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Enantioseparation of D- and L-amino acids by a coupled system consisting of an ion-exchange column and a chiral column and determination of D-aspartic acid and D-glutamic acid in soy products

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Abstract

A coupled system, consisting of a conventional ion-exchange column and a chiral column, was used for separation of the D- and L-enantiomers as constituents of an amino acid mixture. The ion-exchange column was connected to an amino acid analyser and the eluents necessary for the chiral column were delivered by an HPLC pump. With this on-line system the D- and L-forms of eight of the proteinogenic amino acids were separated. An alternative method was also investigated, in which single amino acids or groups of amino acids were fed to the chiral column via a three-way divider, in order to subject the column to less aggressive conditions. By using aspartic acid and/or glutamic acid as an index of racemization and, if necessary, increasing the number of peaks investigated, a simple and fast method for the determination of the degree of racemization was achieved. The method was applied to three soy products. The products contain 3.4–5.8% D-aspartic acid and 1.4–2.4% D-glutamic acid

Keywords: Enantioseparation; Two-dimensional separation; Racemization; D- and L-amino acids; Amino acid analyser; HPLC

1. Introduction

There are still many questions about D-amino acids, concerning both their occurrence and their effects on humans. The reason for this is undoubtedly that hydrolysis and analysis procedures are associated with a number of problems. Investigations performed to date indicate that the occurrence of enantiomeric forms is enhanced in processed food, especially when the process involves high temperatures (Friedman & Liardon, 1985). D-amino acids, probably of bacterial origin, have also been found in fermented foods and ripened cheeses (Brückner & Hausch, 1989; Palla, Marchelli, Dossena, & Casnati, 1989). The human body has a limited capacity to metabolise D-amino acids and therefore most of them will be excreted in the urine (van de Merbel et al., 1995). An abundance of protein-bound D-amino acids may lead to a reduction in the nutritional value of food since the efficiency of the proteolytic enzymes may decrease considerably, not only with regard to the D-amino acid, but also many adjacent bonds (Hayashi & Kameda, 1980; Master & Friedman, 1979).

Conventional acid hydrolysis, preceding amino acid analysis of proteins, employs 6 M HCl at 110 °C for 24 h. These conditions lead to a spontaneous racemization, which cannot be distinguished from the originally occurring D-amino acids without radioactive labelling (Liardon & Hurrell, 1983; Liardon & Lederman, 1986; Manning, 1970). Several studies have been performed to evaluate methods of minimizing this hydrolysis-induced racemization. Csapo et al. (1997) used high-temperature hydrolysis for a short time, while Pe'ter, Laus, Tourwe', Gerlo, and van Binst (1993) used microwave hydrolysis. Stenberg, Marko-Varga, and Öste (2001) considerably reduced racemization by replacing the 6 M HCl by 3 M HCl and, if incomplete hydrolysis could be accepted, a further reduction in racemization was possible with shorter hydrolysis times.

Analysis methods routinely used do not separate the D- and L-enantiomers of amino acids. Therefore, separation systems for D- and L-amino acids must be complemented with chiral selection in one way or another (Buck & Krummen, 1987; Brückner & Lupke,

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1995; Brückner & Schieber, 2001; van de Merbel et al., 1995; Yu & Wu, 2001).

In this study, a method for the determination of D- and L-amino acids as constituents of a protein was evaluated and applied to a number of soy products. The method was based on the combination of a conventional ion-exchange column and a chiral column containing a crown ether as stationary phase. Using an adjustable three-way divider after the analytical column, heart-cuts of the eluting peaks could be diverted onto the chiral column. By choosing aspartic acid or glutamic acid, the amino acids most prone to racemization, it was possible to obtain an index of racemization. If racemization was observed, the number of amino acids investigated was increased.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Those used in the preparation of buffers and ninhydrin reagents, perchloric acid, hydrochloric acid (30%) and phenol were obtained from Merck (Darmstadt, Germany). The L- and DL-amino acid standards were purchased from Sigma Chemical Co. (St. Louis, USA) as was the α-aminoadipic acid used as the internal standard. The water used was purified by Milli-Q purification. The soy products were bought in the local supermarket.

2.2. Apparatus

Qualitative, quantitative and chiral amino acid analyses were performed with an amino acid analyser, Model LC 5001 from Biotronik (München, Germany). The column used for the amino acid analysis (3.2×385 mm) was packed with the resin BTC 2710 (Biotronik). For the chiral separation, a Crownpak CR (+), 150×4 mm column, from Dancel Chemical Industries Ltd (Tokyo, Japan) was used. The recorder used was a Shimadzu CR 3A (Tokyo, Japan). The HPLC pump was from Varian and the three-way divider from Rheodyne (Rohnert Park, USA).

2.3. Protein hydrolysis

To perform complete hydrolysis of the substrate, a sample corresponding to approximately 10 mg protein was hydrolysed in a sealed glass tube with 6 M HCl, containing 0.1% phenol, for 24 h at 110 °C. After hydrolysis and the addition of an internal standard the sample was evaporated to dryness and dissolved in lithium citrate buffer at pH 1.9, as recommended by Biotronik, before separation. To minimize the hydrolysis-induced racemization, one of the samples was also hydrolysed in 3 M HCl for 3 h; otherwise the procedure was the same.

2.4. Amino acid analysis

Amino acid separation was performed with standard lithium citrate buffers of pH 2.85, 2.89, 3.20, 4.02 and 3.49, and the instrument was programmed for physiological fluids. The elution temperature varied between 34 and 61 °C. The injection volume was 50 μl and the post-column derivatization of amino acids was performed with ninhydrin. The buffer flow was 0.25 ml/min and the ninhydrin flow was 0.14 ml/min. The temperature of the reaction vessel was 125 °C. The spectrophotometric estimate of the amino acids was made at 570 nm. Identification was confirmed by retention times and the amount of amino acids was calculated using a standard solution of amino acids as reference.

2.5. Analysis of single D- and L-amino acids (chiral column)

Single D,L- amino acids (500 μg/ml) were separated on the amino acid analyser using the chiral column without the ion-exchange column. The column was installed outside the heating units. Of the solution, 100 μl was diluted in perchloric acid, pH 1.0, and 50 μl of this was injected into the column. Perchloric acid, pH 1.5, and a column temperature of 0 °C (ice-water) were used for the elution of D- and L-aspartic acid, threonine, serine, alanine, valine, arginine, cystine, lysine and histidine. D- and L- glutamic acid were eluted at the same pH, but at room temperature (23 °C). D- and L-phenylalanine, tyrosine, isoleucine and leucine were eluted at room temperature (23 °C) at pH 1.5 using perchloric acid mixed with 10% MeOH (Table 1). The flow rates, the ninhydrin derivatization and the method of detection were the same as in the routine amino acid analysis.

2.6. Analysis of D- and L-amino acids as constituents of an amino acid mixture (coupled system)

In the coupled system, the chiral column was connected to the ion-exchange column by an adjustable three-way divider. Before entering the chiral column the effluent from the ion-exchange column was mixed with perchloric acid delivered by an HPLC pump. An outline of the system is shown in Fig. 1. The parameters for the separation of the different amino acids were the same as in the ordinary amino acid analysis and the injected amino acid concentration was 500 μg/ml. With continuous transportation of the amino acids from the ion-exchange column to the chiral column, the parameters for the chiral column were as follows: perchloric acid pH 0.5, flow 0.1 ml/min for 70 min, perchloric acid pH 0.5 with 15% MeOH, flow 0.3 ml/min for 40 min, and

Table 1
Parameters employed in the separation of enantiomeric forms of single amino acids on a chiral column connected to an amino acid analyser

Amino acid	$HClO_4$ pH	% MeOH	Temp °C	Retention time min[a]		l–d min	Resolution
				d–AA	l–AA		
Aspartic acid	1.5	–	0	8.8	12.2	3.4	1.8
Threonine	1.5	–	0	10.0	13.9	3.9	2.1
Serine	1.5	–	0	9.8	11.2	1.4	0.7
Alanine	1.5	–	0	12.2	21.7	9.5	3.5
Alanine	1.5	–	23	8.1	9.6	1.5	0.9
Valine	1.5	–	0	13.4	16.6	3.2	1.2
Histidine	1.5	–	0	11.7	14.8	3.1	1.3
Arginine	1.5	–	0	14.9	18.7	3.8	1.8
Lysine	1.5	–	0	10.9	14.7	3.8	1.2
Glutamic acid	1.5	–	23	9.5	16.5	7.0	3.5
Methionine	1.5	10	23	9.3	12.5	3.2	1.3
Isoleucine	1.5	10	23	12.4	14.3	1.9	0.5
Leucine	1.5	10	23	14.6	23.3	8.7	2.9
Tyrosine	1.5	10	23	15.8	21.7	5.9	1.8
Phenylalanine	1.5	10	23	22.6	31.2	8.6	2.0

[a] The time deviations between different elutions were a few seconds.

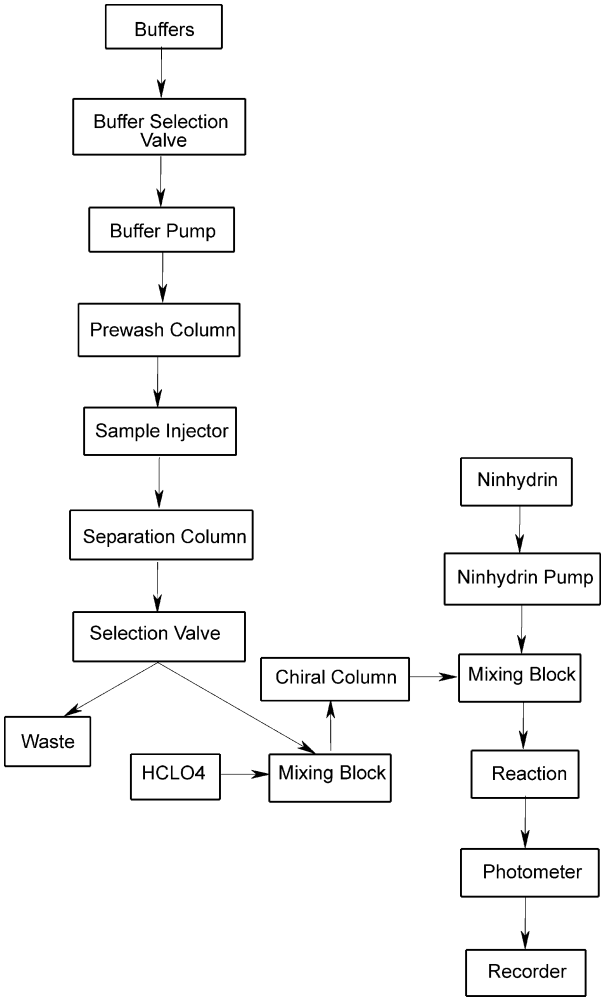

Fig. 1. Schematic drawing of the coupled system employing an ion-exchange column and a chiral column.

perchloric acid pH 0.5, flow 0.1 ml/min for 1 h. The temperature of the chiral column was 0 °C for 36 min, 23 °C for 10 min, 0 °C for 24 min, 23 °C for 27 min and 0 °C for the rest of the run. An ice-water bath and a temperate water bath (23 °C) were used for the fast temperature changes.

An alternative method was to allow only selected amino acids to pass from the ion-exchange column into the chiral column. This was performed manually for 1 min, and to obtain the exact time for this passage, fractions were collected at the inlet to the chiral column and coloured with ninhydrin. Effluent not intended for analysis was led to the waste outlet. The chiral elution parameters were: perchloric acid, pH 1.0, and 0 °C for aspartic acid, threonine, serine, alanine, valine, histidine, lysine and arginine, and the same pH but room temperature (23 °C) for glutamic acid. For methionine, isoleucine, leucine, tyrosine and phenylalanine, perchloric acid, pH 2, was mixed with 10% MeOH and the temperature was 23 °C. The flow throughout was 0.25 ml/min. The parameters are listed in Table 2. Since the separation of the enantiomers is based on complex formation between the crown ether and the protonated amino group (van de Merbel et al., 1995) it was important to maintain the pH of not only the eluent, but also the sample, sufficiently low to ensure that the amino acid was positively charged. It was thus necessary to adjust the high pH of the effluent from the ion-exchange column (= the sample) before entering the chiral column, and to achieve this the pH of the perchloric acid was set to 1 at this moment. For certain amino acids, however, it was necessary to alter the pH of the system to attain satisfactory elution. D- and L amino acids have the same response factors (Stenberg, Marko-Varga, &

Table 2
Parameters employed in the separation of enantiomeric forms of amino acids as constituents of a Dl-mixture[a]

Amino acid	$HClO_4$ pH	% MeOH	Temp °C	Retention time min[b]		l–d min	Resolution
				d–AA	l–AA		
Aspartic acid	1.0	–	0	12.2	18.7	6.5	2.4
Threonine	1.0	–	0	12.4	17.3	4.9	2.4
Serine	1.0	–	0	8.3	10.6	2.3	1.4
Alanine	1.0	–	0	15.1	27.0	11.9	6.0
Valine	1.0	–	0	13.1	17.2	4.1	2.1
Histidine	1.0	–	0	11.0	16.3	6.3	1.6
Arginine	1.0	–	0	16.3	19.9	3.6	0.9
Lysine	1.0	–	0	11.0	15.0	4.0	0.8
Glutamic acid	1.0	–	23	9.7	19.2	9.5	3.8
Methionine	2.0	10	23	11.0	17.5	6.5	1.9
Isoleucine	2.0	10	23	11.5	12.0	0.5	0
Leucine	2.0	10	23	25.0	36.0	11.0	2.3
Tyrosine	2.0	10	23	15.5	24.0	8.5	3.2
Phenylalanine	2.0	10	23	28.0	37.5	9.5	4.2

[a] Fractions of isolated dl-amino acids are fed from the ion exchange column of an amino acid analyser into a connected chiral column.
[b] Calculated from the time of entering the chiral column and the time deviations between different elutions were a few seconds.

Öste, 2001) and a linear response ($r > 0.996$) was found in the concentration range 0.005 to 3.8 μg/ml for all the amino acid forms studied. The amount of D-amino acid was calculated as a percentage of the total amount of amino acid% $D = D/(D + L) \times 100$.

3. Results and discussion

3.1. Single amino acids

Single amino acids could, without any problems, be separated on the chiral column directly connected to the amino acid analyser, as could groups of amino acids when the difference in retention times was sufficiently large e.g. aspartic acid and phenylalanine. The D-enantiomer always eluted before the L-enantiomer and the time lag between D- and L-retention varied between 1.4 min (serine) and 9.5 min (alanine). The resolution was >1.2 for all the acids except serine (0.7) and isoleucine (0.5) (Table 1) and the time deviations between different elutions were a few seconds throughout the experiment.

3.2. Protein hydrolysates

Amino acids as constituents of a protein must be separated prior to chiral separation. For increased capacity a coupled column system is an alternative. In our system the chiral column was connected to the ion-exchange column with an adjustable three-way divider, as described above, and before entering the chiral column the effluents were mixed with perchloric acid necessary for chiral separation. With this on-line system the D- and L- forms of eight of the proteinogenic amino acids were separated (Fig. 2). D- and L-serine, valine and isoleucine could not be separated. The resolution was poor for D- and L-threonine, leucine and tyrosine (<0.6) and besides a relatively new column was required to separate D- and L-threonine. Acceptable resolution was achieved for D- and L-aspartic acid, glutamic acid, alanine, methionine and phenylalanine (>0.9). However, this separation was performed on a standard solution with approximately the same concentrations of D- and L-amino acids. If the determination is intended for small quantities of D-forms in an excess of the L-form the resolution would be expected to increase (van de Merbel et al., 1995). None of the enantiomeric forms of lysine, histidine and arginine was observed. The pH of the lithium buffers (4.02 and 3.49) and the lithium concentration (0.45 M and 1.40 M) necessary for proper elution of these amino acids were probably too high to keep the amino acids protonated.

Another problem encountered in this on-line system was the high pressure over the columns, creating considerable demands on the tightening of the connections. Also, the exposure of the chiral column to the analytical effluent buffers stressed the column and gradually resulted in loss of performance.

The problems described above were completely eliminated when single amino acids were isolated from the mixture and analysed separately. Using this technique, all the common amino acids were separated into their D- and L-forms with a resolution >0.8 for all except isoleucine (zero) (Table 2) and the elutions occurred with the same precision as for single amino acids. It was also possible to obtain good separation on the chiral column feeding groups of amino acids. For example, aspartic acid,

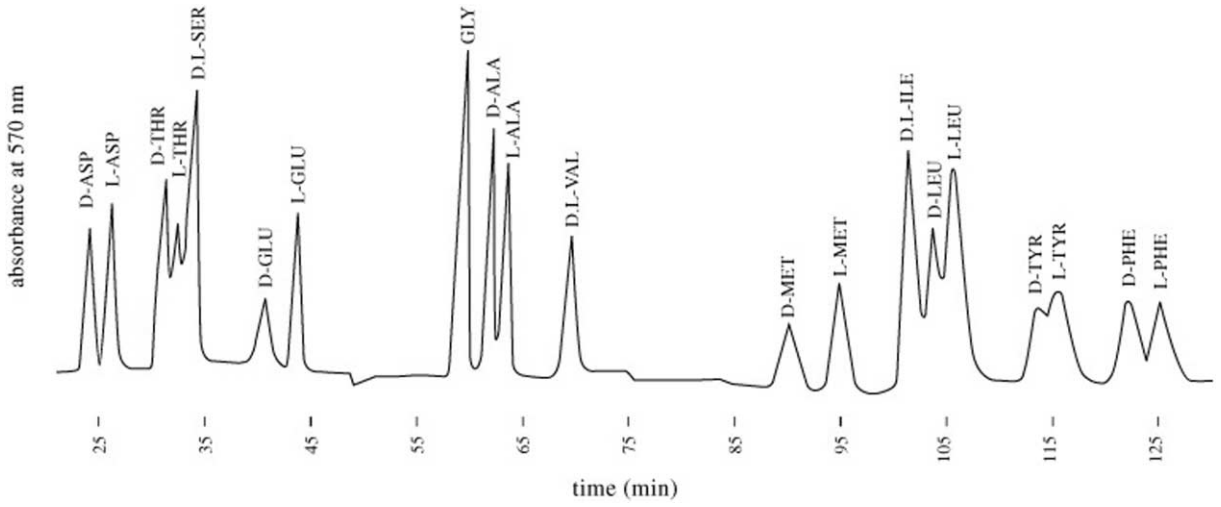

Fig. 2. Enantiomeric separation of an amino acid solution on a coupled two-column system. The acids were continuously transported from the ion-exchange column to the chiral column.

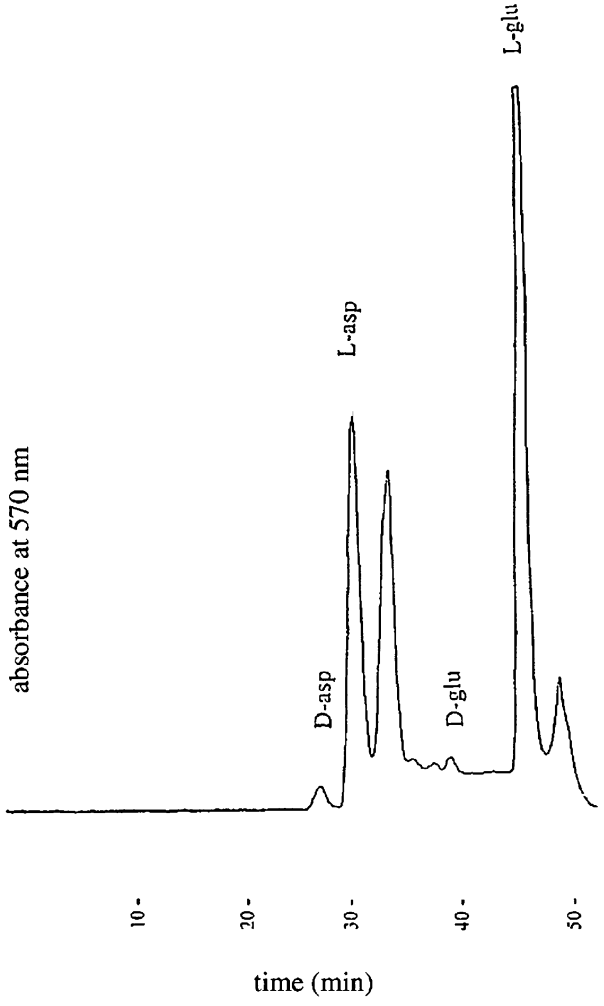

Fig. 3. Enantiomeric separation of aspartic acid and glutamic acid in the hydrolysate of soy milk. The analysis was performed on the coupled column system. Chiral elution parameters: perchloric acid pH 1 and column temperature 0 °C for 30 min, then room temperature (23 °C).

threonine, serine and glutamic acid could easily be fed to the chiral column as one fraction with the same resolution for D- and L-aspartic acid and D- and L-glutamic acid as for single fractions. Then, however, the two forms of threonine and serine were eluated as one peak.

Friedman, Zahnley, and Masters (1981) proposed aspartic acid, the acid most likely to racemize, as an index of racemization. Based on the results of the separation technique described above, we would like to propose glutamic acid, even that prone to racemization, as an additional racemization index. Glutamic acid often occurs in abundant quantities and small amounts of the racemic form will thus be easier to detect. Also, since enantiomeric separation is possible at room temperature, the pressure obtained over the chiral column will be lower sparing the column.

3.3. Products containing processed soy protein

Conventional hydrolysed soy milk, soy meal and soy-based vegetarian meatballs were investigated with respect to racemization using the separation technique developed in this work with aspartic acid and glutamic acid as a racemization index. The fraction containing aspartic acid, threonine, serine and glutamic acid were fed to the chiral column as one group and baseline separation was achieved for aspartic acid and glutamic acid (Fig. 3). The content of D-aspartic acid was highest in the meatballs, 5.8% and lowest in the soy meal, 3.4%, while the highest D-glutamic acid content 2.4% was found in the soy milk (Table 3). The values are the average of two measurements and the result do not indicate a general racemization demanding examination of additional amino acids. The obtained values can be compared with results reported by Man and Bada (1987) and Friedman and Liardon (1985). Man and Bada found 0.5% D-aspartic acid in untreated soy protein

Table 3
The proportion of D-amino acids in soy products expressed as % of the total amino acid hydrolysed with 6 M HCl for 24 h at 110 °C

Sample	% D-asp.acid		% D-glu.acid.	
		Mean		Mean
Soya milk	5.1	4.6	2.4	2.4
	4.1		2.4	
Soya meal	3.4	3.4	1.3	1.4
	3.4		1.5	
Veg. m,eatballs	5.7	5.8	1.9	1.6
	5.9		1.4	

and Friedman and Liardon obtained around 5% aspartic acid and 0.5% D-glutamic acid in alkali-treated (3 h/25 °C) soy protein. The authors do not mention hydrolysis-induced racemization.

In addition, the soy milk was hydrolysed with 3 M HCl for 3 h to test the method on small quantities D-amino acids and in addition obtain an indication of the hydrolysis-induced racemization. Only 0.6% D-aspartic acid and 1.1% D-glutamic acid, with hydrolysis effects of 83 and 69% respectively, were observed. These low values do not provide any indications on a greater process-based racemization of the product, but it cannot be ruled out, that the differences in D-amino acid content observed in the different soy samples are due to process-induced racemization.

4. Conclusions

A flexible coupled system, employing ion-exchange column for separation of the amino acids and a chiral column for subsequent separation of the D- and L-forms, was found to provide a good solution to the problems often encountered in the separation of enantiomeric amino acids. The resolution is good for all the amino acids except isoleucine, and the stress on the columns, as a result of high pressure and contamination by elution solutions, is minimal. Using aspartic acid or glutamic acid as a racemization index this will provide a fast method for the determination of enantiomeric forms of single amino acids, as well as amino acids as constituents of a protein.

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