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Simplified in-line sample preparation for amino acid analysis in carbohydrate containing samples

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Abstract

This report describes 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 is brought onto a short cation-exchange column (trap column) in hydrogen form. Carbohydrates are passing through this column, while only amino acids are retained. Subsequently, the cation-exchange column, holding the amino acid fraction, is switched in-line with the gradient pump and separator column. The mobile phase used at the beginning of the separation (NaOH; pH 12.7) transfers amino acids from the trap column onto the anion-exchange column and the amino acid separation is completed without any interference by carbohydrates. All common amino acids are recovered following the carbohydrate removal step. The average value of their recovery is 88.1%. The calibration plots were tested between 12.5 and 500 pmol (amounts injected). The mean value of correlation coefficients of calibration plots was calculated as 0.99. The mean value of relative standard deviations from five replicates was 3.9%. The usefulness of the method is illustrated with two chromatograms of a carrot juice sample obtained before and after the in-line removal of carbohydrates. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Amino acids; Carbohydrates

1. Introduction

Non-derivatized amino acids and sugars can be separated and detected simultaneously using anion-exchange chromatography in combination with integrated pulsed amperometric detection (IPAD) [1]. The simultaneous separation and detection is advantageous for samples containing approximately equimolar levels of amino acids and sugars. That is the case, for example, with cell culture media [2] and protein hydrolyzates [3]. None of the other current chromatographic techniques is capable of simultaneous analysis of wide range of amino acids and carbohydrates without derivatization.

If amino acids are to be analyzed in samples containing much higher concentrations of sugars (e.g. vegetable or fruit juices), 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.

The amphoteric character of amino acids allows

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their retention by both cation and anion exchange, provided that the pH value of the solution is in a correct range and the ion-exchange materials are in a suitable ionic form. The anion and cation-exchange behavior of amino acids is well understood. The anion-exchange method of the present report helps to clarify anion-exchange interactions of a large number of amino acids. One of the most widely used techniques for chromatography of complex amino acid mixtures is cation-exchange-based [4]. The cation-exchange selectivities are thus also well documented.

While the interactions of carbohydrates with anion exchangers are relatively straightforward and well-documented [5], their interaction with cation exchangers is considerably more complicated, involving several different mechanisms for any given set of experimental conditions. The carbohydrates have been known to complex with a wide range of cations [6]. In consequence, different carbohydrate separations are accomplished with cation exchangers loaded with different cations. For example, calcium and silver ionic forms are recommended for monosaccharides and oligosaccharides respectively [7]. While the main separation mechanism appears to be the ligand-exchange [8], additional mechanisms such as ion exclusion, hydrophilic or hydrophobic interaction, and size exclusion are also discussed [7–9].

In this work, we evaluate the possibility of capturing amino acids on a cation-exchange resin under conditions of no retention for carbohydrates. Our objective is to find conditions allowing a widest possible range of carbohydrates to be completely removed at the cation exchanger. Following the removal of carbohydrate matrix, the captured amino acids are to be eluted from the cation exchanger and separated by anion exchange.

2. Experimental

2.1. Chemicals

Standard mixtures of amino acids and sugars were made by diluting Standard Reference Material 2389 (NIST, Gaithersburg, MD, USA) and corresponding aliquots of single component stock solutions of sugars with a diluent containing 20 mg/l sodium azide. All standard sugars were of 95% purity or better and were purchased as solids from Sigma Aldrich (St. Louis, MO, USA). The single component stock solutions of sugars (1.0 m*M*) were prepared by dissolving solid sugars in 0.1 *M* HCl.

Only 18 megohm water (Milli Q, Millipore, Bedford, MA, USA), 50% sodium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA) and anhydrous sodium acetate (Fluka, Buchs, Switzerland) were utilized for the preparation of mobile phases listed in Table 1. The carrot juice sample was purchased in the local supermarket and diluted 100 fold with the azide containing diluent.

2.2. Anion-exchange chromatography and IPAD detection

We used a Dionex BioLC (Dionex, Sunnyvale, CA, USA) consisting of a GS50 gradient pump, AS50 autosampler, LC30 column thermostat and ED50 electrochemical detector. The chromatographic system control, data acquisition and data reduction were done with PeakNet 6.1 Software. For separations, we chose the AminoPac PA 10 column set consisting of a short Guard column (2×40 mm) and a long analytical column (2×250 mm). Both columns were packed with an identical microporous, polymeric anion-exchange material. The total ion-exchange capacity of the analytical column was 60 μequivalents. The separations were effected with the mobile phases and the gradient program shown in Table 1.

For detection, we connected the outlet of the analytical column to the ED 50 electrochemical cell

Table 1
Gradient conditions (flow rate 0.25 ml/min)

Time (min)	NaOH (m*M*)	NaAc. (m*M*)	Curve
0.00	40		
2.00	40		
12.0	80		8
16.0	80		
24.0	60	400	8
35.0	60	400	
35.1	200		5
37.1	200		
37.2	40		5
60.0	40		

Table 2
Detection conditions

Time (ms)	Potential (V)[a] vs. pH	Integration
0	0.13	
40	0.13	
50	0.28	
210	0.28	Begin
220	0.61	
460	0.61	
470	0.28	
560	0.28	End
570	−1.67	
580	−1.67	
590	0.93	
600	0.13	

[a] Sequence of potentials applied to Au working electrode and referenced vs. Glass/Ag/AgC1 ‘‘combination electrode.’’

containing a gold electrode, pH reference electrode and titanium counterelectrode. The detection waveform is presented in Table 2.

2.3. Cation-exchange columns for in-line experiments

We utilized cation-exchange columns and materials listed in Table 3. The larger diameter (3 and 4 mm I.D.) TCC2, CG3 and CG12A Guard columns were products available from Dionex. The 10×2 mm TCC2 cartridge (PEEK) was vacuum-packed using a suspension of ca. 0.3 g of TCC2 resin per 2 ml of water and placed in a pressure-resistant stainless steel holder (Cartridge and Holder: C270 Assembly, Upchurch Scientific, Oak Harbor, WA, USA).

An aqueous suspension of Dowex 650 C resin (Dow, Midland, MI, USA) was pressure-packed into an empty 50×4 mm Guard column cylinder made from PEEK material (Dionex).

2.4. Valving scheme for in-line experiments

All in-line sample pretreatment experiments were carried out using two inert HPLC six-port valves (Rheodyne, Cotati, CA, USA) interconnected by narrow diameter PEEK capillaries, as shown in Fig. 1. The first of the two valves equipped with a 25 μl sample loop (Valve A in Fig. 1, Rheodyne P/N 9750) was rotated by an electromotor. The second valve (Valve B in Fig. 1 P/N 9156) was pneumatically driven. Port 1, valve A connected to a sampling pump (GP50) and sampling fluid (water) reservoir. The smaller of the two rectangles indicates one of the cation-exchange columns from Table 3. The inlet and outlet of the cation-exchange column were connected to ports 4 and 1, respectively. The larger rectangle, connected to port 2 of valve B, represents the AminoPac PA10 column set. The respective length and diameter of the connecting tubing were 595 and 0.123 mm. The port 2 of valve A was connected to port 6 of valve B by exactly 490 mm of the 0.125-mm I.D. tubing. The length of the 0.125 mm I.D. tubing between port 1 and the cation-exchange column was 180 mm. The section of the 0.125 mm I.D. tubing from port 4 to the cation-

Table 3
Polymeric cation-exchange columns tested for in-line sugar elimination

Ion exchange resin (particle size)	Hydrophobic character of bulk resin	Functional group	Column dimensions [mm] L×I.D.	Capacity [μeq./column]
TCC2 (30 μm)	High	sulfonate	35×3	10
TCC2			10×2	0.13
CG3 (10 μm)	Medium	sulfonate	50×4	10
CG12 (12 μm)	Medium	carboxylate phosphonate	50×4	140
Dowex 650 C (650 μm)	n.a.	sulfonate	50×4	1260

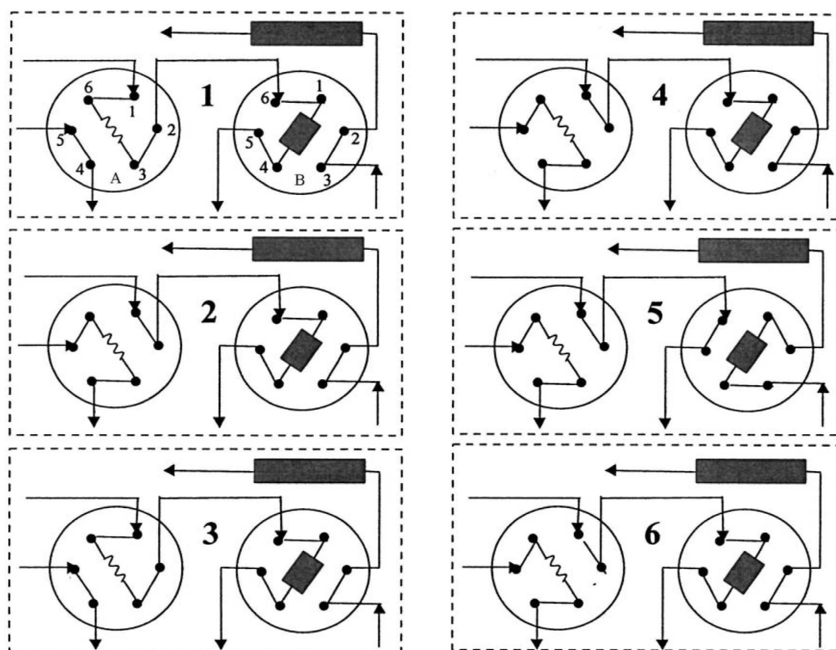

Fig. 1. Column switching scheme for in-line sample preparation of carbohydrate-containing amino acid samples. See Sections 2.4 and 3 for additional detail.

exchange column was 150 mm long. The gradient pump connection to port 3 of valve B was also made of an appropriate length of the 0.125 mm tubing to minimize the gradient delay. Usual rules apply for the choice of remaining connections and capillaries.

3. Results and discussion

Depending on the pH value, amino acids can exist in one of the three forms: cationic, zwitterionic and anionic. This property makes it possible to entrap them as cations on a cation exchanger under acidic conditions and to transfer them from a cation exchanger to an anion exchanger under alkaline conditions. Several parameters must be selected or experimentally optimized to achieve high and reproducible recoveries of amino acids with the instrumental configuration presented in Fig. 1:

1. Choice of liquid and flow rate for transferring samples from the sample loop of valve A to the cation-exchange column of valve B.
2. Dimensions of the cation-exchange column.
3. Type of cation-exchange resin (particle size and material, ion-exchange capacity)
4. Ionic form of the cation-exchange resin.
5. Timing for the execution of six steps in Fig. 1.

The optimal choices of transfer fluid and the ionic form for entrapment are obvious from the selectivity of cation exchange [4]. Only with water as a transfer fluid and the cation exchange in hydrogen form can we expect to completely immobilize amino acids immediately as they enter the column. Any mobility of amino acids within the column, as caused by the transfer fluid, is undesirable because it leads to zone broadening and even losses of more weakly retained amino acids. The cation-exchange selectivity also suggests that hydrophobic cation-exchange particles may retain some of the early eluting analytes (i.e. tyrosine, phenylalanine, leucine, etc.) more effectively.

The use of narrow bore anion exchange and alkaline eluents in the final stage of the analysis

narrows down several of the choices. The cation-exchange column must be as short as possible to generate only a minimal pressure drop. The particle size should ideally be large. However, it should not be so large as to decrease the chromatographic efficiency. The cation-exchange resin must withstand pH values between 12.0 and 14.0. The silica-based materials are thus excluded.

3.1. Preliminary experiments

To clarify the role of different resin types, we carried out amino acid entrapment and recovery experiments with 50 and 35 mm guard columns from Table 3. We compared chromatograms of directly injected standards of common amino acids (312.5x dilution of SRM 2389, see Experimental) with those generated by treating the same standards in the instrument of Fig. 1. The transfer fluid (water) was pumped at 0.25 ml/min. The timing and events of the steps in Fig. 1 were as follows:

Step 1: 30 s, hydrochloric acid (10%) is transferred from the sample loop to the cation-exchange column.
Step 2: ca. 4 min, interstitial hydrochloric acid is rinsed out of the column by the transfer fluid. The autosampler fills the sample loop with dilute (312.5x) SRM 2389 standard.
Step 3: 30 s, amino acid standard is transferred from the sample loop to the cation-exchange column. The pump gradient program for the anion-exchange separation is started at this point.
Step 4: 2 min, non-cationic sample components are removed from the cation exchanger.
Step 5: 50 min, The cation-exchange column is switched into the anion-exchange system. The amino acids are converted to anions (eluent pH ca. 12.7), transferred onto the anion-exchange column to be separated (see gradient in Table 1) and detected (Table 2).
Step 6: ca. 10 min, interstitial anion-exchange eluent (sodium hydroxide) is rinsed off the cation-exchange column. The re-equilibration of the anion-exchange column to initial conditions is completed.

The outcome of the preliminary experiments can be summarized as follows:

The ion-exchange capacity of Dowex 650 was too large for this material to be practical. Only very few of the amino acids were recovered for anion-exchange separation and their recovery was at ca. 10% or less.

Disappointing results were also obtained with the weak cation-exchange column CG12. Only histidine, phenylalanine and tyrosine peaks were recovered and their recovery was less than 50%. There were no anion-exchange peaks for the remaining amino acids. The first encouraging results were obtained with the CG3 column. All amino acids, except isoleucine, leucine, methionine, glutamate and aspartate were observed in the anion-exchange chromatogram. The recovery was ca. 50% when compared with chromatograms by direct injection.

The best results of the preliminary series were with the TCC2 column. Only glutamate and aspartate were missing. The recoveries for the peaks ranged between 50% (valine) and 100% (tyrosine).

As a negative result in both the intitial CG3 and TCC2 experiments, we observed system peaks and peak distortions attributable to an excessively acidic content of the amino acid fraction being transferred onto the anion-exchange column [10].

Only the TCC2 columns were used in subsequent experiments.

3.2. Recovery optimization

Next, we found that an optimized duration of steps 3, 4 and 5 (Fig. 1) made it possible to recover all of the amino acids from the cation-exchange column. The artifacts attributed to the acidic matrix could also be reduced by altering the timing, and could be completely eliminated by using 0.1 *M* instead of 10% HCl for the cation-exchange conditioning in step 1. Still using the 35×3 mm TCC2 column, we decreased the total time of steps 3 and 4 to 1 min (3: 30 s, 4: 30 s). The time intervals of step 5 were varied between 60 and 10 s. We experimented also with the flow rate of the transfer fluid (0.05–0.25 ml/min constant during the entire experiment).

Even though all of the peaks were in evidence in this round of experiments, the range of recoveries was broader than in the preliminary series (see TCC2

in Section 3.1). The best result overall was achieved with 0.1 N HCl, steps 3 and 4: 1 min, step 5: 50 s and 0.05 ml/mm flow rate of transfer fluid. The range of recovery was 40% (glutamate) to ca. 100% (histidine, isoleucine, leucine, methionine).

At this stage, we decided to make the dimensions of the TCC2 column compatible with the narrow-bore format of the anion-exchange separation; and started to use the 10×2 mm column packed with the same resin. Under the conditions of the best experiment with the larger column (except step 5: 20 s), the smaller column delivered more uniform recoveries ranging from 68% (arginine) to 100% (lysine, histidine, tyrosine) with glutamate and aspartate yielding 87% and 70%, respectively.

3.3. Carbohydrate removal

Using the optimum conditions from the preceding paragraph, we experimented with a dilute amino acid standard to which we added an equimolar level of glucose (8 μ*M*). The carbohydrate was only partially removed (ca. 30% residue in the transferred standard) under such conditions. A complete removal of the equimolar level of glucose was achievable only at higher flow rates of the transfer fluid (water: 0.1–0.25 ml/min) with other conditions unchanged. Unfortunately, the higher transfer fluid flow rates were also causing a complete loss of glutamate and aspartate on the shorter TCC2 column. An attempt to improve recovery of the two dicarboxylic amino acids at the higher flow rates by increasing the concentration of HCl to 10% failed.

We also tried increasing the total duration of steps 4 and 5 to 2, 3, 4, and 5 min, with the other conditions from 3.2 unchanged. We succeeded in eliminating the glucose completely in all of those experiments, however, we lost the response for glutamate and aspartate again.

The optimal point for the simultaneous recovery of amino acids and total removal of glucose was, in the end, found to be: 0.1 N HC1 for column conditioning, 0.05 ml/min flow rate of transfer fluid (water), total duration steps 3 and 4: 3 min (step 3: 30 s), step 5: 30 s.

3.4. Evaluation of the new method

Trace 1 in Fig. 2 is a direct separation (no carbohydrate removal) of a 500 fold dilute SRM2389 standard to which we added 10 μ*M* levels of glucose, fructose, sucrose and maltotriose. Also

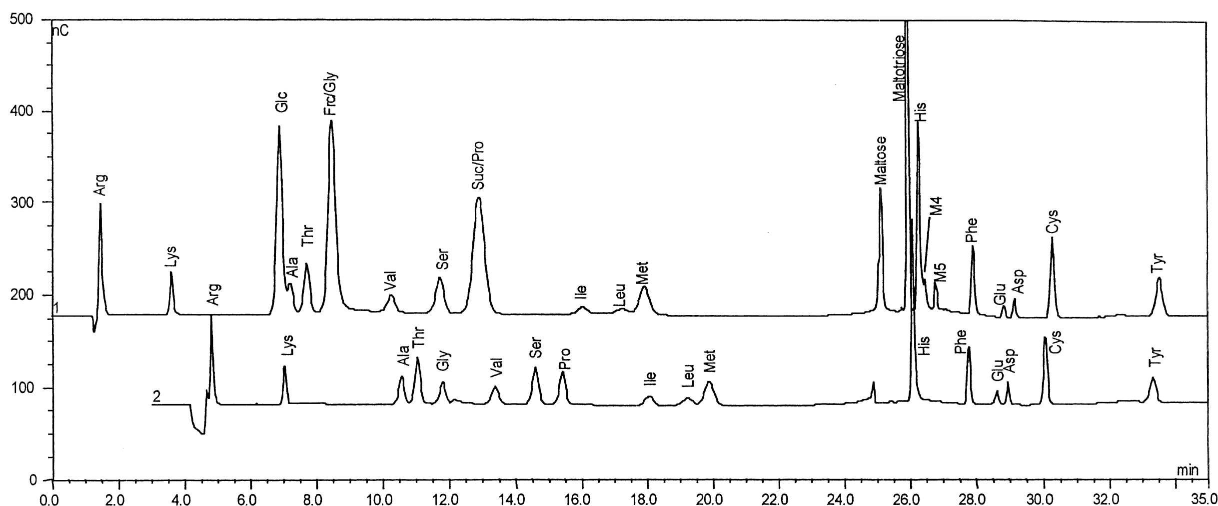

Fig. 2. Direct injection of a carbohydrate-amino acid mixture (Trace 1) and a chromatogram obtained with in-line sample preparation of the same mixture (Trace 2). Note: In the chromatogram of Trace 1, the sample enters the anion-exchange column at time zero of the gradient program. In the chromatogram of Trace 2, the amino acid fraction is transferred to the anion-exchange column at 3.0 min of the gradient program. The retention times are not directly comparable; however, the selectivity of the separation is almost unchanged. See Sections 2.4 and 3.1 for a more detailed description.

Table 4
Summary of method evaluation

Component name	% Recovery (peak areas)	Correlation coefficients	%RSD $n=5$
arginine	87.1	1.00	7.5
lysine	94.8	1.00	5.1
alanine	92.9	0.99	3.1
threonine	91.9	0.98	3.1
glycine	90.2	0.99	5.9
valine	89.4	0.99	3.6
serine	86.4	0.98	2.9
proline	75.9	0.99	3.1
isoleucine	77.1	0.99	4.5
leucine	89.7	0.98	5.0
methionine	73.2	0.98	1.6
histidine	100.6	0.96	1.9
phenylalanine	86.0	0.99	2.7
glutamate	90.2	0.98	5.0
aspartate	102.1	0.99	2.8
cysteine	96.8	0.99	3.7
tyrosine	72.6	0.98	4.3
Mean value	88.1	0.99	3.9

present are peaks of maltose, maltotetraose and maltopentaose introduced as impurities with the maltotriose. A complete co-elution is observed for fructose/glycine and sucrose/proline peak pairs. The peaks of glucose and alanine, histidine and maltotetraose are incompletely resolved.

Trace 2 in Fig. 2 is a chromatogram generated under optimal conditions of Section 3.3. All seven carbohydrates added to the amino acid mixture are completely removed. The possibility of simultaneous removal of mono-, di- and oligo-saccharides is thus demonstrated. We were also able to verify that carbohydrates were completely removed even at a 100 fold molar excess relative to the amino acids (chromatogram not shown). The data in Table 4 were generated with solutions containing 40 μ*M* of the glucose always added to the amino acid mixture and under optimal conditions described in Section 3.3. The recovery values were determined by injecting a 500 fold dilution of SRM2389 (ca. 125 pmol injected).

The calibration plots were tested and correlation coefficients calculated with five levels of amino acids corresponding to 5000, 1000, 500, 250, 125 fold dilution of the SRM2389 standard.

The reproducibility data were obtained from five injections of the 500 fold dilute amino acid standard.

In the final step of method evaluation, we compared chromatograms from a direct injection of dilute vegetable juice with that of the same sample processed in the apparatus of Fig. 1 and by the optimized procedure from Section 3.3.

The direct injection (Trace 1, Fig. 3) reveals several major interfering peaks belonging to glucose, fructose and sucrose. The arginine peak is obscured by an unknown matrix component. Maltose and several minor peaks belonging to oligosaccharides

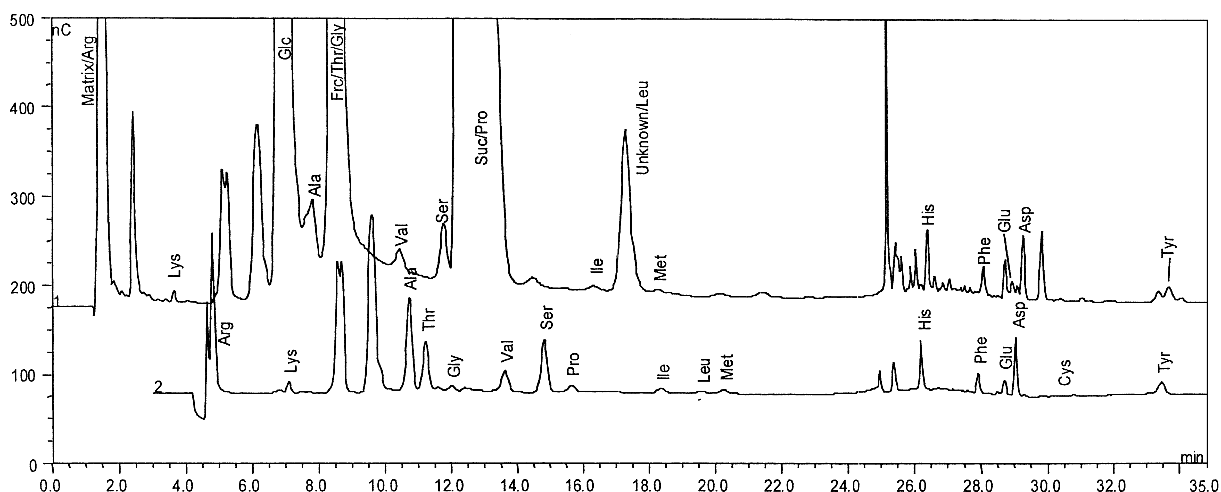

Fig. 3. Amino acids in carrot juice. Trace 1: Direct chromatogram of carrot juice diluted 100:1 with water containing 20 mg/l sodium azide. Trace 2: The same sample after in-line removal of carbohydrates.

are in evidence between 25 and 30 min. The chromatogram obtained with the in-line sample preparation (Trace 2, Fig. 3) demonstrates a complete removal of interfering carbohydrates. The matrix components masking the arginine peak are also eliminated.

4. Conclusions

Complete removal of mono-, di- and oligo-saccharides from amino acid samples is possible using a low capacity, strong cation exchanger with sulfonic groups and a highly hydrophobic bead surface. For each sample injection, the cation exchanger must be converted into the hydrogen form and rinsed thoroughly with water to remove the interstitial acid leftovers from the conditioning step. The sample procedure is completely automated. The reproducibility of the new method is specified in %RSD for 17 common amino acids. The recoveries of amino acids are in the range of 73–100%.

References

[1] A.P. Clarke, P. Jandik, R.D. Rocklin, Y. Liu, N. Avdalovic, Anal. Chem. 71 (1999) 2274.
[2] P. Jandik, A.P. Clarke, N. Avdalovic, D. C Andersen, J. Cacia, J. Chromatogr. B 732 (1999) 193.
[3] P. Jandik, C. Pohl, V. Barreto, N. Avdalovic, Anion exchange chromatography and integrated amperometric detection of amino acids, in: C. Cooper, N. Packer, K. Williams (Eds.), Methods in Molecular Biology, Vol. 159: Amino Acid Analysis Analysis Protocols, Humana Press, Inc., Totowa, NJ, 2001.
[4] D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30 (1958) 1190.
[5] Y.C. Lee, J. Chromatogr. A 720 (1996) 137.
[6] S.J. Angyal, Chem. Soc. Rev. 9 (1980) 415.
[7] G. Bonn, J. Chromatogr. 322 (1985) 411.
[8] J.X. Khym, Analytical Ion-Exchange Procedures in Chemistry and Biology, Prentice-Hall, Englewood Cliffs, NJ, USA, 1974.
[9] U.D. Neue, HPLC Columns, Wiley–VCH, New York, NY, USA, 1997.
[10] P. Jandik, J. Cheng, D. Jensen, S. Manz, N. Avdalovic, Anal. Biochem. 287 (2000) 38.