms of methionine were converted to methionine and the indol group of tryptophan was protected against oxidation [3]. Prior to HPLC analyses fluorescent derivatives can be formed by the reaction of amino acids with *o*-phthaldialdehyde (OPA) [1, 5] in the presence of a reductive thiol-containing reagent, such as 2-mercaptoethanol (ME) in order to improve the limit of detection. The aim of our research was to examine the possibility of using MES-OH not only for hydrolysis of proteins but also for derivatization of amino acids in the subsequent step before analysis by HPLC.

2 Experimental data

Hydrolysis of proteins. Two protein hydrolysis methods were applied:

1. Hydrolysis with hydrochloric acid: 5 ml 6 M hydrochloric acid addition to 100 mg sample and heating in a closed glass vessel at 110±1 °C for 24 h in nitrogen atmosphere.
2. Hydrolysis with MES-OH: 5 ml of 3 M MES-OH solution was added to 100 mg sample and heated in a closed inert vessel at 125±1 °C for 24 h in nitrogen atmosphere.

In case of hydrolysis with hydrochloric acid, the required pH values of the solutions for IEC analysis (pH = 2.2) and for derivatization prior to HPLC analysis (pH = 7.0) were set with 4 M sodium hydroxide solution. After hydrolysis samples were diluted, filtered and frozen at −24 °C. Before analyses samples were filtered through 0.45 μm hidrofil membrane-filters.

Derivatization and analysis. IEC analyses were carried out with an amino acid analyzer (Aminochrom OE-914, LaborMIM, Hungary). Amino acids were separated on a cation-exchange column (230 mm × 4.5 mm, "Kemochrom 9" resin), postcolumn ninhydrin derivatives of amino acids were detected with an on-line fotometer at 570 nm.

Prior to HPLC analyses samples were precolumn derivatized with OPA/ME or OPA/MES-OH with the programmable autosampler. First 200 μl (0.4 M; pH = 9.5) borate buffer was added to 450 μl sample solution, then 100 μl derivatization reagent (100 mg OPA and 100 μl ME /or 100 μl 3 M MES-OH/ in 9 ml methanol and 1 ml borat buffer) was added and mixed. After three min 20 μl of the mixture was injected. The HPLC analyses were accomplished with a MERCK-Hitachi HPLC comprising L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager. The compounds were separated on a 250 mm × 4 mm column packed with Superspher 60 RP-Select B (MERCK, Darmstadt, Germany), the temperature of thermostat was 40 °C. The composition of the mobile phase are shown on *Table 1.* The derivatives were detected with a fluorescence detector (λ_{ex} 330 nm, λ_{em} 450 nm). Reagents were p. a. grade. Solvents (methanol and acetonitril) were HPLC gradient grade and purchased from MERCK (Darmstadt, Germany).

Table 1: Eluent composition applied for the separation of OPA-MES-OH-amino acid derivatives[1]

Time (min)	**Metanol (v/v%)**	**Na-acetate solution**[2] **(v/v%)**	**Acetonitril (v/v%)**
0	4	96	0
10	10	90	0
27	11	84	5
28	8	87	5
37	12	81	7
59	16	68	16
60	18	37	45
71	4	96	0
75	4	96	0

[1]The flow rate was 1 ml/min
[2]50 mM (pH 7.0)

3 Results

Chromatograms of the OPA/MES-OH derivatives of amino acids in standards and in corn, soybean and meatmeal samples can be seen in *Figs 1-4.*

Figure 1: Separation of OPA/MES-OH derivatives of amino acids of a standard solution carried out by RP-HPLC with the gradient shape can be seen at *Table 1*

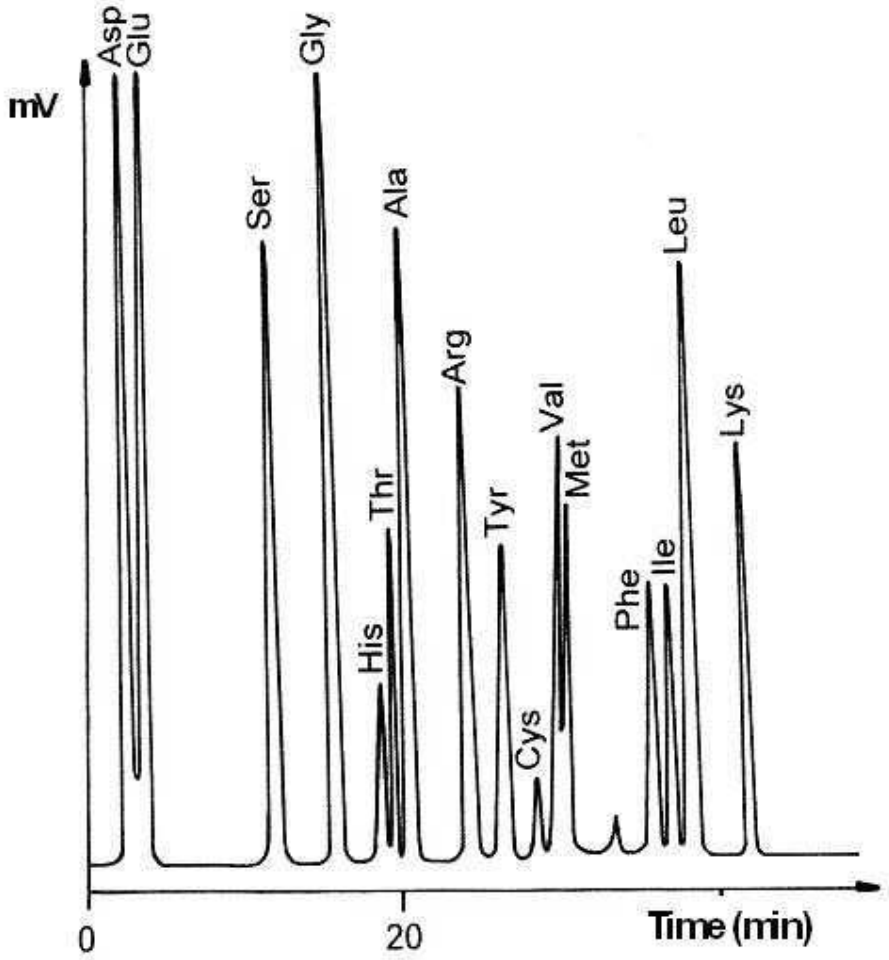


Figure 2: Separation of OPA/MES-OH derivatives of amino acids of a corn sample (see details in *Fig 1)*

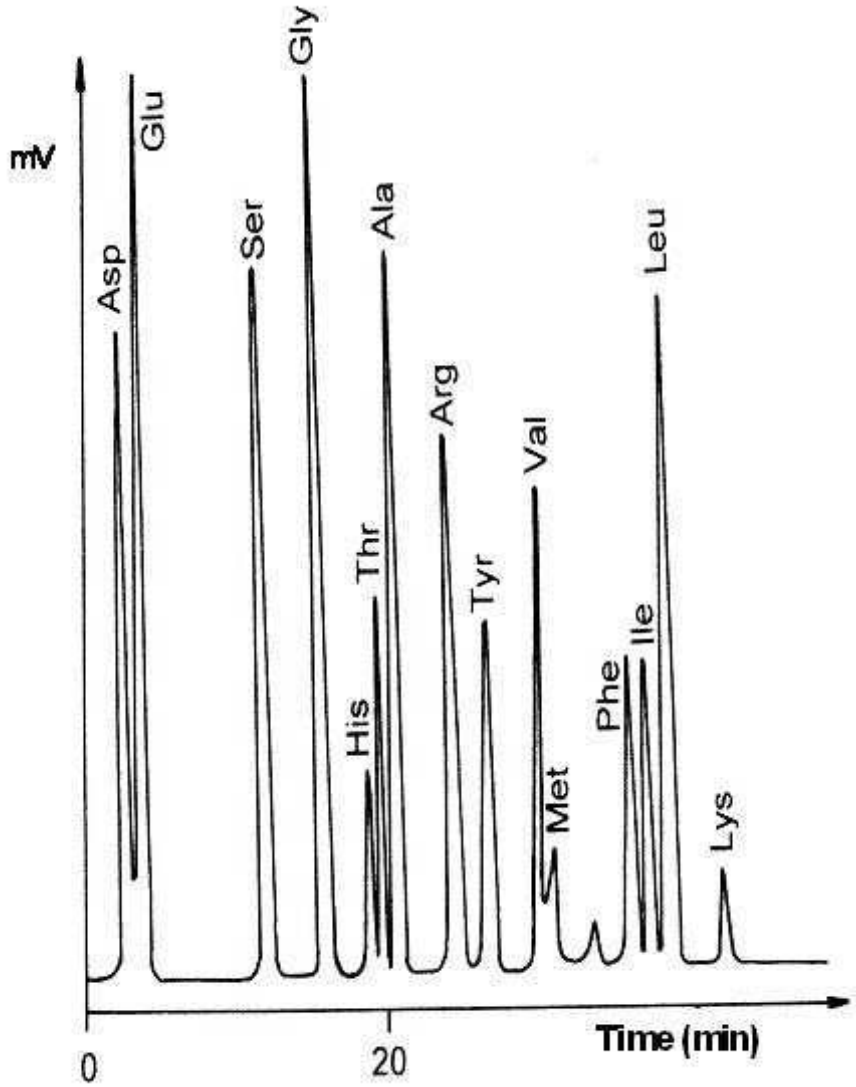


Figure 3: Separation of OPA/MES-OH derivatives of amino acids of a meatmeal sample (see details in *Fig 1)*

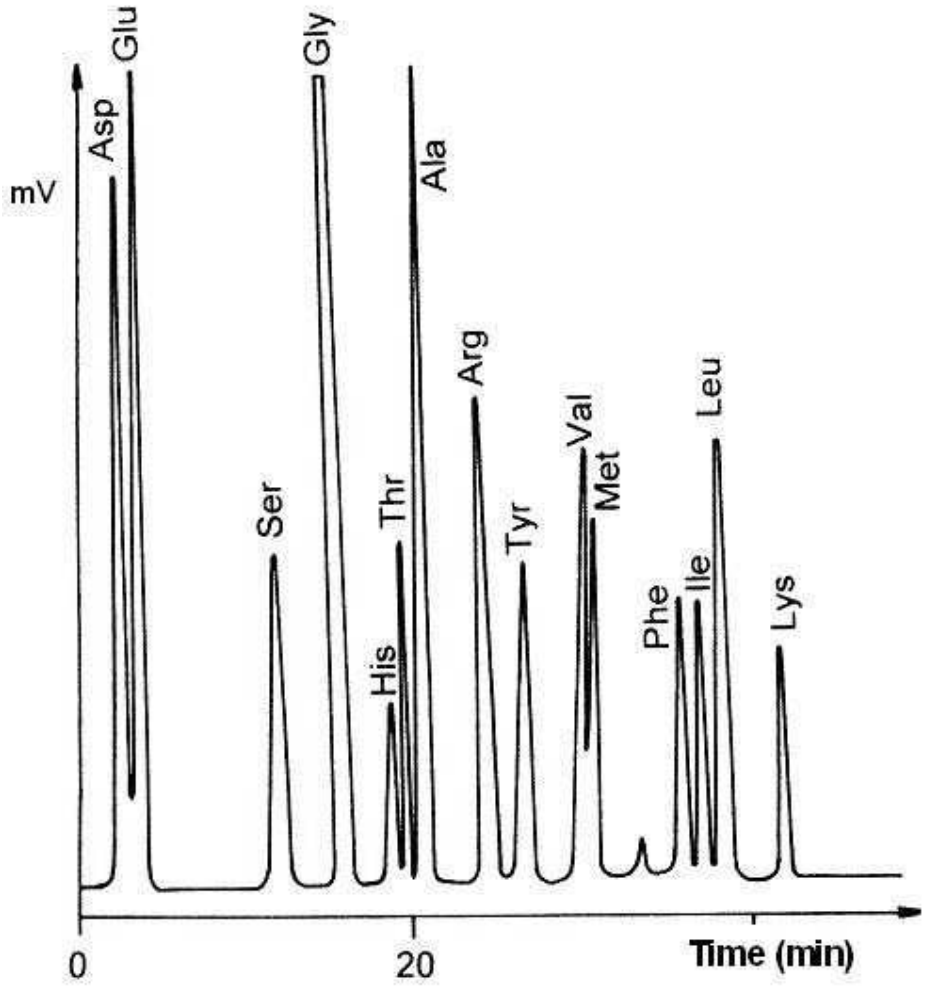
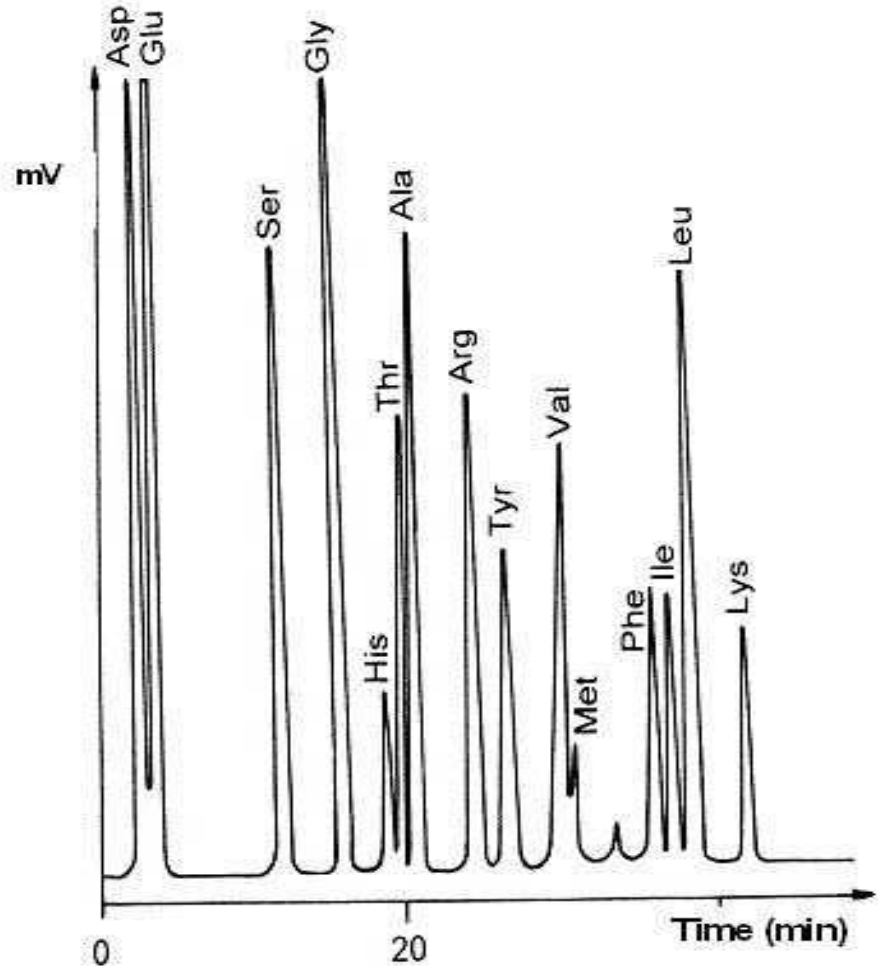


Figure 4: Separation of OPA/MES-OH derivatives of amino acids of a soybean sample (see details in *Fig 1)*



When the OPA/MES-OH derivatization procedure was applied, the sensitivity was about five-fold higher than in case of OPA/ME-method. The amino acid composition of corn, soybean and meatmeal samples analyzed by IEC and HPLC, and hydrolized and derivatized with different methods are shown in *Table 2–4*, respectively.

Table 2: Amino acid content of corn (g/100 g sample) determined with IEC (postcolumn ninhydrin derivatization) and with HPLC (derivatization with OPA/ME after hydrochloric acid hydrolysis; derivatization with OPA/MES-OH without[1] or with[2] an extra MES-OH addition after a MES-OH hydrolysis)

Instrumentation	**IEC**	**HPLC**		
Hydrolysis	**HCl**		**MES-OH**	
Derivatization	**ninhydrin**	**OPA/ME**	**OPA[1]**	**OPA/ MES-OH[2]**
Asp	0.66	0.70	0.69	0.71
Thr	0.32	0.34	0.36	0.36
Ser	0.42	0.47	0.49	0.48
Glu	1.84	1.84	1.82	1.83
Gly	0.34	0.38	0.36	0.37
Ala	0.57	0.61	0.60	0.59
Val	0.42	0.50	0.47	0.49
Met	0.19	0.21	0.23	0.24
Ile	0.36	0.37	0.39	0.40
Leu	0.93	0.94	0.92	0.94
Tyr	0.32	0.36	0.37	0.36
Phe	0.38	0.36	0.37	0.38
Lys	0.29	0.28	0.30	0.29
His	0.22	0.24	0.23	0.25
Arg	0.48	0.54	0.52	0.51

Table 3: Amino acid content of soybean (g/100 g sample) determined with IEC (postcolumn ninhydrin derivatization) and with HPLC (derivatization with OPA/ME after hydrochloric acid hydrolysis; derivatization with OPA/MES-OH without[1] or with[2] an extra MES-OH addition after a MES-OH hydrolysis)

Instrumentation	**IEC**	**HPLC**		
Hydrolysis	**HCl**		**MES-OH**	
Derivatization	**ninhydrin**	**OPA/ME**	**OPA[1]**	**OPA/ MES-OH[2]**
Asp	5.68	5.72	5.69	5.70
Thr	1.63	1.53	1.72	1.74
Ser	2.12	2.35	2.36	2.41
Glu	9.29	9.14	9.23	9.19
Gly	2.14	1.97	2.06	2.11
Ala	1.90	1.98	1.94	1.96
Val	1.90	2.20	1.99	1.98
Met	0.61	0.59	0.68	0.72
Ile	1.73	1.94	1.84	1.86
Leu	3.32	3.17	3.29	3.26
Tyr	1.78	1.55	1.81	1.83
Phe	2.12	2.05	2.07	2.11
Lys	2.96	2.82	2.93	2.92
His	1.19	1.15	1.17	1.20
Arg	3.03	3.09	3.02	3.07

Table 4: Amino acid content of meatmeal (g/100 g sample) determined with IEC (postcolumn ninhydrin derivatization) and with HPLC (derivatization with OPA/ME after hydrochloric acid hydrolysis; derivatization with OPA/MES-OH without[1] or with[2] an extra MES-OH addition after a MES-OH hydrolysis)

Instrumentation	IEC	HPLC		
Hydrolysis	HCl		MES-OH	
Derivatization	ninhydrin	OPA/ME	OPA[1]	OPA/ MES-OH[2]
Asp	3.93	3.85	3.92	3.90
Thr	1.42	1.39	1.43	1.44
Ser	1.60	1.62	1.60	1.59
Glu	5.94	6.02	5.97	6.01
Gly	6.92	6.72	6.83	6.79
Ala	3.98	3.95	3.91	3.97
Val	1.89	2.26	2.16	2.21
Met	0.75	0.73	0.84	0.82
Ile	1.29	1.33	1.31	1.31
Leu	2.65	2.64	2.67	2.63
Tyr	1.06	1.04	1.22	1.21
Phe	1.44	1.36	1.41	1.42
Lys	2.40	2.33	2.37	2.41
His	0.79	0.81	0.82	0.81
Arg	3.32	3.31	3.34	3.29

In fact, there were no significant differences among the results of IEC and HPLC analyses irrespective of choice of the hydrolysis or derivatization methods. Although in case of samples analyzed by HPLC and derivatized with the OPA/ME or with the OPA/MES-OH methods the concentration of some amino acids (threonine, serine, methionine, tyrosine) were slightly higher than in case of IEC analysis with ninhydrin derivatization. When samples were hydrolized with MES-OH, in most cases the concentration of MES-OH after dilution was enough also for derivatization and therefore the addition of OPA was solely required. If the solution after hydrolysis contains at least 1 μl of 3 M MES-OH that is, the protein content of the sample was below 50%, an extra addition of MES-OH was not necessary for derivatization. In case of

corn samples with crude protein content of 9-10% the amount of MES-OH in diluted sample (approx. 4 μl 3 M) was sufficient for derivatization, and also for meatmeal and soybean samples with 46.6–45.2% crude protein content. If the protein content of the sample is less than 5% the peak of MES-OH can disturb the evaluation of acidic amino acids in IEC.

The intensity of OPA/MES-OH derivatives on chromatograms began to decrease after 10–12 hours of the reaction, therefore analysis should be achieved within this time period. After that time a significant decrease was detected mostly in case of aspartic acid and glutamic acid derivatives.

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Separation and determination of the tryptophan enantiomers

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Abstract. Diastereoisomers of L- and D-tryptophan were formed with a chiral reagent 1-thio-β-D-glucose tetraacetate and *o*-phthaldialdehyde and they were separated from the derivatives of the other amino acids that occur in food proteins on an achiral column by high performance liquid chromatography. Mercaptoethanesulfonic acid that is an adequate agent for hydrolyzing proteins made the derivatization with *o*-phthaldialdehyde and 1-thio-β-D-glucose tetraacetate impossible, contrary the reaction completed in the presence of *p*-toluenesulfonic acid, but the oxidative losses during hydrolysis is significant. During boiling, the racemization of tryptophan can be detected above pH = 9 after 12 hours, but the rate of conversion was lower than expected (<1%). The concentration decrease of L-tryptophan after 24 h was 2–5% depending on pH. Besides racemization other reactions e.g. oxidative deterioration may played a role in the loss of L-tryptophan.

Key words and phrases: racemization, tryptophan, mercaptoethanesulfonic acid, p-toluenesulfonic acid

1 Introduction

The knowledge of the exact amino acid demand of the animals and the available amino acid content of the fodder is becoming a crucial point in animal nutrition due to its economical and environmental aspects. In the case of the domestic animal species the digestibility and the bioavailability of the D-enantiomer of a given amino acid is usually lower than that of the L-enantiomer. With the knowledge of the ratio of the amino acid enantiomers within the proteins of fodder, the amino acid requirements of the animals could be better satisfied. That could be especially important for essential amino acids like tryptophan (Trp). The determination of the amino acid enantiomers is also a question of importance in the human nutrition when the health effects due to the consumption of D-amino acids are studied.

In order to determine the amount of Trp in food samples the hydrolysis of the proteins is necessary. Under the most often used acidic hydrolysis conditions (6 M hydrochloric acid, 110 °C, 24 h) the amount of this amino acid partially decompose due to oxidative processes and the loss is even higher in real food samples when carbohydrates are present. Hydrolysis in alkaline solutions (e. g sodium hydroxide) has been reported to preserve almost the whole Trp content of the sample [6]. Later collaborative studies clarified that the use of an internal standard is important in order to avoid the underestimation of the amount of Trp [9]. The AOAC method uses NaOH hydrolysis method [1] if the ratio of the enantiomers is not in the scope of the interest. In the case of the determination of D- and L-Trp, the enhanced degree of the racemization of Trp under alkaline conditions theoretically excludes this sort of solution for hydrolysis. Among acidic hydrolysis methods the highest recoveries were reported when 3 M mercaptoethanesulfonic acid [11] and 3 M *p*-toluenesulfonic acid containing 0.2% tryptamine [10] was used.

Besides the deliberation of the amino acids from the peptide chain the separation of the Trp enantiomers should be accomplished. In the case of high performance liquid chromatography (HPLC) there are three main possibilities: using columns with a chiral stationary phase, using an achiral stationary phase column with a chiral mobile phase, or derivatization with a chiral reagent and separation of the diastereoisomers formed on an achiral stationary phase column. Precolumn derivatization can be accomplished with *o*-phthaldialdehyde (OPA) and N-isobutyryl-L-cysteine (IBLC) [2] or N-isobutyryl-D-cysteine (IBDC) [3], with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [7], or with OPA and 1-thio-β-D-glucose tetraacetate (TATG) [8].

The primary purpose of the research was to achieve an analytical method

that renders practicable the detection of the racemization of Trp. First the separation of the Trp enantiomers in the form of diastereoisomers was accomplished on an achiral column in the presence of the other amino acids occurring in food proteins. Later on the applicability of acidic hydrolysis methods was investigated to clarify how they can be used prior to derivatization and analysis when protein bound amino acids has to be analyzed. Finally the racemization kinetics of free L-Trp of during heat treatments at different pH was studied.

2 Material and methods

Derivatization and analysis. Diastereoismers were produced with OPA (*o*-phthalaldehyde) and TATG (1-thio-β-D-glucose tetraacetate) based on the methods of Einarsson et al. [8] and Csapó et al. [4]. 200 μl sodium-tetraborate buffer (pH = 9.5) was added to 460 μl hydrolized protein solution or free amino acid standard solution containing 0.16 mg/ml amino acid, then 20 μl derivatization reagent (8 mg OPA and 44 mg TATG were dissolved in 1000 μl methanol) was added. The solution was mixed and after 6 minute-standing 20 μl was injected into the HPLC. OPA was obtained from Sigma Chemica Co. St Louis USA, and TATG was purchased from Aldrich-Chemie Gmbh, Steinheim, Germany. The separation was performed with a Superspher 60 RP-8e column or with a Purospher RP-18e 125×4 column (MERCK, Darmstadt, Germany); the temperature of the oven was 40 °C. The organic solvents were gradient grade methanol and acetonitril (MERCK, Darmstadt, Germany). The pH of the 39 mM phosphate buffer was set to 7.0 with 6 M hydrochloric acid solution, and ultrapure water for the preparation of the buffer was obtained from a Millipore Direct-Q instrument (Millipore, Molstein, France). The derivatives were detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm). Derivatization and analysis were carried out with a MERCK-Hitachi HPLC containing the following modules: L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, AIA data conversion utility for D-7000 HPLC system manager (MERCK, Darmstadt, Germany).

Two sorts of amino acid standards were used for the HPLC method development. The first solution did not contain Trp. This solution consisted of 40 nmol/ml of the following protein constructed amino acids dissolved in water: D-Asp, L-Asp, D-Thr, L-Thr, D-Ser, L-Ser, D-Glu, L-Glu, D-Ala, L-Ala, D-Val, L-Val, D-Met, L-Met, L-D-, D-L-, L-L-, D-D-Ile, D-Leu, L-Leu, D-Tyr, L-Tyr, D-Phe, L-Phe, D-Lys, L-Lys, D-His, L-His, D-Arg, L-Arg and Gly. Secondary amino acids as Pro are not derivatized by OPA. Cys is deriva-

tized, but the fluorescence quantum yield is very low. The amide group of Asn and Gln hydrolyze during the hydrolysis of proteins and form Asp and Glu, therefore these four amino acids were not included into the standard solution. The second standard solution consisted of 40 nmol/ml L-Trp and 20 nmol/ml D-Trp. The D- and L-amino acids were purchased from Sigma-Aldrich, St Louis, USA).

Hydrolysis. In order to eliminate the reaction of MES-OH and TATG three sorts of trials were applied. The aim was to remove or destroy MES-OH. First 5 ml 0.5 M $CuSO_4$ solution was added to 5 ml mercaptoethanesulfonic acid solution (0.01 mmol D- and 0.01 mmol L-Trp and 4.5 mmol MES-OH) then the solution was centrifuged at 4000 g for 20 min. The pH of the supernatant was set between 5 and 6 with 4 M NaOH solution.

For the second time the mercaptoethanesulfonic acid solution (3 ml 3 M MES-OH and 0.01 mmol D- and 0.01 mmol L-Trp) was diluted three-fold, then 3 ml oxidising solution (the mixture of one part of 30 (w/v)% H_2O_2 and nine part of 85 (w/v) % formic acid) was added to 1 ml solution. The mixture was heated at 50 °C for 5 minutes. After cooling the remaining performic acid was reacted with 0.52 g sodium-metabisulfite. The same procedure was accomplished with D- and L-alanine (0.01 mmol of each).

For the third time 1 ml aliquots of mercaptoethanesulfonic acid solution (1 ml 3 M MES-OH solution and 0.01 mmol of D- and 0,01 mmol of L-Trp in 5 ml; 0.6 mmol MES-OH in each ml) was placed into a 25-ml-volumetric flask. The pH was set to 2, 6 or 9 with 4 M NaOH (a control sample also was prepared without pH setting) then 20 ml distilled water and 1 ml 0.1138 g /ml (0.612 mmol) iodoacetic acid solution was added and the volume was set with distilled water. Mercaptoethanesulfonic acid was obtained from Fluka Chemie Gmbh, Buchs, Switzerland.

In the case of *p*-toluenesulfonic acid hydrolysis 5 ml 3 M *p*-toluenesulfonic acid solution containing 0.2% 3-(2-aminoethyl)indole (tryptamine) was added to the sample containing 15 mg protein. The hydrolysis was carried out in a closed ampoule under nitrogen at 110 °C for 24 h. Lyophilized sheep hemoglobin samples were hydrolyzed with this method. The *p*-toluenesulfonic acid was obtained from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany. Neutralization was carried out with 4 M NaOH and the solution was diluted 125-fold with water.

Boiling. Free L-Trp-solutions (1 mg L-Trp /ml) with different pH values (pH = 3; 5; 7; 9; and 11) were prepared. The solutions were acidified with 6 M hydrochloric acid solution and the alkaline pH was set with 4 M NaOH solution. The ampoules with solutions were sealed after purging with nitrogen for 2 min. The L-Trp solutions were kept at 100 °C±1 °C for 0; 5; 10; 20; 40; 60 min and 2; 4, 8; 12; 24; 48 hours.

3 Results

The separation of the derivatives of L- and D-Trp. Though the hydrolysis of proteins is prior to the analysis of the amino acids, the studying of the hydrolysis methods cannot be achieved without an analytical method for the separation of the Trp enantiomers. To reach the aim, D- and L- Trp were reacted with OPA and TATG and the resulting diastereoisomers were separated on an achiral stationary phase column following fluorescence detection with high performance liquid chromatography. An amino acid standard solution that contained the amino acids that are present in the food proteins with the exception of Trp and another standard solution of D- and L-Trp was used for method development in order to avoid interferences.

Table 1: Eluent composition applied for the separation of OPA-TATG derivatives of L- and D-Trp from the derivatives of the other amino acids that occur in food proteins

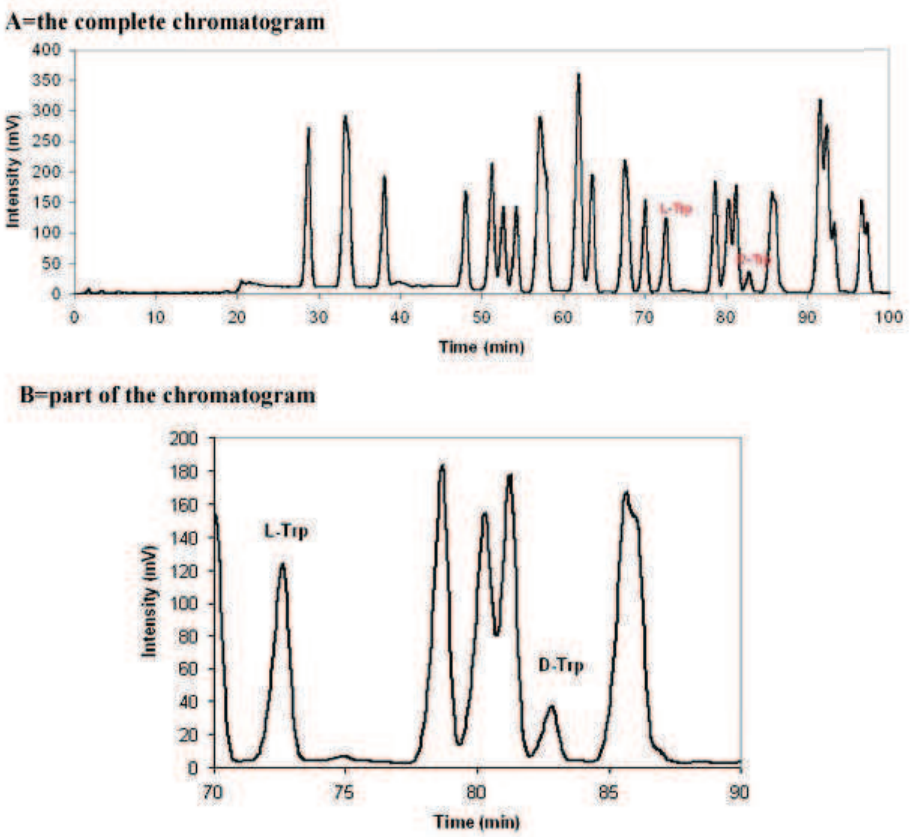
Time (min)	**Metanol (v/v%)**	**Phosphate buffer (39 mM, pH=7.0) (v/v%)**	**Acetonitril (v/v%)**
0	20	80	0
120	20	45	35
130	20	45	35
135	20	80	0
140	20	80	0

(Column: Purospher RP-18e; 125 mm × 4 mm; flow rate: 1 ml/min)

During the optimization of the separation the sort of the stationary phase; the type (methanol, acetonitril or both) and the ratio of the organic solvent was changed. The best separation (see *Figure 1* for chromatogram containing

both Trp and the other amino acids) was achieved on the RP-18 column and the composition of the mobile phase can be seen in *Table 1.*

Figure 1: Separation of the OPA-TATG derivatives of L- and D-Trp from the derivatives of the other amino acids that are occur in food proteins



(Column: Purospher RP-18e; 125 mm × 4 mm; flow rate: 1 ml/min)

There was a significant difference between the fluorescence factors of the enantiomers. With the same concentration increase the relative fluorescence of L-Trp was 2.4-fold higher than that of D-Trp. The data of calibration graphs

for the enantiomers can be seen in *Table 2.* The detector response was linear between 14 and 336 ng L-Trp / injection, and 14–700 ng D-Trp / injection. The limit of detection was 0.9 ng / injection for L- and 0.7 ng / injection for D-Trp. The limit of quantification for L- and D-Trp was 9.1 and 7.5 ng / injection, respectively. The relative standard deviation (RSD) in the low concentration range (14 ng L-Trp and 28 ng D-Trp / injection) were 1.1 and 4.1%; in the medium concentration range (140 ng L-Trp and 280 ng D-Trp / injection) were 6.8 and 1.9%; and in the high concentration range (276 ng L-Trp and 552 ng D-Trp / injection) were 1.6 and 2.3%, respectively. The number of replicates was 6.

Table 2: The calibration graph data of the OPA-TATG derivatives of the tryptophan enantiomers detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm)

Amino acid	Linear range nmol/ml	Correlation coefficient	Slope (A) (x,y see below)	Intercept (B)	LOQ nmol/ml
L-Trp	3 – 72	0.9998	12.6	−2.6	2.2
D-Trp	3 – 150	0.9996	5.3	−3.0	1.8

X = height of the peak (mV) / concentration (nmol/ml)
Y = concentration (nmol/ml)

Investigation of hydrolysis methods. Before the analyses of real samples different hydrolysis methods were tried out with standard Trp-solutions in order to examine if the following step the derivatization with OPA-TATG can be carried out.

Mercaptoethanesulfonic acid hydrolysis. In the presence of mercaptoethanesulfonic acid (MES-OH) D- and L-Trp did not form derivatives with OPA and TATG. Probably, instead of the bulky molecule of TATG, the MES-OH molecule reacts with OPA and the amino acid, and an achiral derivative of Trp forms. In the case of the other amino acids that occur in the protein MES-OH was applied for both hydrolysis and formation of achiral derivatives with OPA [5]. In the case of Trp OPA/MES-OH derivatives can also be formed and used for the determination of the amount of (L+D) Trp. When the knowledge

of the ratio of the Trp enantiomers is important, the building of MES-OH into the derivative should be eliminated. When hydrolysis is completed and the solution with free Trp is ready to derivatization, MES-OH should be converted into nonactive form with respect to the above-mentioned derivatization. To reach the aim three trials were conducted:

Elimination of MES-OH in the form of metal salt: The copper-mercaptide precipitate was separated from the suspensoid with centrifugation and the clear supernatant was used for derivatization.

Performic acid oxidation of MES-OH: The thiol group of MES-OH was converted into sulfonic acid group. The remaining amount of performic acid was reacted with sodium-metabisulfite.

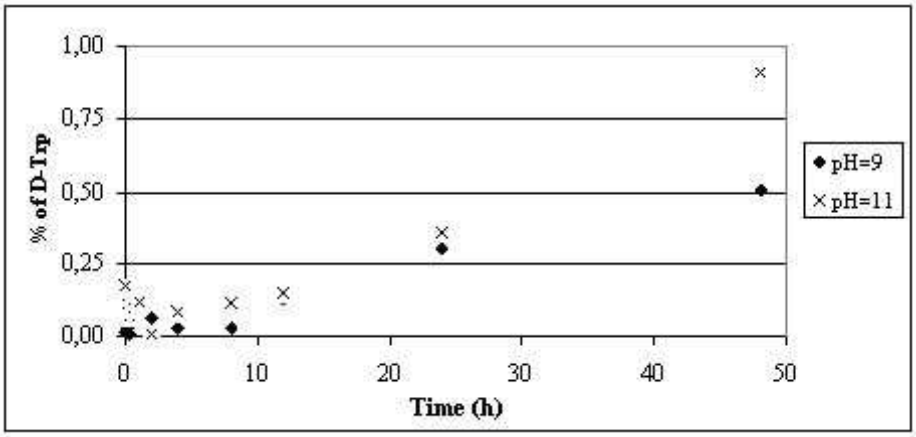
Reaction of MES-OH with iodoacetic acid: In order to block the thiol group, carboxyl-methyl derivative of MES-OH was formed.

In the first and the second cases we cannot detect OPA-TATG derivatives of D- and L-Trp. In the third trial there were some formation of the required derivatives, but the conversion rate of D-and L-Trp were poor. The amount of TATG was increased in order to provide enough TATG for derivatization, because TATG can also react with the remaining iodoacetic acid, but the level of conversion did not increase.

***p*-Toluenesulfonic acid hydrolysis.** First *p*-toluenesulfonic acid was added to standard solutions of L- and D-Trp and it can be ascertained that in the presence of *p*-toluenesulfonic acid the reaction of D- and L-Trp into OPA-TATG derivatives was completed. When samples are heat-treated during hydrolysis the addition of tryptamine is necessary [10] because this molecule is a protecting agent again oxidative conversion of Trp. In the presence of 0.2% tryptamine in the *p*-toluenesulfonic acid solution the derivatization of Trp is blocked due to the amine group of tryptamine. The recovery of *p*-toluenesulfonic acid hydrolysis without tryptamine was reported to be low [10]. The same tendency was observed when sheep hemoglobine was hydrolized without tryptamine and the recovery of L+D Trp was 44±2%. Despite of the low recovery the ratio of the L-and D-Trp can be established. The next step should be the use of another protecting agent such as 3-(3-indolyl)propionic acid that does not contain amine group but suitable for the protection of the indole ring of Trp from oxidative deterioration.

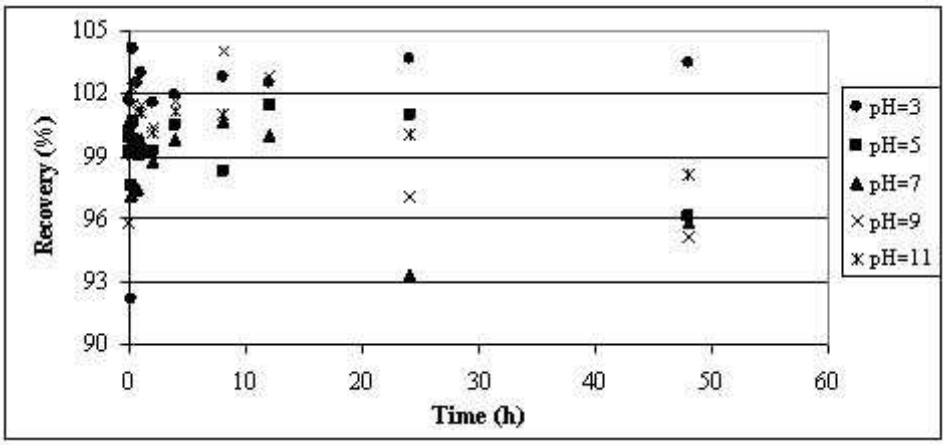
The influence of boiling on the Trp content in the function of pH and time. The loss of L-Trp due to racemization was not significant at lower pH values (pH = 3–7). The amount of the D-Trp increases at pH = 9 and pH = 11 after twelve hours of boiling *(Figure 2)*, but the rate of conversion (<1%) is lower than expected.

Figure 2: The increase of D-Trp content during boiling at 100 °C at pH = 9 and pH = 11 (% of Trp content)



After 24 h the amount of L-Trp tended to decline slightly *(Figure 3)*. When the boiling exceeds one day, the loss of Trp can be 2–5%. Beside racemization other reactions e.g. oxidative deterioration of Trp indole-ring can be responsible for the loss of L-Trp.

Figure 3: Change in the L-Trp content during heat treatment at 100 °C in aquatic solutions at different pH



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Investigation of performic acid oxidation in case of thiol-containing amino acid enantiomers

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Abstract. Performic acid oxidation of cysteine and methionine resulting in the formation of cysteic acid and methionine sulphon has been applied in order to avoid the loss of these two sulphur containing amino acids during the acidic hydrolysis of proteins that is necessary prior to amino acid analysis. The aim of the research was assigned by the increasing demand for the determination of the amount amino acid enantiomers: the applicability of performic acid oxidation was evaluated in this point of view. Racemization of L-cysteine and L-methionine was found not

Key words and phrases: performic acid oxidation, cysteine and methionine enantiomers, racemization

significant during oxidation with performic acid, therefore this process can be applied before hydrolysis during quantification of cysteine and methionine enantiomers. Additionally, the quantification of cysteic acid and methionine sulphon enantiomers was accomplished in the form of their diastereoisomer derivatives via the development of a reversed phase high performance liquid chromatography method.

1 Introduction

The determination of the amount of sulfur containing amino acids in foods and feeds involves some difficulties because under the generally used protein hydrolysis conditions (6 M hydrochloric acid solution, 110 °C, 24 hours) a part of these amino acids undergo oxidative deterioration [4]. In order to prevent these losses the thiol group of these amino acids was suggested to be converted into more stabile groups. With performic acid oxidation cysteine and methionine can form cysteic acid and methionine-sulphon [5] and the loss of these molecules during hydrolysis is negligible related to that of the initial amino acids. This method has been used for decades for the determination of sulfur containing amino acids and cysteic acid can be analyzed rapidly by an ionic exchange liquid chromatography system [1]. Nowadays there is an increasing demand for the determination of the amount of the L- and D-enantiomers of the amino acids. The question arises if this sort of analysis needed to be operated whether the extent of racemization during performic acid oxidation is negligible or not. The purpose of the research was to investigate whether performic acid oxidation can be used when the aim is to determine the amount of methionine and cysteine enantiomers, and besides an RP-HPLC method was developed in order to separate the derivatives of these oxidized amino acids. In a preliminary research the separation of cysteic acid enantiomers has been accomplished [6]. In the present work the aim was to extend the separation and the investigation of performic acid oxidation to the other sulfur containing amino acid, methionine in order to determine the amount of methionine and cysteine in one single analysis.

2 Material and methods

Oxidation with Performic Acid. A sample of cysteine and that of methionine (approx. 0.1 mM) was weighed into a vial. Five cm^3 performic acid, produced based on the method of Hirs [3] was added and the mixture was heated at 50 °C for 15 minutes then it was cooled down immediately and lyophilized

at −5 °C. If the sample contains only free amino acids the dried sample is washed with water into a 50 cm^3 volumetric flask. The pH was adjusted to 7 with 4 M sodium hydroxide, and the solution was ready for analysis.

Hydrolysis. For protein containing samples the oxidized and lyophilized sample was dissolved in hydrochloric acid (6 M; 5 cm^3) and hydrolyzed at 110 °C for 24 h. After cooling the solution was neutralized (pH = 7) with sodium hydroxide solution (4 M).

Derivatization and Analysis. Diastereoisomers were produced with OPA (*o*-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) by the method of Einarsson and co-workers [2]. OPA and TATG were obtained from Sigma (St. Louis, MO, USA). The compounds were separated on a 125 mm × 4 mm i.d. column packed with LiChrospher 100 RP-18. At the beginning of the experiments, the mobile phase consisted of 5% (v/v) tetrahydrofuran and 95% phosphate buffer (39 mM, pH = 7.05), as in the case of the separation of OPA-TATG derivatives of cysteic acid enantiomers [6]. The temperature of the oven was 40 °C. The derivatives were detected with a fluorescence detector (λ_{ex} 325 nm, λ_{em} 420 nm). Derivatization and analysis were carried out with a MERCK-Hitachi HPLC comprising L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager. Reagents were pro analysis grade. Solvents (tetrahydrofuran and water) were HPLC gradient grade and purchased from MERCK (Darmstadt, Germany).

3 Results

Separation of the enantiomers of sulfur containing amino acids. The aim of the method development was to achieve an acceptable resolution within a reasonable range of retention factor k ($1<k<10$). The adequacy of resolution was the most important point of view of the method development because the amount of the D-enantiomer can be less with two orders of magnitude than the amount of L-enantiomer in foods and feeds. When the separation method of the OPA-TATG derivatives of D- and L-cysteic acid was developed, the type of organic solvent used, the organic solvent/buffer ratio and the stationary phase of the column were optimalized among analytical conditions. In order to separate the OPA-TATG derivatives of D- and L-methionine-sulphon the strength of the mobile phase had to be changed and some part of the resolu-

tion of the first diastereoisomer pair had to be sacrificed to elute the second diastereoisomer pair in time. Increasing the initial tetrahydrofuran volume ratio with only two percent (that means 7% tetrahydrofuran, 93% phosphate buffer) halved the retention of cysteic acid derivatives while the resolution also dropped significantly, from 2.1 to 1.4. But this value is still acceptable in case of diastereoisomer pairs. Separation of cysteic acid and methionine-sulphon derivatives in one analysis cannot be achieved using isocratic condition in this system thus a gradient program was developed. After an initial period when the cysteic acid derivatives were to be separated (0-14 minutes) the ratio of tetrahydrofuran was increased in the eluent. The changes of the resolution and the retention time of the methionine-sulphon derivatives in the function of the tetrahydrofuran-phosphate buffer composition of the mobile phase from the 20^{th} minutes of analysis can be seen in *Table 1.*

Table 1: The influence of eluent composition on the resolution and retention time of OPA-TATG derivatives of methionine sulphon

Eluent composition (% v/v)		**Retention time (min) OPA-TATG derivative of**		**Resolution**
Tetrahydro-furan	**Phosphate buffer (39 mM, pH = 7.05)**	**L-methionine sulphon**	**D-methionine sulphon**	
20	80	29.4	29.7	0.87
19	81	29.6	30.1	1.02
18	82	29.9	30.5	1.09
16	84	31.2	32.4	1.31

Fine-tuning of the tetrahydrofuran volume ratio from 20% (v/v) to 16% from the 20 min resulted in a retention increase of L- and D-methionine-sulphon to some extent but the resolution of these two peaks improved considerably (from 0.87 to 1.31).

The final mobile-phase gradient is given in *Table 2.*

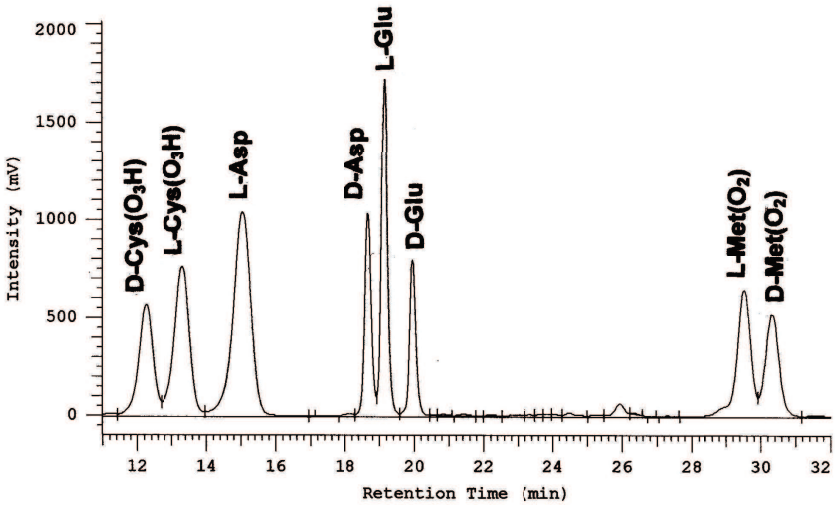
Table 2: The mobile phase gradient for the separation of sulphur containing amino acids

Time (min)	Gradient composition (v/v%)	
	Phosphate buffer (39 mM. pH = 7.05)	Tetrahydro-furan
0	93	7
13	93	7
14.5	85	15
20	84	16
31	84	16
35	60	40
45	60	40
47	93	7
50	93	7

The flow rate was 1 cm^3/min.

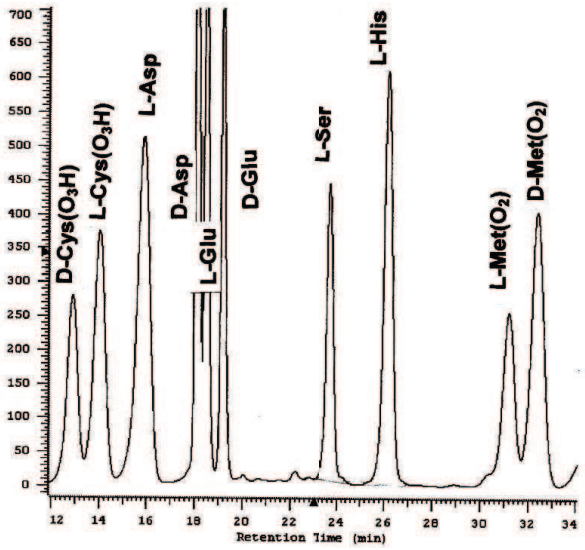
With the use of the above gradient program the OPA-TATG derivatives of acidic amino acids can also be separated besides that of the sulfur containing amino acids *(Figure 1)*.

Figure 1: Separation of OPA-TATG derivatives of cysteic acid, aspartic acid, glutamic acid and methionine-sulphon enantiomers with RP-HPLC.



Based on the results of the preliminary research L-serine and L-hystidine derivatives were considered to elute in the same time period as the above amino acid derivatives therefore the possible interference was investigated. Derivatives of L-serine and L-hystidine were separated from the derivatives of the sulfur containing amino acids and separation was also acceptable for the derivatives of the acidic amino acids as *Figure 2* shows. The detection limit for methionine-sulphon was 0.61 nmol/injection. The detector response was linear between 5.5 and 250 nmol/injection. At 50 nmol methionine-sulphon/injection the RSD (n = 3) was calculated to be 8.5%.

Figure 2: Separation of OPA-TATG derivatives of cysteic acid, aspartic acid, glutamic acid, and methionine-sulphon enantiomers, L-serine and L-histidine with PR-HPLC.



Investigation of Performic Acid Oxidation. In case of cystein no significant racemization was observed during performic acid oxidation. For the other sulphur containing amino acid L-methionine standard of high optical purity was used to detect whether racemization occurred during oxidation. Solutions of L-metionine were oxidized like samples, and the quantity of D-

and L-methionine-sulphon was measured. The D/(D+L)×100 ratio, corrected with the fluorescence factors of the corresponding OPA-TATG derivatives, proved to be less than 10^{-4}. This ratio is not significant when it is compared to the D/(D+L)×100 ratios occurs in food analysis, therefore it can be concluded that the extent of racemization of methionine during oxidation with performic acid is negligible.

To study the rate of conversion, that is the extent of the other losses during performic acid oxidation of the amino acid, L-methionine in standard solutions were oxidized and analyzed. The quantity of the product was determined by use of calibration curves of methionine-sulphon standard solutions. The rate of conversion from methionine to methionine-sulphon seemed to be higher than that of cysteine to cysteic acid (96±3% and 71±3% (n = 3) respectively). Certainly the determination of the recovery needs to be accomplished separately in case of each substance under study.

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Quantitative determination of the protein of bacterial origin based on D-amino acid contents

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Abstract. In recent years, several methods have been developed for determination of the proportion which is of microbial origin of the nitrogen-containing substances passed from the rumen into the abomasum or small intestine. These methods are based on analysis of components which were believed could only be of microbial origin, or which were known to be very closely related to protein of microbial origin. Assays have also been developed to use nucleic acids and adenosine triphosphate as indicators. Radioisotopes ^{35}S, ^{15}N, ^{32}P and ^{33}P have been incorporated into bacterial protein and phospholipids to estimate bacterial protein content. Duodenal amino acid composition, amino-ethylphosphonic acid (AEP), diaminopimelic acid (DAPA) and D-alanine (D-Ala) contents of the duodenum have been investigated as indicators.

Key words and phrases: D-aspartic acid, D-Asp, D-glutamic acid, D-Glu, diaminopimelic acid, DAPA, bacterial protein synthesis, protein of bacterial origin

Recently, the D-amino acid content of foodstuffs, particularly milk and milk products, has been determined and it has been observed that, in addition to D-alanine (D-Ala), D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) were also detected in products which are associated with bacterial activity. This suggested the possibility of using D-Glu and D-Asp content of bacteria extracted from the rumen of cattle and of chyme from the same cattle to estimate protein of bacterial origin. The efficiency of these indicators would need to be compared with that of an established indicator such as 2,6-diaminopimelic acid (DAPA).

DAPA, D-Asp and D-Glu content of duodenal chyme from five growing bulls and of ruminal bacteria from the same bulls was determined by means of amino acid analyser and high performance liquid chromatography. Based on linear regression, coefficients of correlation among these indicators were 0.778 and 0.703 respectively for DAPA and D-Asp and for DAPA and D-Glu in chyme. Corresponding values in ruminal bacteria were 0.758 and 0.808. The r values between the crude protein content of ruminal bacteria and the markers were: DAPA, 0.737; D-Asp, 0.725; D-Glu, 0.614. In the model experiment performed to estimate percentage of protein derived from bacteria the values determined on the basis of D-Asp and D-Glu content were approximately 8% lower than the value based on DAPA. It is recommended that, in addition to DAPA, these two amino acids be considered to be markers for estimating bacterial protein.

1 Introduction

A common characteristic of new protein evaluation systems introduced in cattle nutrition in the past decade is that protein content of diets is evaluated on the basis of quantity of amino acids absorbed in the small intestine. In addition to endogenous amino acids, which are only a small proportion, there are two substantial sources of absorbed amino acids. These are microbial protein synthesised in the rumen and dietary protein not broken down in the rumen (by-pass protein). Approximately 70% of the protein content of the diet is broken down into amino acids in the rumen, which may be utilised for synthesis of amino acids or microbial proteins, or broken down still further to provide ammonia for the construction of microbial body proteins. Ammonia not utilised for synthesis of bacterial protein is absorbed from the rumen and converted into urea via the ornithine cycle in the liver. The quantity of microbial protein produced in the rumen must be established if we are to determine the proportion of by-pass protein in the diet.

In order to define the quantity of microbial protein synthesised in the rumen, it is essential that microbial protein in duodenal chyme be separable from the

dietary by-pass protein and protein of endogenous origin. This is possible only if components characteristic solely of microbial protein can be identified in the total protein.

Recently several methods have been developed for determination of the proportion which is of microbial origin of nitrogen-containing substances passed from the rumen into the abomasum. Attempts to estimate the proportion of microbial origin nitrogen-containing substances have been based on nucleic acid, vitamin B_{12} and sulphur 35 isotope. A critical evaluation and summary of these methods was given by Stern and Hoover [19].

Czerkawski [2] was successful in estimating protozoan nitrogen by means of the measurement of 2-amino-ethylphosphonic acid (AEP) and estimating nitrogen content of bacterial origin by measuring 2,6-diaminopimelic acid (DAPA). The AEP was found to occur almost exclusively in protozoa, while DAPA occurs exclusively in peptidoglycans in the bacterial cell wall. Despite the fact that the quantity of DAPA in the cell wall is strongly dependent on the species of bacterium, the ratio of DAPA to total bacterial protein does not vary under constant dietary conditions. Therefore, in comparative experiments, DAPA can be used effectively to estimate the proportion of protein of bacterial origin found in the contents of the intestine.

Schleifer and Kandler [17] discovered that, like DAPA, D-alanine (D-Ala) also occurs only in peptidoglycans in the bacterial cell wall. This compound was also suitable for use as a marker of proteins of bacterial origin and their quantitative determination. Garrett et al. [12] succeeded in determining nitrogen of bacterial origin by measuring the quantity of D-Ala in ruminal fluid. Garrett et al. [13] performed comparative experiments using DAPA and D-Ala to investigate the precision of estimating nitrogen of bacterial origin. They established that D-Ala was a better indicator of nitrogen of bacterial origin, since the coefficient of variation resulting from the data based on D-Ala was substantially lower than that for data based on DAPA. In addition, greater accuracy was achieved in determinations based on D-Ala than in those based on DAPA. In a series of experiments the authors [7] also established that both DAPA and D-Ala are suitable for use in the estimation of the quantity of protein of bacterial origin. No difference was observed between the two substances with respect to error either in analytical method (ion exchange column chromatography for DAPA and high performance liquid chromatography for D-Ala) or in determination of protein of bacterial origin.

Edols [10] determined DAPA in a hydrolysate from the ruminal fluid by the application of a two-column method using an automated amino acid analyser. By the optimization of the composition of buffers DAPA appeared between

methionine and isoleucine in the chromatogram, well separated from these two amino acids, in the form of a sharp, easily quantifiable peak. Csapó et al. [6]. oxidized samples with performic acid prior to protein hydrolysis, as a consequence of which – the disturbance effects of neighbouring amino acids having been eliminated – DAPA, even if present only in trace quantities, could be determined accurately. Subsequently to this, exploiting the similar representation on chromatograms of methionine and DAPA, the authors developed a fast method for the determination of DAPA alone by means of ion exchange column chromatography [4].

Analysis of data in the literature suggests that solutions have been found with respect to analytical methods for DAPA, allowing even trace quantities of the compound to be determined. The investigation of D-amino acid content of foodstuffs, particularly milk and milk products [3, 8], revealed that, in addition to D-Ala, D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) could be detected in similar quantities. This was primarily true for products which were produced through bacterial activity. These findings suggested that D-Asp and D-Glu content of ruminal bacteria and duodenal chyme could provide additional markers for estimating the percentage of protein derived from bacteria.

2 Material and method

2.1 Methodology for the animal experiment.

Chyme and ruminal bacteria samples were taken from two experiments using five growing bulls (crossbred Hungarian Simmental × Holstein Friesian) weighing 480–500 kg. Each was fitted with ruminal and duodenal fistulae. The main objectives of these experiments were to determine ruminal protein degradability of various diets, and to establish the effect of various types of feed additives on ruminal degradability of proteins. After a ten-day adaptation period duodenal chyme samples were taken through the duodenal fistula and ruminal fluid was taken through the ruminal fistula on several occasions during the same experimental period; the average chyme and ruminal fluid samples were analyzed for DAPA, D-Asp and D-Glu.

2.2 Preparation of samples for chemical analysis.

Ruminal fluid was centrifuged at 3000 g to separate the feed particles from infusorians. The bacterial mass was then separated by centrifugation of the

fluid phase at 10 000 g. The bacterial mass was dried by lyophilization. Aliquot parts of the chyme samples taken from the duodenum were also lyophilised.

2.3 Chemical analysis of samples.

DAPA content was determined, by means of the procedure developed by Csapó et al. [9], using Aminochrom-II (Labor MIM, Hungary) type amino acid analyser. Protein was oxidized with performic acid followed by 24 hour hydrolysis with 6 M hydrochloric acid containing 0.1% phenol.

Prior to determination of D-Asp and D-Glu, the protein was hydrolysed with 6 M hydrochloric acid for 30 minutes at 170 °C in order to minimize the degree of racemization [5]. Separation and determination of the enantiomers was performed by means of high performance liquid chromatography in accordance with the method described by Einarsson et al. [11]. Derivatization and analysis were carried out with a MERCK-Hitachi (Darmstadt, Germany) HPLC containing the following modules: L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, AIA data conversion utility for D-7000 HPLC system manager.

For derivative formation, *o*-phthaldialdehyde (OPA) and 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG) were purchased from Sigma (St. Louis, Mo). Enantiomer separation was performed on a reversed phase (250 $\times$ 4.6 mm internal diameter (i.d.), 5 μm particle size, Kromasil octyl (C-8)) analytical column. In order to extend the life of the column a guard column (RP-8, Newguard, 25 $\times$ 3.2 mm i.d., 7 μm particle size, Brownlee) was connected between the injector and the analytical column and a cleaning column (C-18, 36 x 4.5 mm i.d., 20 μm particle size, Rsil) was fitted between the pump and the injector. A gradient system consisting of two components was used for enantiomer separation, the composition of this system being the following: A = in 40% methanol phosphate buffer (9.5 mM, pH = 7.05); B = acetonitrile. The rate of flow used was 1 ml per minute. The derivatives were detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm).

Since, in this series of experiments, only the quantities of D-Asp and D-Glu were of interest to the authors, determination was restricted to the enantiomers of these two amino acids. By this method D-Asp and D-Glu present in very small quantities can be detected and determined alongside L-amino acid present in large quantities.

3 Results

Table 1 presents the associations between DAPA and D-Asp, DAPA and D-Glu and D-Asp and D-Glu in both chyme and ruminal bacteria samples. Analysis of the data established that there were very high correlations between DAPA and D-Asp and between DAPA and D-Glu content. In both bacterial protein and chyme the value observed between DAPA and D-Glu content of the chyme was the lowest, at 0.703, while the same correlation in ruminal bacteria was the highest, at 0.808. The highest correlation was that between D-Asp and D-Glu for chyme (r = 0.949) and for ruminal bacteria (r = 0.843).

Table 1: Linear regression parameters and correlations among DAPA, D-Asp, and D-Glu contents of chyme and ruminal bacteria[a]

Parameter	Chyme			Ruminal bacteria		
statistical characteristic	DAPA-D-Asp mg/kg	DAPA-D-Glu mg/kg	D-Asp-D-Glu mg/kg	DAPA-D-Asp mg/kg	DAPA-D-Glu mg/kg	D-Asp-D-Glu mg/kg
A	0.313	0.440	-0.168	1.121	2.686	2.346
sd	0.089	0.142	0.074	0.560	0.436	0.437
B	0.655	0.830	1.331	0.840	0.771	0.729
sd	0.094	0.148	0.078	0.186	0.145	0.120
S.D.	0.118	0.188	0.083	0.238	0.185	0.169
n	34	34	34	17	17	17
r_{xy}	**0.78**	**0.70**	**0.95**	**0.76**	**0.81**	**0.84**
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]Derived from regression equation Y=A+B×X, where Y is dependent and X is independent variable. In each column, first variable listed is Y and second is X.
A = The intercept of the value of Y when X = 0.
B = The slope of the line of the units change in Y per one unit change in X.
sd = Standard deviation of A and B.
S.D. = Standard deviation
n = number of observations
r_{xy} = correlation coefficient
P = Probability

Figure 1 illustrates the relationships of D-Glu and D-Asp to DAPA content of ruminal bacteria, and *Figure 2* shows the same relationships for chyme. *Figure 3* demonstrates regression of D-Glu on D-Asp content of the chyme.

Figure 1: Linear regressions of D-Asp and D-Glu on DAPA in ruminal bacteria

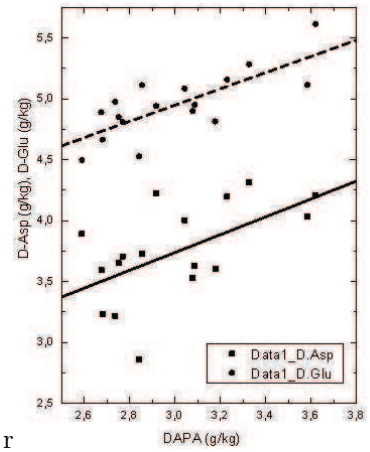

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Figure 2: Linear regressions of D-Asp and D-Glu on DAPA in chymus

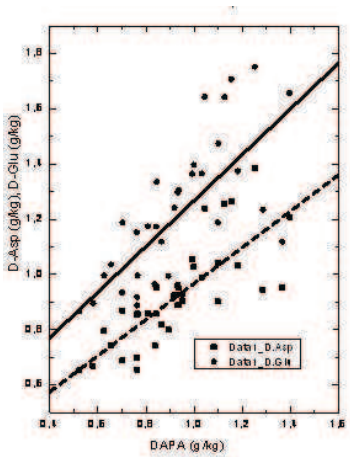


Figure 3: Linear regressions of D-Glu on D-Asp in chyme

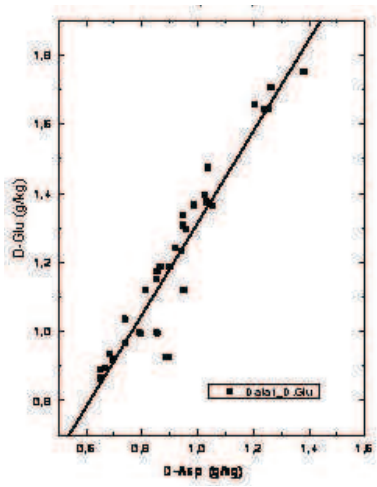


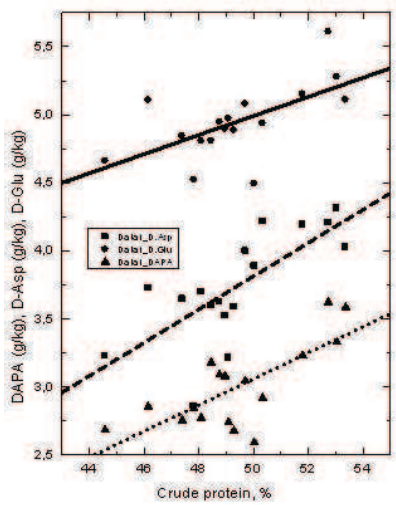
Table 2: Linear regression parameters for crude protein regressed on DAPA, D-Asp and D-Glu for ruminal bacteria and chyme[a]

	Crude protein %					
Parameter	**Chyme**			**Ruminal bacteria**		
statistical characteristic	**D-Asp mg/kg**	**D-Glu mg/kg**	**D-Glu mg/kg**	**D-Asp mg/kg**	**D-Glu mg/kg**	**D-Glu mg/kg**
A	−0.698	−1.746	1.485	1.073	1.267	1.309
sd	1.704	1.125	1.152	0.322	0.379	0.453
B	0.088	0.096	0.070	−0.006	−0.014	−0.004
sd	0.035	0.023	0.023	0.013	0.015	0.018
S.D.	0.329	0.217	0.222	0.187	0.221	0.263
n	17	17	17	34	34	34
r_{xy}	**0.73**	**0.74**	**0.61**	**−0.08**	**−0.16**	**−0.04**
P	<0.01	<0.001	<0.01	0.644	0.381	0.832

[a]Derived from regression equation Y=A+B×X. where Y is dependent and X is independent variable. In each column, first variable listed is Y and second is X.
Abbreviations: Identical as at *Table 1.*

Table 2 shows relationships between crude protein content of the ruminal bacteria and chyme and D-Asp, DAPA and D-Glu for the same samples. *Figure 4* displays the relationships of DAPA, D-Asp and D-Glu with crude protein content of the ruminal bacteria.

Figure 4: Linear regression of DAPA, D-Asp and D-Glu content on crude protein content of ruminal bacteria



It was established that, for ruminal bacteria, the closest correlation was between DAPA and crude protein content ($r = 0.737$); the r value between D-Asp and crude protein content was only very slightly lower ($r = 0.725$), while the relationship between D-Glu and crude protein content was somewhat lower ($r = 0.614$). However, the same analysis of data on chyme samples did not produce a close correlation between the markers and crude protein content. In all three examinations, linear regression analysis demonstrated a very weak negative correlation (r values varying between -0.038 and -0.155). This lack of correlation could be explained by the fact that only part of the protein present in the chyme is derived from bacteria, the rest being comprised of dietary proteins which do not undergo bacterial degradation in the rumen, plus small amounts of endogenous protein. Ruminal degradability of dietary proteins is on average 70%. However, there is a very wide range of reported values. Results from experiments performed under *in vivo* and *in vitro* con-

ditions have demonstrated very great differences among individual proteins with respect to ruminal degradability [15]. There are feedstuffs whose protein content undergoes almost total degradation in the rumen, while the ruminal degradability of other dietary proteins is only 15–20%. The great deviations are attributed to protein structure, primarily with the number of disulphide bonds present [16]. The quantity and ratio of protein fractions influence ruminal degradability [17] along with protein amino acid composition [18] and the chemical and heat treatment of protein [19, 20]. This implies that variations in the proportions of microbial protein in the chyme and non-degradable dietary protein in the rumen will be dependent on the dietary proteins. In the experiment from which the chyme and ruminal bacteria samples were taken the animals were fed diets of different ruminal degradability, which resulted in different ratios of microbial protein to by-pass dietary protein in the chyme for individual experimental periods. In the opinion of the authors, this is the fundamental reason for low associations of protein in the chyme with levels of DAPA and the two D-amino acids.

In the next step the average crude protein content and the average DAPA, D-Asp and D-Glu content were calculated from the analysis data for 17 ruminal bacteria produced by the method described in the section Material and method. The composition of the lyophilized ruminal bacteria (100% dry matter) was: 49.50% crude protein, 0.300% DAPA, 0.366% D-Asp and 0.494% D-Glu. By means of these results and with the use of crude protein content of the lyophilised bacteria, the DAPA, D-Asp and D-Glu content of the bacterial protein was calculated. The protein of the ruminal bacteria contained on average 0.606% DAPA, 0.739% D-Asp and 0.998% D-Glu. No possibility exists to compare values for D-Asp and D-Glu with literature values because, as far as the authors are aware, no other researchers have investigated these components of bacterial protein. The value of 0.606% obtained for DAPA is lower than the value of 1.0 $\pm$ 0.25% reported for bacterial protein by Orskov [21]. Estimates in the literature vary from 0.6 to 1.4%, and may be influenced by differences in the quality of diets fed to experimental livestock. Since the objective of these investigations was to evaluate new markers for bacteria, it was necessary to establish that the deviation of DAPA content from data in the literature did not influence the results obtained for D-Asp and D-Glu in this study.

After analysis of ruminal bacteria for DAPA, D-Asp, D-Glu and crude protein content, multiplying factors were calculated to enable the proportion of protein of bacterial origin in an unknown sample to be estimated on the basis of DAPA, D-Asp and D-Glu content. The multiplying factor used for DAPA was

100/0.606 = 165; for D-Asp, 100/0.739 = 135; and for D-Glu, 100/0.998 = 100. By the application of the multiplying factors the quantity of the protein of bacterial origin can be calculated very easily. In order to establish the applicability in practice of the multiplying factors calculated in this study, two experiments were performed. In the first, these multiplying factors were applied to various chyme samples. The results obtained are presented in *Table 3.* It can be seen that values estimated on the basis of DAPA content (the average of the 10 estimates being 12.774%) were on average 8% higher than the microbial protein quantities based on D-Glu (11.705%) or D-Asp (11.800%) content. The explanation for this may be that the DAPA content of ruminal bacteria was measured at a slightly lower value when the multiplying factors were calculated. When protein content determined on the basis of D-Glu was compared with that based on D-Asp, the level of concordance was obvious, and in most cases, the values obtained concurred almost exactly.

Table 3: Examples of the application of multiplying factors in the determination of protein of bacterial origin content in chyme samples

Chyme sample	Analysis results*			Protein of bacterial origin, % calculated on the basis of		
	D-Asp %	DAPA %	D-Glu %	D-Asp	DAPA	D-Glu
1.	0.08546	0.07630	0.11513	11.54	12.59	11.67
2.	0.06681	0.05814	0.08935	9.03	9.59	9.06
3.	0.12546	0.11276	0.16415	16.96	18.61	16.64
4.	0.07402	0.06560	0.10342	10.01	10.82	10.49
5.	0.06519	0.05249	0.08644	8.81	8.66	8.77
6.	0.10546	0.09933	0.13637	14.26	16.38	13.83
7.	0.08565	0.07666	0.09952	11.58	12.64	10.09
8.	0.08671	0.07041	0.11865	11.72	11.61	12.03
9.	0.08591	0.08090	0.11730	11.61	13.34	11.89
10.	0.09230	0.08189	0.12402	12.48	13.50	12.58
			Mean	11.800	12.774	11.705

*Different chyme samples were analysed for D-Asp, DAPA and D-Glu, and the protein of bacterial origin was calculated by applying multiplying factors.

After the multiplying factors were tested for chyme samples, a mean sample

was produced from 17 lyophilized ruminal bacteria samples, and the multiplying factors were also tested for ruminal bacteria. The crude protein content of this mean sample was 49.5%, its DAPA content was 0.325%, its D-Asp content was 0.364% and its D-Glu content was 0.492%. By application of the multiplying factors crude protein content values of 53.62% (0.325% × 165), 49.14% (0.364% × 135) and 49.20% (0.492 × 100) respectively were estimated.

Subsequently, the authors lyophilized beef to produce a meat meal of zero DAPA content. The D-Asp and D-Glu content was below 0.01% after hydrolysis of its protein. Racemization testing was performed during protein hydrolysis. A mixture of 1 g meat meal and 1 g bacterial sample was analyzed for DAPA, D-Asp and D-Glu content using 5 parallel analyses. This was then repeated using a mixture of 9 g meat meal and 1 g bacterial sample. The results are presented in *Table 4*. From the data given in *Table 4* it can be established that the S.D.% for the first sample, containing a higher quantity of bacterial protein, are in each case below 5; thus, the standard deviation of the results meets the requirements for a reliable analytical method.

The second sample contained only 20% of the quantity of bacterial protein contained by the first sample. S.D.% for D-Asp and D-Glu was below 5, but that calculated for DAPA slightly exceeded 5. When compared with the values calculated for bacterial origin protein content (24.76% and 4.95%), the only values to deviate significantly ($P<0.05$) were three individual values and the two mean values based on DAPA content *(Table 4).* DAPA estimates (26.58% and 5.54% protein of bacterial origin) averaged 9.5 and 13.5% respectively, higher than those for D-Asp (24.63 and 4.89%) and D-Glu (24.82 and 4.87%). DAPA estimates averaged 7.3 and 11.9%, higher than the percentages calculated (24.76 and 4.95%) for samples one and two.

Table 4: Model experiment to investigate the accuracy of determination of protein of bacterial origin

1:1 mixture of meat meal and bacterium with 24.76% calculated protein of bacterial origin						
Parallel analysis	**Analysis results**			**Protein of bacterial origin, % calculated on the basis of**		
	D-Asp %	**DAPA %**	**D-Glu %**	**D-Asp**	**DAPA**	**D-Glu**
1.	0.182	0.162	0.247	24.60	26.71	25.05
2.	0.181	0.159	0.239	24.47	26.22	24.24
3.	0.179	0.155	0.251	24.20	25.56	25.45
4.	0.184	0.164	0.241	24.87	27.04*	24.44
5.	0.185	0.166	0.246	25.01	27.37	24.94
Mean	0.1822	0.1612	0.2448	24.63	26.58	24.82
SE $\overline{X}$				0.144	0.318	0.217

9:1 mixture of meat meal and bacterium with 4,952% calculated protein of bacterial origin						
Parallel analysis	**Analysis results**			**Protein of bacterial origin, % calculated on the basis of**		
	D-Asp %	**DAPA %**	**D-Glu %**	**D-Asp**	**DAPA**	**D-Glu**
1.	0.037	0.037	0.047	5.001	6.101*	4.766
2.	0.035	0.035	0.049	4.731	5.772	4.969
3.	0.038	0.032	0.051	5.136	5.277	5.171
4.	0.034	0.031	0.046	4.596	5.112	4.664
5.	0.037	0.033	0.047	5.001	5.442	4.766
Mean	0.0362	0.0336	0.0480	4.893	5.541*	4.867
SE $\overline{X}$				0.099	0.118	0.091

SE = Standard error of mean.
*Differs from calculated protein of bacterial origin ($P<0.05$).

4 Conclusions

These investigations have provided evidence that both D-Asp and D-Glu may be used to estimate percentage of protein of bacterial origin. The results obtained using these two markers proved to be approximately 8% lower than those obtained using DAPA. This deviation was not attributable to error associated with the new markers, but rather to unreliability of determination using DAPA. The analyses performed on samples of known bacterial protein content indicate that D-Asp and D-Glu produced practically identical values for bacterial protein content, which were very close to the theoretical (calculated) values.

The preparation process for DAPA analysis is lengthy due to the treatment with performic acid required. However, without performic acid treatment, the determination of DAPA present in small quantities is unreliable, due to interference of other amino acids in concentrations which may, in some cases, be higher by several orders of magnitude. In addition to the large amount of work required for the process, determination using DAPA also requires more time and more chemical agents, and is therefore considerably more expensive. An amino acid analyser operating on the principle of ion exchange column chromatography is suitable for analysis with DAPA, but separation of the D-amino acids cannot be performed with an amino acid analyser operating on the traditional principle.

Determination using D-Asp and D-Glu can be carried out with precision by means of high performance liquid chromatography. By the application of the fast procedure developed by the authors, determination of the quantity of D-Asp and D-Glu can be performed in 25% the amount of time required for ion exchange column chromatography analysis used for DAPA.

When determining D-amino acids, racemization may occur during protein hydrolysis, which can falsify the results obtained. Due to racemization, quantities of D-Asp and D-Glu greater than those actually present may be measured and lead to overestimation of the amount of protein of bacterial origin present. Two methods are recommended for the elimination of this source of error. One is the application of a hydrolysis procedure which allows only a very low degree of racemization to take place (for example, the protein hydrolysis method developed by the authors, performed at high temperature and for a short period, i.e. 160–170 °C for 30–45 minutes; [5]. The other method is to determine, by means of the method to be applied, the D-Asp and D-Glu content of bacteria obtained from the rumen, and developing multiplying factors for use in estimating the quantity of protein of bacterial origin (this being our new method).

In the latter case, racemization occurring during protein hydrolysis is regarded as a constant error, in both calculation of multiplying factors and analysis of real samples. As a result, it exerts no substantial influence on the accuracy of the determination procedure. Thus, the use of D-Asp or D-Glu is associated with minimum error if a protein hydrolysis procedure involving a low degree of racemization is used, and if multiplying factors are determined and applied.

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Rapid method for the determination of diaminopimelic acid using ion exchange chromatography

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Abstract. A new method for the determination diaminopimelic acid (DAPA) from rumen fluid was developed. The concentration of DAPA is used as an indicator for the estimation of protein content of bacterial origin. Due to the performic acid oxidation preceding hydrolysis of proteins, the neighbouring amino acids do not interfere in the determination of DAPA. As a result, even trace concentrations of DAPA may be accurately determined. Since, following the performic acid oxidation the sample does not contain methionine, the buffers developed for rapid determination of methionine may be used to advantage. As a result of this, DAPA may be determined by ion exchange column chromatography in ca. 18 minutes.

Key words and phrases: diaminopimelic acid, DAPA, fast method, oxidation with performic acid, bacterial protein

Following the development of the analytical method, it was applied to the determination of DAPA in the rumen fluid and the bacterial proteins prepared from the rumen fluid of cattle, goats and sheep. Based on the results, a method for evaluation of protein content of bacterial origin, based on the DAPA content, was derived.

1 Intoduction

In the case of ruminants, a large proportion of the protein content of feeds decomposes in the rumen. Microorganisms of the rumen use the ammonia derived from decomposition to build up new proteins. As a consequence, a sizeable proportion of the protein content of feeds is converted into microbial protein. This conversion may be advantageous if starting from low grade proteins or from NPN materials, because the synthesised bacterial proteins have higher biological value. In contrast to this, in many cases the decomposition of feed proteins of high biological value in the rumen is undesirable. In the future it will become more important to be able to ascertain the proportion of the feed proteins which is decomposed in the rumen. It will also be desirable to estimate the proportion of proteins arriving into the duodenum which originate from the feed and from bacteria of the rumen.

In recent years, several methods were developed for estimation of the proportion of microbial origin nitrogenous materials which enters the small intestine. The determination of nucleic acids, and the tracing of B_{12} and S^{35} isotopes have been proposed as methods for evaluation of the proportion of nitrogenous compounds of microbial origin. Stern and Hoower [12] compiled a critical review of these methods. Schleifer and Kandler [11] have shown that DAPA and D-alanine may be found only in the peptidoglycan of the cell walls.

Both of the above mentioned compounds proved to be adequate for the marking and quantitative determination of the protein of bacterial origin. Czerkawski [2] estimated the proportion of proteins of bacterial origin by measuring the DAPA content of protein and the determination of 2-amino-ethylphosphonic acid was used for the calculation of protozoa nitrogen. Garrett et al. [4] reported that D-alanine was a better indicator of bacterial-origin protein than was DAPA. They attributed this difference in accuracy to the fact that the DAPA position in the peptidoglycan of the cell wall may often be occupied by lysine or ornithine [11]. Garrett et al. [5] also reported that prior to analysis of DAPA, it was necessary to pretreat samples with performic acid to convert methionine to methionine sulfone. Ibrahim et al. [9] reported that the peaks for both DAPA and methionine occurred at 134 minutes while

the peak for methionine sulfone occurred at 50 minutes.

Besides those described above, other methods have been developed for the determination of DAPA. Hutton et al. [8] used an automatic amino acid analyser with photometric detection at 420 nm, Czerkawski [2] used acid ninhydrin and Pongor and Baintner [10] combined a video densitometer with thin layer ion-exchange chromatography for the determination of DAPA. Edols [3] used an automatic amino acid analyser combined with a dual column technique for the determination of DAPA. Following the optimisation of the composition of buffers, it was possible to obtain a sharp and well defined DAPA peak which was located between the methionine and isoleucine peaks, and was distinctly separated from these. This latter method ensures a good separation of DAPA as long as it's concentration is of the same order of magnitude as that of the amino acids. Another requirement is that the concentration of the two amino acids that show chromatographic peaks adjacent to that of DAPA, methionine and isoleucine, should not exceed more then tenfold the concentration level of DAPA. Should the concentration of the above mentioned amino acids exceed the specified limit, the DAPA chromatographic peak will appear as a shoulder on the methionine and isoleucine peaks. Such a situation makes uncertain or impossible the evaluation of the chromatogram. In order to avoid the problem described above, an improved method [1] for the determination of DAPA was developed. The method is based on oxidation with performic acid preceding the hydrolysis of proteins. During the oxidation process, methionine is converted into methionine-sulfone. During the ion-exchange column chromatographic separation, methionine-sulfone elutes between aspartic acid and threonine, making available the space between valine and isoleucine for the elution of DAPA.

Following oxidation with performic acid, the sample does not contain free methionine and by a judicious change in the composition of buffers, one may achieve a situation in which the DAPA peak appears on the chromatogram in the place that is normally reserved for methionine. Since the ion-exchange column chromatographic properties of DAPA are very similar to those of methionine, the methods developed for the determination of methionine content of foods and feeds were used as the basis for developing a rapid method for the determination of DAPA in samples of biological origin.

2 Material and methods

Materials tested The DAPA contents of rumen fluid and rumen bacteria (the latter being separated by centrifugation) were determined on samples from three Holstein-Friesian cows fitted with fistulae, 12 Hungarian improved white goats and 9 Hungarian combing merino ewes. The rumen contents of the goats and ewes were taken from the rumen immediately after the animals were slaughtered at the experimental slaughter house. During the experimental period, the cows consumed maize silage, good quality lucerne and meadow hay ad libitum, and about 0.5–1.0 kg/cow/day of a mixture of farm grains. Before slaughter, the goats consumed wheat-, barley-, pea- and vetch straw and good quality maize silage ad libitum, occasionally supplemented with fodder cabbage or meadow and leguminous hay. The sheep were on pasture and also consumed about 0.4 kg/ewe/day of a mixture of farm grains and plus wheat and barley straw ad libitum.

The traditional method [3] was compared with the rapid, new method involving oxidation with performic acid [1]. Edols [3] used a TSM amino acid analyser equipped with 8% cross linked cation exchanged resin. Two columns were used: column A for the basic and column B for the acidic and neutral amino acids, with resin bed sizes of 9.5×0.4 cm I.D. and 23.5×0.5 cm I.D., respectively. Three different buffers were used. An aliquot (50 μl or 100 μl) of the hydrolysate sample was applied to both columns. Column A was run first with buffer 1 (pH = 5.25; sodium ion concentration = 0.405 M) for 36 min. Fractionation on column B was then effected for 24 min with buffer 2 (3.25 pH and 0.2 M) and 24 min with buffer 3 (4.10 pH and 0.2 M). Both columns were run at 60 °C with a flow rate of 30 ml/h. With each sample, 50 nmol of norleucine were used as an internal standard.

The rumen fluid and the rumen bacteria were prepared for analysis by filtering through three layers of gauze in order to eliminate the larger feed particles from the rumen fluid. Separation from the protozoa was achieved by centrifugation for 20 minutes at 500 g. The DAPA content of the resulting fluid was determined. The rumen bacteria were separated from the same fluid by centrifugation for 30 minutes at 15.000 g. After discarding the supernatant of the centrifuged fluid, the residue was washed thrice with acetone, and thrice with distilled water. The remaining bacterial mass was dried at 40 °C.

Hydrolysis and processing of the hydrolysate Rumen fluid, 0.1 ml, was pipetted into a Pyrex hydrolysis tube with I.D. 8 mm. The fluid was brought to dryness in a lyophilising installation. The residue left after lyophilization

of rumen fluid and a separate sample of 10 mg of air dried rumen bacteria were oxidised with performic acid, according to the method described by Hirs [6]). Following oxidation, the tubes were dried in the lyophilising installation at −55 °C. The residue was subjected to hydrolysis for 24 hours at 110 °C in the presence of 6 M HCl. The remaining HCl was eliminated by lyophilization. The residue was dissolved in a buffer with pH = 2.2 and $[Na^+] = 0.2$, the solution was filtered and DAPA was determined on the material after the necessary dilution.

Determination of DAPA Following the oxidation with performic acid, the DAPA content of the hydrolysate was determined with an LKB 4101 Amino Acid Analyser. The analysis conditions were:

Ion exchange column:	length	10 cm	
	diameter	6 mm	
	temperature	55 °C	
Ion exchange resin	CHROMEX UA-8		
Buffer A:	pH = 3.28	0.2 M $[Na^+]$	5 min
Buffer B:	pH = 4.25	0.2 M $[Na^+]$	20 min
NaOH:		0.4 M	3 min
Equilibration:	Buffer A	10 min	

The flow rates of the buffers were 50 ml/h, and the flow rate of ninhydrin was 25 ml/h. The column pressure did not exceed 3.5×10^6 Pa for buffers and the ninhydrin pressure was 5×10^5 Pa after the column. The other parameters were those recommended by the manufacturer of the amino acid analyser for amino acid analysis.

Statistical analysis Twenty seven samples (two from each of three fistulated cows, 12 from goats and nine from sheep) of rumen fluid and corresponding samples of rumen bacteria were analysed for DAPA contents. Each of the 54 samples were analysed by the rapid method, by the traditional method of Edols [3] without oxidation and by the traditional method with oxidation [1]. Preliminary analysis of the data showed that variation among samples within a cow was as great as that among cows. Therefore, the cow samples were treated as six samples.

The data were subjected to analysis of variance within main effects of species (cow, goat, sheep), type of sample (fluid, bacteria), and method of analysis

(rapid, traditional and traditional-oxidation) in the statistical model. All interactions were also in the model.

3 Results

In *Fig. 1,* a typical chromatogram obtained by adding 25 nmol of a DAPA standard is shown. This chromatogram was developed using the experimental conditions described above. The peaks of some acidic and neutral amino acids overlap in the initial part of the chromatogram. However, after valine, the DAPA peak appears well separated and distinct from both valine and isoleucine. In the chromatographic conditions used, the chromatogram may be also used for the evaluation of valine, lysine and histidine. If the purpose of the analysis is only the determination of DAPA, then after 18 minutes, following the appearance of the DAPA peak, the residual amino acids left in the column may be washed away with 0.4 M sodium hydroxide. After subsequent equilibration with Buffer A, the analysis of new samples may be started.

Figure 1: Amino acid composition of rumen fluid + 25 nmol DAPA

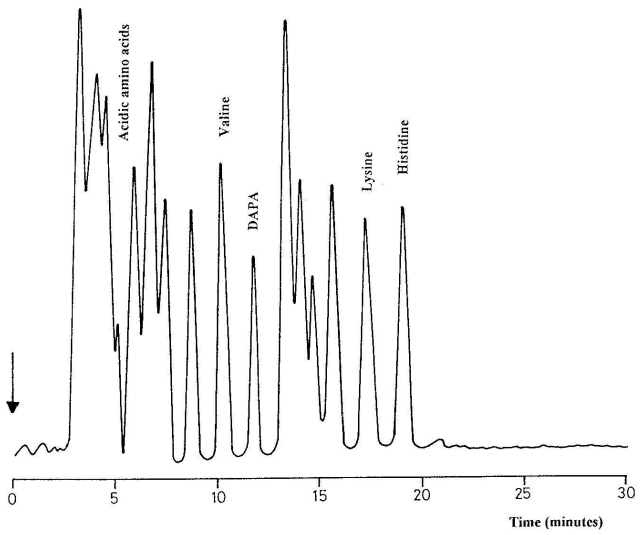
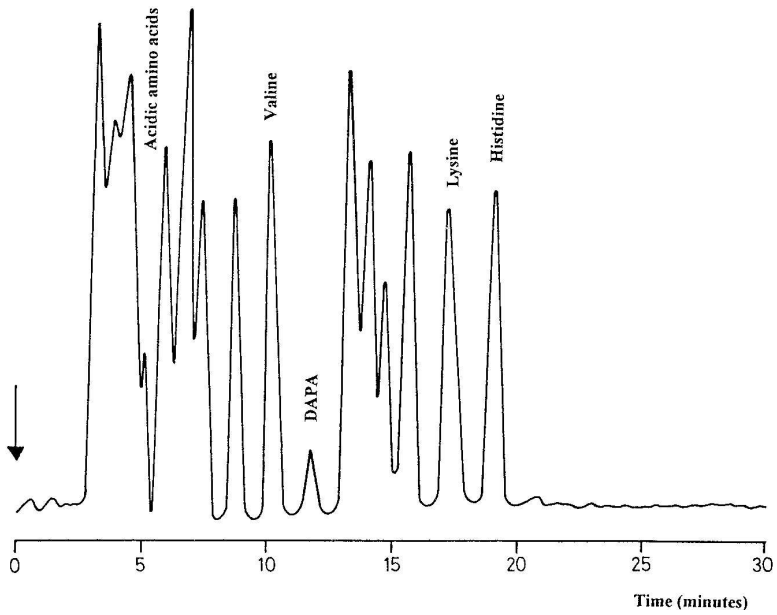


Fig. 2 shows a typical chromatogram of the determination of DAPA in rumen fluid. Comparing this to the previous chromatogram, where a DAPA standard was added to the sample, it may be concluded that low concentrations of DAPA may be quantified adequately. High concomitant concentrations of valine and isoleucine do not interfere with the determination.

Figure 2: Amino acid composition of rumen fluid



The analysis of variance revealed that the only significant effect ($P < 0.01$) was the difference between DAPA contents of rumen fluid and rumen bacteria. Since there were no significant interactions, the means can be pooled across species and/or analytical methods.

Means and standard errors for DAPA contents of rumen bacteria are shown in *Table 1.* Means, both within a species and pooled across species, clearly show that the three analytical methods were equivalent. The variance among samples within species were lowest when the rapid method was used. However, the test for homogeneity of variances did not indicate ($0.10 < P_{\chi^2} < 0.20$) lack of homogeneity. The small apparent improvement of precision could be

attributed to oxidation and consequent elimination of methionine. The overall average of 0.711 g DAPA/100 g protein provided a basis for estimating bacterial protein. The factor (100 / 0711 = 140.6) can be used to convert g DAPA/100 g protein in unknown samples to g bacterial protein/100 g protein.

Table 1: Means ± standard errors for DAPA contents (g AA/100 g protein) of rumen bacteria samples

		Analytical methods			
Species	**No.**	**Rapid**	**Trad-ox**	**Trad**	**Pooled**
Cows	6	0.697	0.712	0.710	0.706
		± 0.018	± 0.026	± 0.048	± 0.019
Goats	12	0.708	0.709	0.712	0.710
		± 0.014	± 0.021	± 0.026	± 0.012
Sheep	9	0.724	0.709	0.717	0.717
		± 0.023	± 0.023	± 0.031	± 0.015
Pooled	27	0.711	0.710	0.713	0.711
		± 0.011	± 0.013	± 0.019	± 0.008

Trad = traditional or Edols [3]
Trad-ox = traditional following oxidation with performic acid

DAPA contents of the corresponding rumen fluid samples are shown in *Table 2.* Since neither species nor analytical method influenced DAPA content of samples, the pooled value of 0.604 g DAPA/100 g protein, when multiplied by 140.6, yields a value of 84.9 g bacterial protein/100 g protein in the rumen fluid.

It is well known from university text-books that, if the diet of the ruminant animal changes, the quality and the quantity of the bacteria will also change [4, 7]. Hungate [7] stated that, if cattle are fed with only forage diets, the gram-negative bacteria will be predominant in the rumen. However if cattle consume more concentrate, the proportion of gram-positive bacteria will increase. The different bacteria have different peptidoglycans in the cell wall, which would lead to higher or lower DAPA concentration. For example the gram-positive bacteria contain 30–70% peptidoglycan in the cell wall, and the gram-negative bacteria contain only 10%. Moreover the amino acid composition (involving DAPA) of the peptidoglycan is almost identical among gram-negative bacteria, independent of species. The comparable composition is highly variable among gram-positive bacteria [4, 7].

Table 2: Means ± standard errors for DAPA contents (g AA/100 g protein) of rumen fluid samples

		Analytical methods			
Species	**No.**	**Rapid**	**Trad-ox**	**Trad**	**Pooled**
Cows	6	0.588	0.598	0.590	0.592
		± 0.051	± 0.033	± 0.047	± 0.026
Goats	12	0.603	0.600	0.601	0.601
		± 0.030	± 0.032	± 0.034	± 0.018
Sheep	9	0.610	0.622	0.612	0.615
		± 0.015	± 0.026	± 0.029	± 0.014
Pooled	27	0.601	0.607	0.602	0.604
		± 0.018	± 0.018	± 0.021	± 0.011

Trad = traditional or Edols [3]
Trad-ox = traditional following oxidation with performic acid

For this reason when DAPA is used as an indicator of rumen micro flora, one must consider all factors which might change the bacterial population after the indicator is used. If the forage is changed, this may cause a change of bacterial species, and therefore peptidoglycan content, and DAPA content. The factors for calculating the bacterial protein synthesis must be determined after major changes in diet.

Diets for cattle, goats and sheep were not identical in this study, in fact, had little overlap in dietary ingredients. Diet for all three species were, however, primarily composed of forage ingredients. Since there were no significant species differences in DAPA contents of bacteria, these data indicate that a single conversion factor can be used to estimate microbial protein derived from forages.

4 Acknowledgement

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Age determination based on amino acid racemization: a new possibility

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Abstract. A method has been developed to determine the age of fossil bone samples based on amino acid racemization (AAR). Approximately one hundred fossil bone samples of known age from Hungary were collected and analysed for D- and L-amino acids. As the racemization of amino acids is affected by temperature, pH, metal content of the soil, and time passed since death, these factors were eliminated by comparing the estimated age to age determined by the radiocarbon method. Determining the D- and L-amino acid contents in samples of known age, determining the half life of racemization and plotting the D/L ratio as a function of time, calibration curves were obtained. These curves can be used for the age estimation of samples after determining their D- and L-amino acid content. The D/L ratio for 2 to 3 amino acids was determined for each sample and the mean value of estimated ages based on calibration curves was considered to estimate age of the fossil samples.

Key words and phrases: Amino acid racemization, D-amino acids, age determination, D-Ala, D-Asp, D-allo-Ile, D-Glu, D-Phe, D-Val

1 Intoduction

Amino acid contents in fossil shell, bone and tooth samples from early ages were reported first by Abelson [1]. Hare and Abelson [6] reported that D-amino acids in fossils resulted from conversion of L- amino acids of protein. It was found that the older the fossil the higher the D/L ratio and, after a certain age, amino acids occurred in racemic form. The ratio of D-allo isoleucine and L-isoleucine content in a fossilised shell sample was found to be 0.32 and the fossil was estimated to be 70 000 years old, as reported by Hare and Mitterer [7]. It is considered as the first application of amino acid racemization – or rather epimerization – in geochronology.

Subsequently, racemization of amino acids was used for age determination of various materials containing protein. Isoleucine and aspartic acid were given special attention because L-isoleucine can be easily separated from D-allo isoleucine by an amino acid analyser and aspartic acid, being the most acidic of amino acids, is the first to come off of the ion exchange column. However, some errors of age determination based on AAR were reported by Williams and Smith [9]. Temperature, pH, soil composition and various contaminants should also be considered when estimating the age of fossil bone samples. Recently Marshall [8] established that the bones are not reliable materials for AAR testing, particularly if they come from a warm environment. The statement was based on differences observed between the age of the California bones determined by C^{14} accelerator mass spectrometry (5 000–6 000 years) and by AAR (50 000–60 000 years). Milford Wolpoff, paleoanthropologist, expressed the opinion [8] that many people currently regard AAR as "some kind of joke".

Since various changes in temperature during the past and other conditions influencing dead biological organisms are not well known, the reaction temperature of racemization can only be estimated and not accurately determined. This is the reason that – in this study – contents of D- and L- amino acids and their ratio were determined in samples of known age (as determined by the radiocarbon method). These data were then compared with data obtained from the analysis of amino acids in samples of unknown age. To make the comparison more accurate, the antecedents of samples of known age when analysed were the same as or similar to those of unknown age. Therefore, 100 fossil bone samples previously analysed by the radiocarbon method were collected from various Hungarian museums, and their D- and L-amino acid contents were determined. The D/L ratio was calculated and plotted against time which produced a calibration curve. This curve can be used for age estimation of samples of unknown age after their D- and L-amino acid contents have been

determined. The D/L ratio for 2 to 3 various amino acids was determined for each sample and the mean value of ages estimated from calibration curves was considered the true age of the fossil sample.

2 Material and methods

Sample preparation The samples were washed in running- and distilled water, dried in a vacuum drying oven and ground to produce powder material as fine as flour. Apolar contaminants were removed with petroleum ether in a Soxhlet extractor for 3 hours at 40 °C. The free amino acids were extracted by 0.1 M HCl solution for 16 hours. The nitrogen content of the residue was determined by Kjel-Foss nitrogen analyser. Sample size (200–2 000 mg residual material containing app. 10–20 mg protein) was dependent on nitrogen content. Samples were weighed and hydrolysed with 6 M HCl at 110 °C for 24 h. HCl was removed by lyophilization, the residue was dissolved in water, and the precipitated silicate compounds were separated from the liquid containing free amino acids using a centrifuge. The solution was alkalised to pH = 9 for a moment and precipitated metal hydroxides were filtered. The hydrolysed solution was neutralised and evaporated to dryness by lyophilization.

Determination of amino acids An aliquot of hydrolysed material was dissolved in a citrate buffer solution of pH = 2.2 and isoleucine and D-allo isoleucine were determined by LKB 4101 type amino acid analyser as described by Csapo et al. [2]. The other D- and L-amino acids were separated in the form of alanyl- [4] and 2-sulphonylic acid alanyl diastereomerisomer dipeptides [3] by ion exchange column chromatography and by the method of Einarsson et al. [5] with reversed-phase HPLC using precolumn derivatization with the chiral reagent *o*-phthalaldehyde/2,3,4,6,-tetra-O-acetyl-1-thio-β-glucopyranoside.

Prior to conducting analyses of all samples by HPLC, the D- and L-amino acids of three samples were determined by both HPLC and ion exchange column chromatography (IEC). The results are in *Table 1,* and the D/L ratios determined by the two methods were in excellent agreement.

Table 1: D/L ratios for various amino acids determined by ion exchange column chromatography (IEC) and by high performance liquid chromatography (HPLC)

Age of samples	Analytical method	The D/L ratios for various AA			
		Phe	Asp	Ala	Ile
15 600					
	IEC	0.568	0.367	0.153	-
	HPLC	0.553	0.389	0.163	-
38 450					
	IEC	-	-	0.395	0.123
	HPLC	-	-	0.401	0.121
46 900					
	IEC	-	-	0.487	0.146
	HPLC	-	-	0.492	0.149

3 Results

The analyses data on 24 fossil bone samples from various Hungarian museums of known age are summarised in *Table 2.* Seven amino acids (His = histidine, Phe = phenylalanine, Asp = aspartic acid, Glu = glutamic acid, Ala = alanine, Ile = isoleucine,
Val = valine) are presented. These may be considered as being the most suitable for age determination because some of them show very fast racemization (His, Phe, Asp), while others show very slow racemization (Ile, Val). Analytical data for other analysed amino acids are not presented in *Table 2* in order to make it more synoptic. None of the ratios lower than 0.1 or higher than 0.7 are presented in *Table 2* because, in these cases, the accuracy of age determination was doubtful. Half lives of AAR were also calculated from the data of *Table 2* and are presented in *Table 3.*

From the data of *Table 2,* His, Phe, Asp, Glu and Ala contents can be used for the age determination of samples which are 2–12 000, 3–20 000, 5–35 000 and 10–80 000 years old, respectively. Age of samples older than 30 000 and 50 000 years can be determined on the basis of Ile and Val content, respectively. Data in *Table 2* were corrected (reduced) with the D-amino acid content of a fresh pig bone to eliminate the errors of analysis. When fresh pig bone

Table 2: D/L ratios for various amino acids concerning ages of fossil samples determined by the radiocarbon method

Age of	The D/L ratios for various amino acids						
samples*	His	Phe	Asp	Glu	Ala	Ile	Val
2 200	0.138	-	-	-	-	-	-
2 800	0.162	0.101	-	-	-	-	-
3 110	0.181	0.109	-	-	-	-	-
3 240	0.199	0.128	-	-	-	-	-
4 630	0.253	0.179	0.109	-	-	-	-
5 460	0.312	0.225	0.128	-	-	-	-
6 850	0.419	0.252	0.171	0,091	-	-	-
11 200	0.618	0.442	0.271	0,126	0.112	-	-
12 400	0.682	0.473	0.289	0,143	0.131	-	-
15 600	-	0.561	0.378	0,178	0.158	-	-
18 600	-	0.654	0.432	0,209	0.192	-	-
20 200	-	0.689	0.491	0,233	0.209	-	-
22 600	-	-	0.543	0,256	0.228	-	-
25 400	-	-	0.580	0,275	0.246	-	-
28 600	-	-	0.621	0,311	0.289	-	-
30 400	-	-	0.643	0,325	0.321	-	-
32 500	-	-	0.702	0,355	0.343	0.099	-
36 900	-	-	-	0,395	0.381	0.118	-
44 600	-	-	-	0,481	0.465	0.134	-
46 800	-	-	-	0,500	0.483	0.142	-
54 300	-	-	-	0,527	0.510	0.169	0.100
62 200	-	-	-	0,606	0.586	0.188	0.115
65 000	-	-	-	0,634	0.613	0.199	0.119
72 400	-	-	-	-	0.652	0.221	0.136

Age deterined by the ^{14}C corrected method (year)

was hydrolysed with 6 M HCl for 24 h at 110 °C, the forms of glutamic and aspartic acids, respectively, represented 1.9 and 1.3% of the totals due to racemization during processing. Concentrations of the D-form for the other amino acids were negligible. However, all analyses were corrected for the small concentrations present in fresh pig bone.

Table 3: Half lives of racemization and epimerization of various amino acids found in Hungarian fossil bone samples

Amino acids	Half life (year)
His	500
Phe	8 500
Tyr	8 600
Asp	13 500
Ser	16 500
Thr	17 000
Glu	28 500
Ala	32 000
Ile	110 000
Leu	140 000
Val	180 000

Studying the calibration curves, it can be concluded that, in the case of D/L ratio being lower than 0.1, the D-amino acid content is too low and age determination is uncertain. Both curves may be considered to be linear in the D/L range of 0.1–0.5. It is obvious that the calibration curves can be used for age determination most satisfactorily in the linear range, (D/L between 0.1 and 0.5) where D-amino acids are present in well detectable amounts. The optimum D/L ratio can be found for each sample by analysing the amino acids best suited for age determination. E. g., for fossil bone samples of 11 200 years the D/L ratio for His, Phe, Asp and Ala is 0.682, 0.473, 0.271 and 0.112, respectively. In this case the D/L ratios of Phe and Asp are recommended for determining the age of samples, however the D/L ratios of His and Ala can be used to confirm the estimate based on the ratios of Phe and Asp.

Known age (Y) was regressed on D/L ratio (X_1) and ln[(1+D/L)/(1-D/L)] (X_2) for each of four amino acids (Phe, Asp, Ala and Ile) to produce prediction equations of the form $\hat{Y} = a + bX$. All eight regression equations produced r^2 values greater than 0.99. In each amino acid, was greater than 0.99 which means that X_2 was simply a coded value of X_1. The standard deviation of deviations from regression (standard error of estimate= $s_{Y.X.}$) can be used to

calculate the standard error of an individual estimate as

$$s^2_{\hat{Y}} = s^2_{Y.X.}[\frac{1}{n} + \frac{(X - \bar{X})^2}{\sum (X - \bar{X})^2}]$$

with n = number samples used in estimatingregression and $\sum (X - \bar{X})^2$ being the sum of squares of deviations from the mean X. The value, $\hat{Y}$ was calculated for each regression for two situations (X=$\bar{X}$ and X=an extreme value). For Phe, Asp and Ala, mean values for D/L were 0.35 to 0.41 and extremes were approximately ±0.30. Corresponding means for $\ln(X_2)$ were 0.75 to 0.90 and extremes were ±0.75. For Ile, means were 0.16 and 0.32 with corresponding extremes at ±0.06 and ±0.12. The two $\hat{Y}$ values for each amino acid mean and extreme were averaged to produce the following values:

Amino acid	Mean	Extreme
Phe	189	329
Asp	226	458
Ala	382	988
Ile	311	514

A mean of estimates based on two amino acids would have a standard error of S.E. = $\sqrt{(s^2_{\hat{Y}_1} + s^2_{\hat{Y}_2})/4}$ and 95% confidence limits can be established as C.I. = Mean of two $\hat{Y}$ values ± S.E. ($t_{0.05}$).

Since the average based on the smallest number of samples would have 15 degrees of freedom, the value of $t_{0.05}$ used in the following estimates was 2.13. The ± deviations were calculated for each pair of amino acids and are shown below. The confidence intervals at mean values are shown above the diagonal and confidence intervals at extreme values are below the diagonal:

	Phe	Asp	Ala	Ile
Phe	-	313	454	388
Asp	601	-	473	409
Ala	1109	1160	-	524
Ile	650	733	1186	-

If both D/L values were near the mean, we would be 95% confident that our estimate was in the range of mean $\hat{Y}$±313 to 524 years. If both estimates were based on extreme values of D/L, we would be 95% confident that our estimate

was in the range of mean $\hat{Y} \pm 601$ to 1 186 years. The confidence interval for each estimate of age of an unknown sample would be calculated individually.

Finally, the applicability of calibration curves is presented. As an example, one bone sample of unknown age was analysed for L- and D-amino acids and the following results were obtained:

L-His:	0.0697 mg, D-His: 0.0289 mg, D/L-His= 0.428
	Age calculated from calibration curve: 7 100 year; S.E. = 337
L-Phe:	0.0543 mg, D-Phe: 0.0138 mg, D/L-Phe=0.254
	Age calculated from calibration curve: 6 950 year; S.E. = 191
L-Asp:	0.1346 mg, D-Asp: 0.0245 mg, D/L-Asp=0.182
	Age calculated from calibration curve: 6 900 year; S.E. = 465

The estimated age of the sample is the mean value of the above estimates or 6 980 years. This mean value has a standard error of 202 years and the 95% confidence interval would be 6 554 to 7 406 years.

Conclusion The D- and L-amino acid composition was determined in fossil bone samples of known age. Ages were determined by the radiocarbon method. The D/L ratio was plotted as a function of time which resulted in a calibration curve which can be used for age estimation after the D- and L-amino acid contents in samples of unknown age have been determined. However, this method includes the analytical error of age estimation by the ^{14}C method, but the effects of temperature, pH and the composition of soil on AAR can be eliminated. The D/L ratio for 2 to 4 amino acids should be determined for each sample, and the mean value of estimated ages based on calibration curves is considered the best estimate of age of the fossil sample.

We have utilised this method very successfully for dating fossil bone samples from Hungary. The difference between the data from the calibration curve and those from ^{14}C dating was generally negligible. We were very cautious with both sample selection and preparation; the unknown samples were mainly of origin similar to those from which the calibration curves were formulated and sample preparation was carried out exactly the same for samples of known and unknown ages.

We are aware of the weak points of this method and the possible errors associated with ^{14}C dating. However, the results support the reliability of this method. Our calibration curves should not be used in other environments because of different conditions (e.g. temperature, pH, soil composition). How-

ever, based on these results, other calibration curves can be formulated for each environment based on methods described here.

4 Acknowledgement

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