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ORIGINAL PAPER

Isotachophoretic determination of amino acids after their conversion to hydroxy acids

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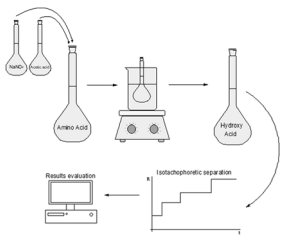
Abstract

In this article, determination of amino acids by capillary isotachophoresis based on their derivatization (van Slyke reaction) is presented. Hence, the suggested method deals with reaction of amino acids with nitrous acid forming corresponding hydroxy acids. This reaction was used in isotachophoretic analysis for the first time. Optimal conditions represented by electrolyte system for separation of hydroxy acids, 0.01 mol/dm^3 HCl, 0.05% hydroxyethyl cellulose, with β-alanine (leading electrolyte of pH 3.6) and 0.01 mol/dm^3 valeric acid with sodium hydroxide (terminating electrolyte of pH 7.24), were chosen; furthermore, other critical details of the analysis are also described. Developed method brings original results and shows applicability on analysis of amino acids in relatively simple mixtures, especially in food supplements.

Graphical abstract



Keywords Amines · Diazo compounds · Electrophoresis · Zwitterions

Introduction

Nowadays, it is generally known that consumers, producers, and authorities have raised interests to know which ingredients and nutrients are present in food. According to this, a lot of methods for determination of various nutrients were proposed. One of the common studied groups is biologically active compounds, namely, amino acids which are essential for human nutrition due to their presence in proteins.

Concerning the physico-chemical properties, amino acids are ionized in water, but their molecules have amphoteric character. For that reason, their mobility in electric field is rather low within the pH range from 3 to 10 which is recommended for isotachophoretic (ITP) analysis. These conditions bring problems in direct ITP separation of most amino acids. Nevertheless, there are some exceptions such as amino acids of acidic character (glutamic and aspartic acid) and basic amino acids (histidine, lysine, and arginine) which can be easily separated by isotachophoresis (ITP) without derivatization; hence, their mobilities in electric field is satisfactory.

One of the possibilities of amino acid determination in foodstuffs and food supplements is use of isotachophoretic analysis which has been demonstrated by Jastrzebska et al.

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[1]. They optimized direct isotachophoretic separation for determination of free amino acids in cheese samples using both modes—anionic mode in combination with alkaline electrolyte systems for determination of tyrosine and phenylalanine and cationic mode for lysine, arginine, histidine, and ornithine. Moreover, cationic mode was also used for analysis of histidine derivates in meat samples [2]. Another works dealing with ITP analyses of amino acids using anionic mode and alkaline electrolyte systems were performed by Kvasnička et al. [3], Everaerts et al. [4], Hirokawa et al. [5], and Prest et al. [6]. However, ITP analysis using alkaline electrolyte brings a problem connected with migration of carbonate (originated from dissolved atmospheric carbon dioxide) which cause prolongation of analysis time together with decrease of column separation capacity. The main problem of carbonate is creation of mixed zones which have negative influence on separation process. The content of carbonate is usually decreased by the addition of barium hydroxide, hence formation of insoluble barium carbonate. However, carbonate cannot be completely removed [7].

More often, acidic or neutral conditions are in ITP analyses frequently used, but in case of amino acids analyses, only glutamic and aspartic acid can be separated directly under acidic conditions. As an example, work of Zgola-Grześkowiak [8] can be mentioned, they dealt with determination of these two acids in tomato juice using anionic mode.

One of the possibilities how to determine other amino acids under acidic conditions is using non-aqueous solvents, for example, dimethylsulfoxide [9] and propionaldehyde [4]. The other possibility is their derivatization, hence conversion of amino acid to other compounds, which are not ampholytes. There are several ways of derivatization suitable for ITP analysis, e.g., reaction with formaldehyde in alkaline conditions [10], esterification [11]. In other methods employed in determination of amino acids such as liquid chromatography or electrophoresis, derivatization is not inevitable, but has many advantages (for example, better separation or decreasing of detection limit). Recently, derivatization of amino acids (reaction with 2,4-dinitrofluorobenzene [12], 9-fluorenylmethyl chloroformate [13, 14], benzoyl chloride [15], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [16–18]) was performed.

The other option, which has not yet been used in ITP analysis, is reaction with nitrous acid [19, 20] (see Scheme 1, as an example reaction of valine was used).

In this contribution, we focused on isotachophoretic method for determination of amino acids based on conversion of amino group to diazo group which is subsequently hydrolyzed to form hydroxy acid. According to author's knowledge, such method of derivatization has been employed for the first time in ITP analysis of various amino acids. The aim of developed method is finding the best conditions for sufficiently sensitive determination of amino acids in food supplements with regard to preserving the main benefits of isotachophoresis such as simple sample preparation, low costs, or relatively low time consuming.

Results and discussion

Conversion of amino acids to hydroxy acids

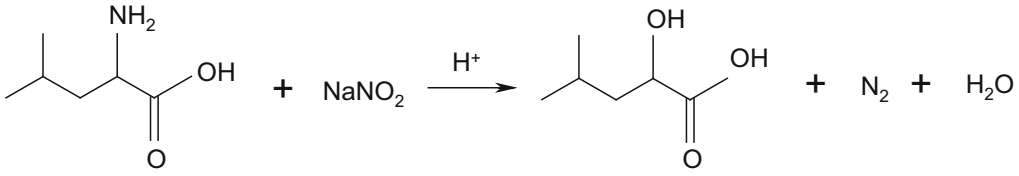
Reaction of primary amino group with nitrous acid forming diazonium salt which immediately decomposes to appropriate amount of gaseous nitrogen is the basis of manometric determination of amino groups elaborated by van Slyke [19, 20]. Reactivity of primary amino groups increases with temperature and together with the presence of carboxylic group in the neighborhood. On the contrary, the reaction slows down with increasing distance between amino and carboxylic group [21]. Such properties were applied for optimization of the suggested method, described below.

Most important role in van Slyke method plays nitrous acid (formed by reaction of sodium nitrite with acetic acid) employed as derivatization agent. Amount of nitrous acid in reaction mixture also influences the course of reaction as mentioned later. The other important parameter is pH of reaction mixture, which should be sufficiently low to form nitrous acid (pK_A = 3.15 [22]), but also high enough for quick quantitative decomposition of diazonium salt to gaseous nitrogen, or to prevent side reactions which can decrease the yield of emerging α-hydroxy acid, respectively. This is the reason why optimization of reaction conditions (i.e., composition of reaction mixture, way of stirring, and influence of temperature and reaction time) was necessary. Moreover, because the determination was applied to analysis of foodstuffs and food supplements containing leucine, isoleucine, and valine (hence, amino acids containing non-polar chain bound on the same carbon as amino group), the whole optimization was done only for these amino acids.

Concerning the mechanism, leucine is converted to 2-hydroxyisocaproic acid; hence, the resulting conversion efficiencies were assessed based on ratio of zone length achieved by analysis of converted leucine and zone length of 2-hydroxyisocaproic acid standard solution at the same concentration.

As the first parameters of optimization, influences of stirring, temperature, and time of conversion efficiency at fivefold excess of sodium nitrite and acetic acid were investigated (Fig. 1). It should be mentioned that at laboratory conditions, the kinetics of conversion is very slow

Scheme 1



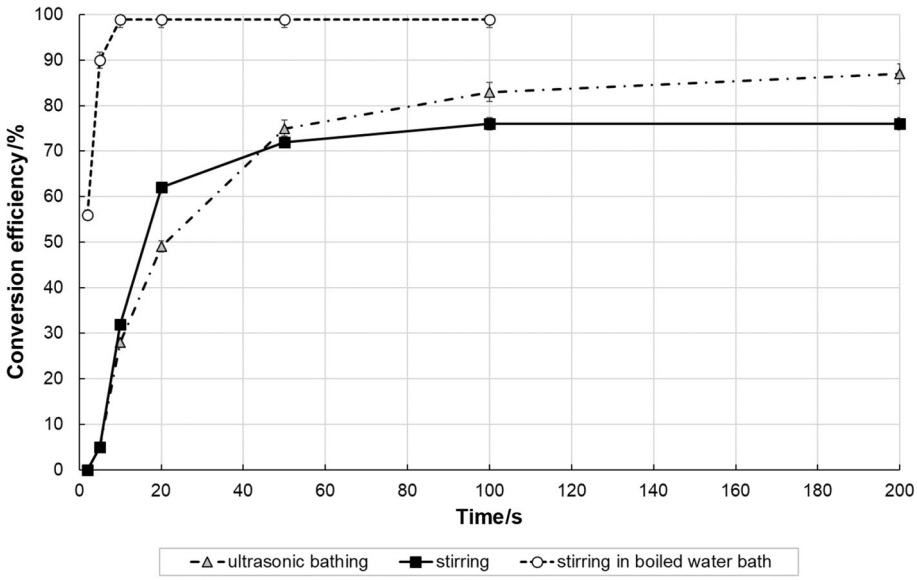


Fig. 1 Relationship between leucine conversion efficiency and time for different reaction support; composition of reaction mixture: 100 mmol/dm^3 sodium nitrite, 100 mmol/dm^3 acetic acid, and concentration of leucine 20 mmol/dm^3

while using mechanical stirring and ultrasound. To significantly increase conversion, efficiency elevated temperature and simultaneous stirring was applied—conversion efficiency 99.5 ± 2.9% was achieved using a boiling water bath for less than 10 min. Increase of conversion rate during elevated temperature and stirring can be explained by decrease of diazonium salt stability as well as of nitrogen solubility.

Composition of reaction mixture was the second optimized parameter, and varied concentration of sodium nitrite reacting with solution either of acetic acid or of sulfuric acid was tested. Conversion efficiency 100.0 ± 1.8% was achieved only in case of acetic acid considering more than fivefold excess of sodium nitrite versus leucine, whereas in case of sulfuric acid was 83.5 ± 2.5% (see Fig. 2).

Similar experiment, but this time with varied concentration of several acids by constant concentration of sodium nitrite was performed (see Fig. 3). Sulfuric acid, acetic acid, valeric acid, and acetate buffer (pH 4.76) were tested. As can be seen in Fig. 3, it was again confirmed that sulfuric acid is not suitable for this purpose, because maximum of conversion efficiency was 83.5 ± 2.5% when molar ratio 1:1 of acid and leucine was used. Decrease of conversion efficiency at higher concentrations of sulfuric acid is caused by decomposition of formed α-hydroxy acid in concentrated sulfuric acid. Products of decomposition are corresponding carbonyl compound (aldehyde or ketone) and formic acid. In case of acetic acid conversion efficiency 100.0 ± 1.8% was obtained at a theoretic ratio of acetic acid and leucine 1:2. However, for very weak valeric acid, the ratio of this acid and leucine is opposite in comparison with the previous case. Moreover, valeric acid can also be used as terminating electrolyte. Further experiment dealing with acetate buffer was performed. The main reason of testing this buffer was the control of pH value which can influence formation of hydroxy acids. Nevertheless, these experiments can be considered as unsuccessful, due to very low conversion efficiency. The better results can be probably achieved using a more acidic buffer.

Acidification of reaction mixture by strong and weak catex provided similar results as acidification by strong and weak acid (Fig. 4). Meanwhile, after adding sufficient amount of weak catex, 94.0 ± 2.8% conversion efficiency can be achieved, and a small excess of strong catex causes significant decrease of conversion efficiency.

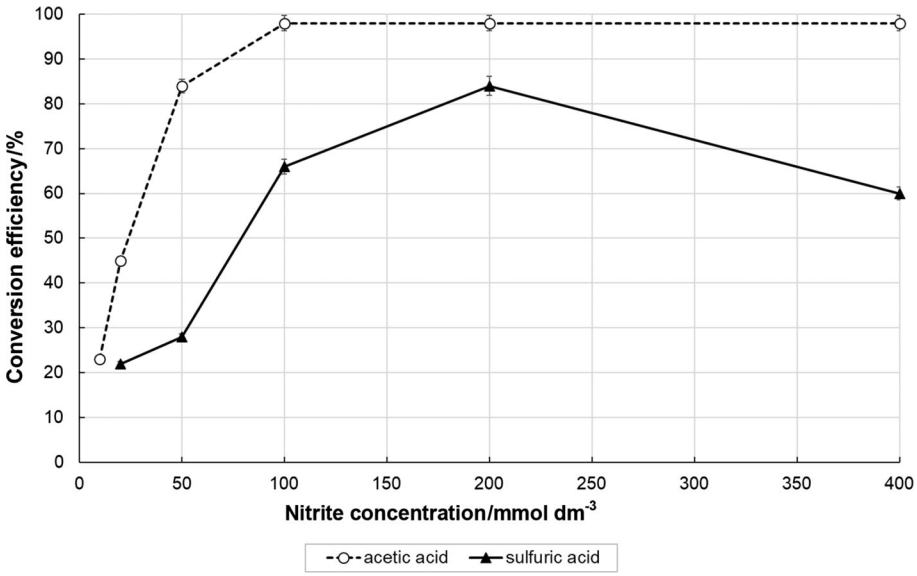


Fig. 2 Dependence of leucine conversion efficiency on sodium nitrite content for 100 mmol/dm^3 acetic acid, 100 mmol/dm^3 sulfuric acid, and 20 mmol/dm^3 leucine, reaction mixture was 10 min stirred in boiling water bath

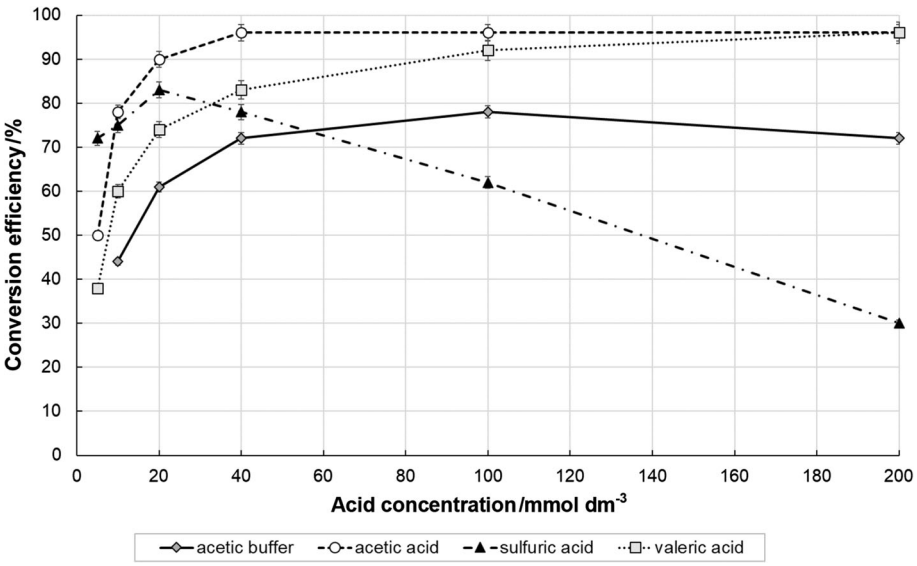


Fig. 3 Dependence of leucine conversion efficiency on concentration of various acids, concentration of sodium nitrite was 100 mmol/dm^3, concentration of leucine was 20 mmol/dm^3, acetic buffer (pH 4.76), and reaction mixture was 10 min stirred in boiling water bath

Use of catex can be advantageous because of no increase of anions amount in the solution which can prolongate analysis. However, it has some disadvantages, i.e., more difficult manipulation and destruction of catex during analyte preparation as well (catex quickly loses its capacity and it is not worth to regenerate it).

The optimization revealed that the best molar ratio of leucine, sodium nitrite, and acetic acid is 1:5:5 when solution was stirred in boiling water bath for 10 min. Under these conditions, almost 100% conversion efficiency for 1 mmol/dm^3 and higher concentration of leucine solution was achieved.

Results of optimization were practically the same for valine, leucine, and isoleucine (even abovementioned results of optimization are shown on analysis of leucine).

Isotachophoretic separation

Reaction mixture after conversion contains apart from hydroxy acid also other anions, which increase the separation capacity requirements and prolong analysis time. These anions are, namely, nitrites, nitrates formed by oxidation of nitrites, and ions of acids as well. Mobility of acetate is usually lower than mobility of anions formed from leucine, isoleucine, and valine. Nitrate is created during conversion and after long standing of reaction mixture. Its mobility is much higher than mobilities of desirable hydroxy acids, so nitrates only prolong the analysis time, but they do not disturb it. Nitrites can be removed using several agents, the most recommended is urea and amidosulfonic acid. Use of both agents relates to

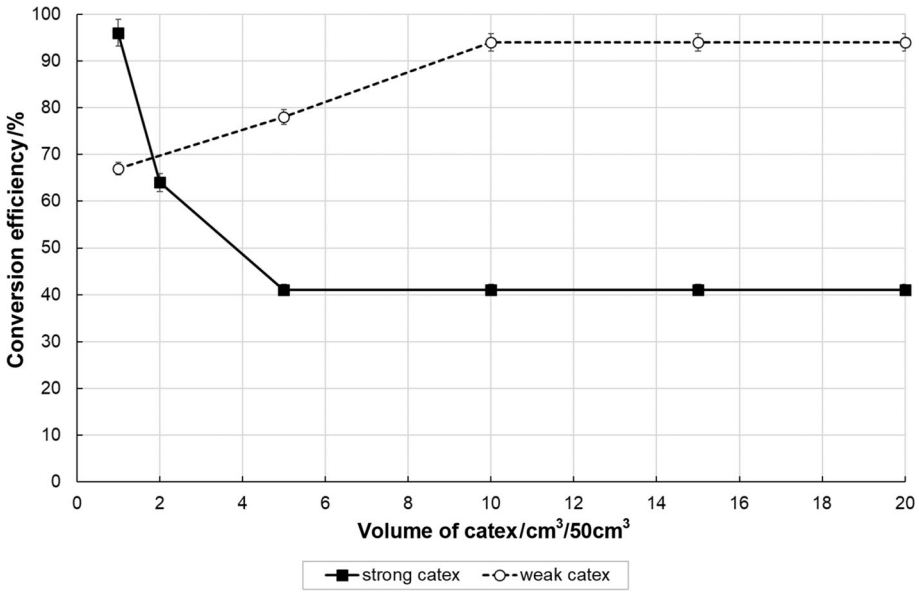


Fig. 4 Dependence of leucine conversion efficiency on volume of strong and weak catex adding to reaction mixture, concentration of sodium nitrite was 100 mmol/dm^3, concentration of leucine was 20 mmol/dm^3, and reaction mixture was 10 min stirred in boiling water bath

formation of undesirable ions—urea works only under very acidic conditions and amidosulfonic acid is converted to sulfates and creates its own zone as well. Because of that reason, it is better to not remove nitrites but modify the separation conditions, so that its zone does not disturb the determination. It was decided to do not remove nitrites for our experiments. Extraordinarily, nitrites can be converted to nitrates by heating of solution with the addition of strongly acidic catex, which is placed to boiling water bath for 1 h.

The isotachophoretic analysis can be performed with leading electrolyte of various pH. System which we chose consisted of chloride as leading ion, β-alanine as counter ion, and acetate as terminating ion. The pH of leading electrolyte was 3.6 (see "Experimental" part). Analyses were performed in analytical column. Analytical parameters of determination are listed in Table 1 for amino acids valine, leucine, and isoleucine. Determination limit was calculated as concentration of amino acid which provides zone with 1 s length. Subsequently, detection limit is a third of the determination limit. Detection and determination limit can be lowered by decreasing of concentration of leading ion and decreasing of separation current. We tried to use 2 mmol/dm^3 HCl and separation current 10 μA which causes decreasing of detection and determination limit approximately to half concentration for all three amino acids. Repeatability was calculated as relative standard deviation (multiplied by 100 to obtain % value) of ten consecutive measurements. For determination of repeatability amino acid, standards of 1 mmol/dm^3 concentration were used. Recovery was determined from analysis of converted 0.1 mmol/dm^3 amino acid standard solution and was calculated as ratio of measured and real concentration multiplied by 100 to achieve value in %. Regression equation of calibration curve—influence of zone length (L [s]) on concentration of converted amino acid standard (c [mmol/dm^3])—is mentioned in Table 2. Calibration curves showed good linearity over minimally two concentration orders.

It was found that after conversion, leucine and isoleucine gave products with the same relative signal highs (*RSH*s). Fortunately, several ways of isomers mixture separation [23–27] were previously published. According to this, the addition of α- and β-cyclodextrine into the leading electrolyte was tested. Meanwhile, β-cyclodextrine had no influence on separation of leucine and isoleucine

Table 1 Method parameters for determination of converted amino acids [leading electrolyte: 10 mmol/dm^3 HCl + β-alanine (pH 3.6), separation current 30 μA, analytical column]

	Valine	Leucine	Isoleucine
Detection limit	5.3 μmol/dm^3 (0.62 mg/dm^3)	5.0 μmol/dm^3 (0.66 mg/dm^3)	5.0 μmol/dm^3 (0.66 mg/dm^3)
Determination limit	16.0 μmol/dm^3 (1.87 mg/dm^3)	15.0 μmol/dm^3 (1.97 mg/dm^3)	15.0 μmol/dm^3 (1.97 mg/dm^3)
Repeatability	2.1%	1.8%	1.9%
Recovery	97.0 ± 2.5%	97.8 ± 1.4%	97.6 ± 1.7%

Table 2 Calibration data of converted valine, leucine, and isoleucine

	Regression equation (L/s, c/mmol dm^{-3})	R^2	Linearity range/mmol dm^{-3}
Valine	$L = 152.3c + 4.3$	0.9985	Min 0.016–1
Leucine	$L = 181.1c + 0.02$	0.9994	Min 0.015–1
Isoleucine	$L = 184.6c + 0.04$	0.9992	Min 0.015–1

conversion products, and the addition of α-cyclodextrine exhibited better results. The effect of presence of α-cyclodextrine in the leading electrolyte is evident from Fig. 5, where with the higher concentration of α-cyclodextrine increased the detector response of leucine, isoleucine, and of terminating electrolyte (acetate) as well. The most significant influence of α-cyclodextrine is to isoleucine conversion product. Optimum amount 20 mmol/dm^3 of α-cyclodextrine for separation of isoleucine and leucine was selected based on the maximum distances between signals. Isotachophoreogram of converted standard mixture, which contain 0.1 mmol/dm^3 valine, 0.1 mmol/dm^3 leucine, and 0.1 mmol/dm^3 isoleucine and is shown in Fig. 6.

Real sample analysis

Developed method was tested on food supplements containing L-valine, L-leucine, and L-isoleucine (see "Experimental" part). Their content in sample was determined by two methods—calibration curve and standard addition (50 mg of each relevant hydroxy acid was added to 50 cm^3 volumetric flask with converted sample). It was found that contents of all amino acids agree with declared contents. Better results were achieved with standard addition method. Measured data for sample are given in Table 3.

Determination of other amino acids

Under abovementioned optimized conditions, other encoded amino acids were converted and analyzed. Number of isotachophoretic zones, their lengths, and behavior of solution during conversion were the most monitored parameters. Conditions for conversion and isotachophoretic separation were not optimized, and it is probable that after optimization better results could be achieved. The most of amino acids provided minimally one isotachophoretically determined product. However, it must be mentioned, that the situation is more complicated with rising number of products as follow: proline and lysine (two products), methionine and tyrosine (three), and cysteine (four). Products of tryptophan conversion (two) and histidine conversion (one) cannot be detected in given system; however, they can be analyzed in different systems (pH 7.0 of leading electrolyte: 10 mmol/dm^3 HCl + imidazole; terminating electrolyte: 10 mmol/dm^3 HEPES). Furthermore, guanidine group in arginine structure is probably as resistant as it cannot be converted to anion.

In determination of those amino acids providing only one measurable product (Fig. 7), different relative heights of signal (*RSH*) were obtained for most of them. Leucine with isoleucine and glutamic acid with glutamine were the exceptions. Glutamic acid and glutamine gave the same product—2-hydroxyglutaric acid. Leucine and isoleucine

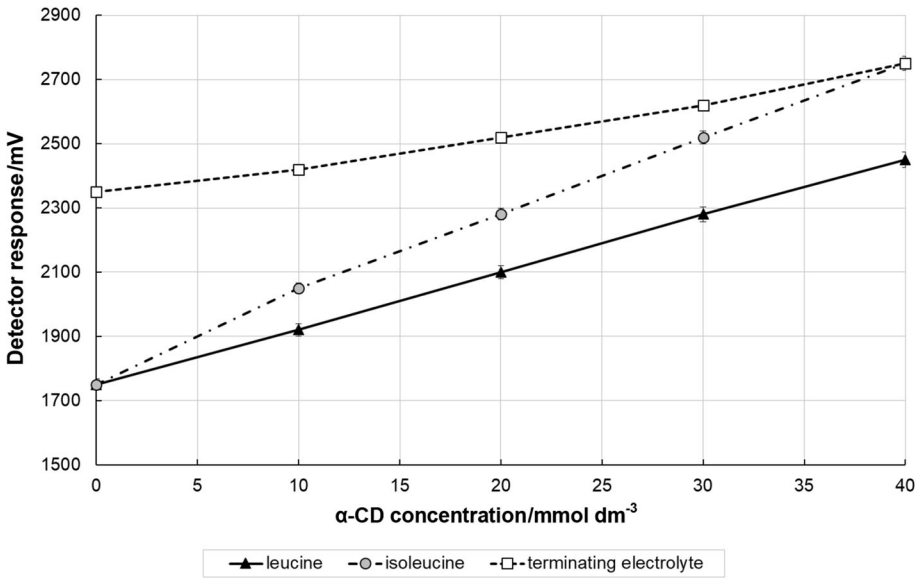


Fig. 5 Dependence of conductivity detector response on α-cyclodextrine concentration in leading electrolyte (pH 3.6)

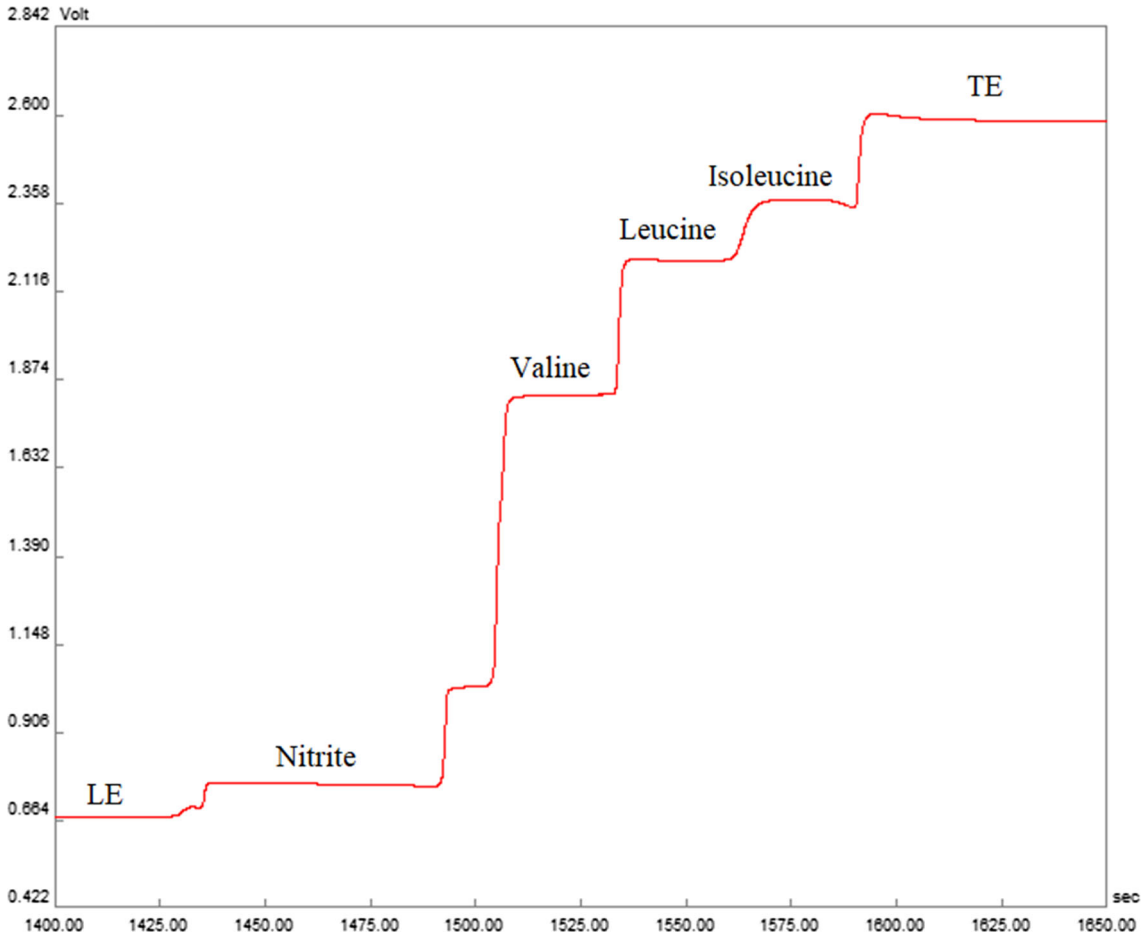


Fig. 6 Isotachophoreogram of standard mixture containing 0.1 mmol/dm^3 valine, 0.1 mmol/dm^3 leucine, and 0.1 mmol/dm^3 isoleucine after conversion, *LE*: 0.01 mol/dm^3 HCl + β-alanine (pH 3.6), *TE*: 0.01 mol/dm^3 acetic acid, analytical column

Table 3 Measured and declared content of amino acids in BCAA Mega Capsules

Analyte	Calibration curve	Standard addition	Declared content
Valine/mg/tablet	134.4 ± 4.1	157.7 ± 4.9	150
Leucine/mg/tablet	282.6 ± 13.4	311.0 ± 4.5	300
Isoleucine/mg/tablet	467.1 ± 7.2	465.8 ± 5.5	450

after conversion is possible to separate with some addition of α-cyclodextrine to leading electrolyte as it was mentioned.

However, those amino acids having more than one conversion product (Fig. 8) complicated the analysis, because some products created mixed zone (products which create mixed zone have the same color in Figs. 7 and 8, for example, conversion product of glycine and first conversion product of proline have the same *RSH*; therefore, they are marked with yellow color; conversion products marked with light grey color have not got the same *RSH*s with others). For that reason, developed method is not suitable for determination of all encoded amino acids in one analysis. Fortunately, relatively simple samples, especially food supplements, can be analyzed by the ITP.

Conclusion

Isotachophoretic determination of amino acids converted to corresponding hydroxy acids was done. Optimum concentration of sodium nitrite and acetic acid was 100 mmol/dm^3. As the most suitable way necessary for already mentioned amino acids conversion, boiling water bath for 10 min simultaneously with stirring was used; hence, under these conditions, the conversion efficiency was almost 100%. Optimal separation of corresponding hydroxy acids

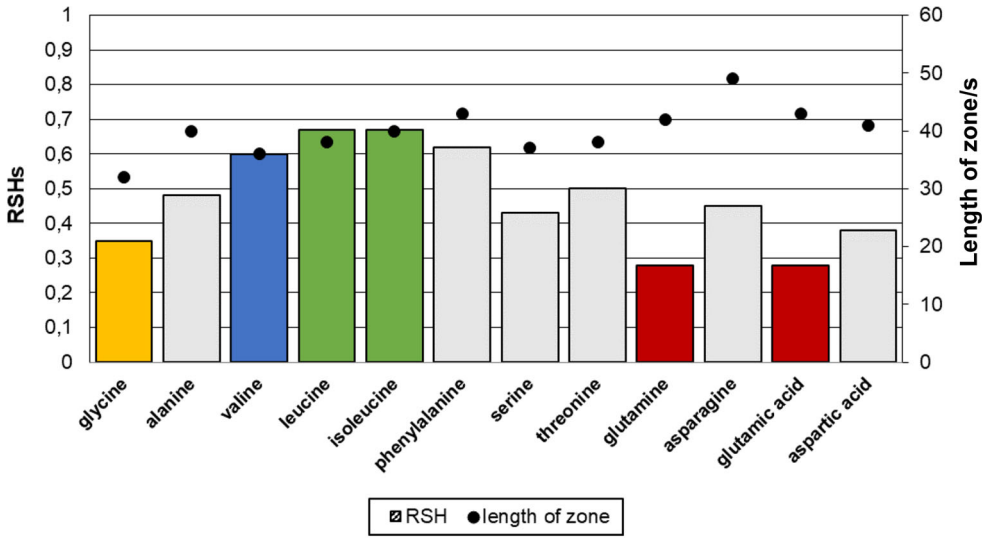


Fig. 7 Amino acids (all 0.25 mmol/dm^3) with one conversion product—their relative signal highs (*RSH*s) and lengths of zones in analytical capillary

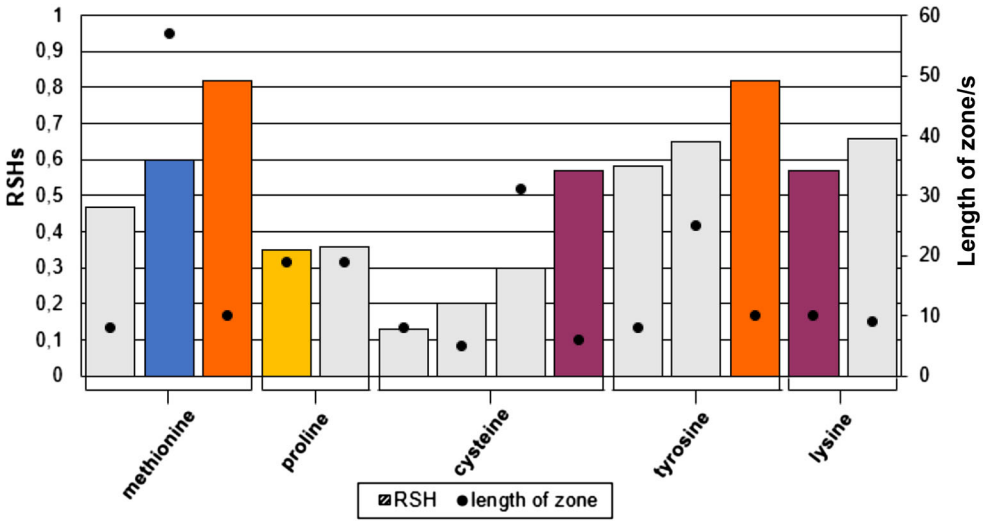


Fig. 8 Amino acids (all 0.25 mmol/dm^3) with the more conversion products (two, three, or four)—their *RSH*s and lengths of zones in analytical capillary

was performed in the system containing: 0.01 M HCl, 0.05% HEC, and β-alanine (pH 3.6) as the leading electrolyte and 0.01 M valeric acid and sodium hydroxide (pH 7.24) as the terminating electrolyte. Parameters of the method for determination of valine, leucine, and isoleucine were determined. It was found that limit of determination for these three amino acids was about 2 mg/dm^3 and reproducibility of measurements was lower than 3% with recovery close to 100%. The method was tested on real sample of commercially available capsules which contained valine, leucine, and isoleucine showing that the obtained content is in good agreement with the declared one. Method is suitable for analysis of simpler mixture which contains most of amino acids, but it is complicated by the presence of methionine, proline, cysteine, tyrosine, and lysine. These five amino acids during conversion formed more isotachophoretically determined products which disturb analysis of remaining amino acids. This problem can be maybe solved by use of different pH electrolyte systems, where some products may not be visible for isotachophoresis. To conclude, the developed method is not discerning on sample preparation and is suitable for determination of most of amino acids in the various food supplements.

Experimental

All used amino acids, 2-hydroxyisocaproic acid, both catexes (strong catex Amberlite IR-120 and weak catex Amberlite IRC86), and both cyclodextrines (α- and β-cyclodextrine) were purchased from Sigma-Aldrich (St.

Louis, USA). Sodium nitrite, acetic acid (99%), sulfuric acid (98%), hydrochloric acid (36%), urea, amidosulfonic acid, valeric acid, and sodium hydroxide was purchased from Lachema (Brno, Czech Republic). Hydroxyethyl cellulose (4000) and β-alanine were purchased from Serva (Heidelberg, Germany). Deionized water was used to dilution of solutions. As a real sample, we used commercially available food supplement BCAA Mega Capsules, Amino 1000 (declared content 150 mg of L-valine, 300 mg of L-leucine, 450 mg of L-isoleucine per tablet, producer Vitalmax s.r.o., Frýdlant v Čechách, Czech Republic).

Amino acid conversion

Amino acid (less than 1 mmol) was quantitatively transferred into 50 cm^3 volumetric flask. In addition, 5 cm^3 of 1 mol/dm^3 sodium nitrite and 5 cm^3 of 1 mol/dm^3 acetic acid were added into the same volumetric flask. In the end, whole flask was filled in by deionized water. It was placed on the plate of magnetic stirrer and boiled in water bath at 100 °C for 10 min. After that, this solution was 20 times diluted before isotachophoretic analysis (ITP).

Sample preparation

One capsule of BCAA Mega Capsules was dissolved in 100 cm^3 of distilled water in volumetric flask. 5 cm^3 of the solution was given into 50 cm^3 volumetric flask, 5 cm^3 of 1 mol/dm^3 acetic acid, and 5 cm^3 of 1 mol/dm^3 sodium nitrite was added. Volumetric flask was placed into water bath on plate of magnetic stirrer and was stirred and boiled for 10 min. After this, solution was cooled and afterwards ten times diluted and analyzed by ITP.

Instrumentation

EA 102 (Villa-Labeco, Spišská Nová Ves, Slovakia) was used as isotachophoretic analyser. This analyser is equipped with two polytetrafluorethylene capillary columns—pre-separation (160 × 0.8 mm) and analytical (160 × 0.3 mm). Individual zones are detected by conductivity detector. Isotachophoreograms were evaluated in a software ITP Pro supplied with the analyzer.

Analysis was performed in anionic mode with leading electrolyte (*LE*) containing 10 mmol/dm^3 hydrochloric acid, 0.05% hydroxyethyl cellulose (HEC), with β-alanine (pH 3.6) and terminating electrolyte (*TE*) consisting of 10 mmol/dm^3 valeric acid with sodium hydroxide (pH 7.24). Sodium hydroxide was added to increase of valeric acid solubility. Separation of leucine and isoleucine was performed in anionic mode with electrolyte system consisting of 10 mmol/dm^3 hydrochloric acid, 20 mmol/dm^3 α-cyclodextrine, and β-alanine as leading electrolyte of pH 3.6 and 20 mmol/dm^3 acetic acid as terminating electrolyte. Acetic acid was used to acceleration of analysis, because it has higher mobility than valeric acid. Use of valeric acid as terminating electrolyte is necessary only for determination of methionine and tyrosine, because they have lower mobility than acetic acid. Analyses were performed in analytical column. The driving current used for the pre-separation capillary was 250 μA and for the analytical capillary 50 μA, respectively. During detection, last mentioned current was decreased to 30 μA. Each analysis required maximally 35 min.

RSH values were calculated according to the following formula:

$$RSH = \frac{\text{analyte signal high} - LE \text{ signal high}}{TE \text{ signal high} - LE \text{ signal high}}.$$

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