Isotachophoretic Determination of Amino Acids After Their Conversion to Hydroxy Acids

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Abstract
Determination of amino acids by capillary isotachophoresis is presented. Developed method is suitable for separation of amino acids from relatively simple mixtures, especially for dietary supplements. The most of amino acids is not possible to separate directly by isotachophoresis. Thus, they had to be converted to other compounds. Chemical conversion to corresponding hydroxy acids in presence of nitrite acid was used. This necessary nitrite acid was prepared by reaction of sodium nitrite with acetic acid. Under optimization, concentration 0.1 mol/L sodium nitrite and acetic acid was found. As the best electrolyte system for separation of hydroxy acids, 0.01 mol/L HCl, 0.05% hydroxyethyl cellulose, with β-alanine (leading electrolyte of pH = 3.6) and 0.01 mol/L valeric acid with sodium hydroxide (terminating electrolyte of pH = 7.24) were chosen.

1. Introduction

Nowadays, it is generally known that consumers, producers, and supervisory bodies 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 are developed. This study was focused on determination of amino acids. These biologically active compounds are necessary for human nutrition because they represent the basic building component of proteins.

Many various methods for determination of amino acids exist. High-performance liquid chromatography and capillary electrophoresis are the most popular separation techniques for simultaneous determination of amino acids. Moreover, several papers also deal with isotachophoretic determination of amino acids [1–3].

Generally, amino acids are ionized in water solutions, but their molecules are almost electroneutral due to presence of positive and negative charge. For that reason, mobility of amino acids is low in electric field. This phenomenon causes

impossibility of direct separation. There are some exceptions: amino acids with two –COOH groups (glutamic and aspartic acid) and basic amino acids (histidine, lysine, and arginine) can be separated by isotachophoresis without pre-treatment, because their mobility in electric field is high enough.

One of the possibilities how to separate amino acids by isotachophoresis can be their conversion to hydroxy acids using nitrite acid

$$HOOC–CHR–NH_2 + HNO_2 \rightarrow HOOC–CHR–OH + N_2 + H_2O \quad\quad (1)$$
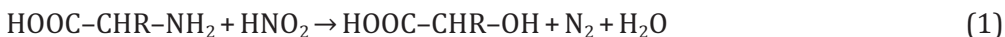
This reaction of primary amino groups [4] was used.

2. Experimental

2.1 Reagents and chemicals

All used amino acids (L-valine, L-leucine, L-isoleucine, L-tyrosine, L-lysine, L-tryptophan, L-arginine, L-histidine, L-cysteine, L-serine, L-alanine, glycine, L-phenylalanine, L-asparagine, L-glutamine, L-glutamic acid, L-aspartic acid, L-threonine, L-proline) and α-cyclodextrine were purchased from Sigma-Aldrich (USA). Sodium nitrite, acetic acid (99%), sulfuric acid (98%), hydrochloric acid (36%), valeric acid, and sodium hydroxide was purchased from Lachema (Czech Republic). Hydroxyethyl cellulose (4000) and buffer β-alanine were purchased from Serva (Germany). Deionized water was used to dilution of solutions.

2.2 Amino acids conversion

Amino acid (less than 1 mmol/L) was quantitatively transfered into 50 mL volumetric flask. Additionally, 5 mL of 1 mol/L sodium nitrite and 5 mL of 1 mol/L 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 minutes. After that, this solution was twenty times diluted before isotachophoretic analysis.

2.3 Instrumentation

EA 102 (Villa-Labeco, 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 analyser.

Analysis was performed in anionic mode with leading electrolyte consisting 10 mmol/L hydrochloric acid, 0.05% hydroxyethyl cellulose, with β-alanine (pH = 3.6) and terminating electrolyte consisting 10 mmol/L valeric acid with

sodium hydroxide (pH = 7.24). Separation of leucine and isoleucine was performed in anionic mode with electrolyte system consisting 10 mmol/L hydrochloric acid, 20 mmol/L α-cyclodextrine, with β-alanine as leading electrolyte of pH = 3.4 and 20 mmol/L acetic acid as terminating electrolyte. 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 45 min.

3. Results and discussion

### 3.1 Conversion of amino acids to hydroxy acids

Working conditions necessary for amino acids conversion had to be optimized. Whole optimization was done on non-polar amino acid leucine.

Concentration of sodium nitrite was the first parameter which was optimized. Six concentrations of sodium nitrite were selected, such as 10, 20, 50, 100, 200, and 400 mmol/L. For achievement of acidic conditions to form nitrite acid, 100 mmol/L acetic or 100 mmol/L sulfuric acid was used. From Fig. 1 it is evident that acetic acid represents better choice because conversion efficiency was higher by 15% at acetic acid. The lowest concentration of sodium nitrite to achieve the highest efficiency was 100 mmol/L for acetic acid. However, maximal efficiency about 85% at concentration 200 mmol/L sodium nitrite was found for sulfuric acid.

Selection of suitable acid was the next optimized parameter. Sulfuric, acetic, and valeric acid were tested. Dependence of leucine conversion efficiency on these acids of concentrations 5, 10, 20, 40, 100, 200, and 400 mmol/L was measured. It was found that sulfuric acid is not suitable for this purpose, because maximum
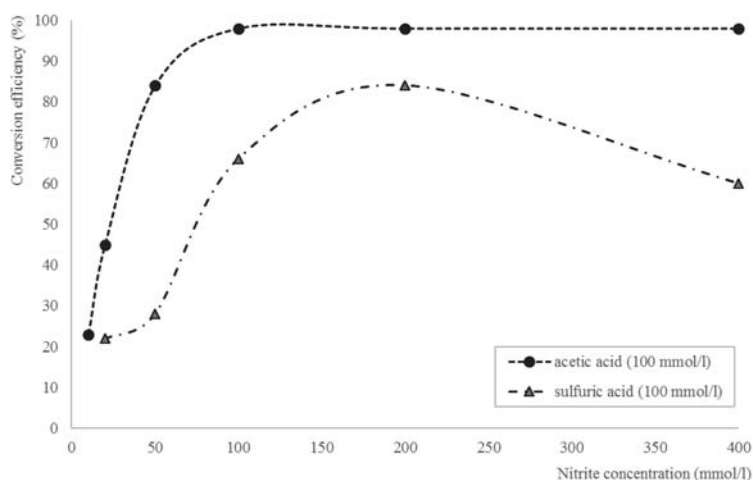
Fig. 1 Dependence of leucine conversion efficiency on sodium nitrite concentration for acetic and sulfuric acid.
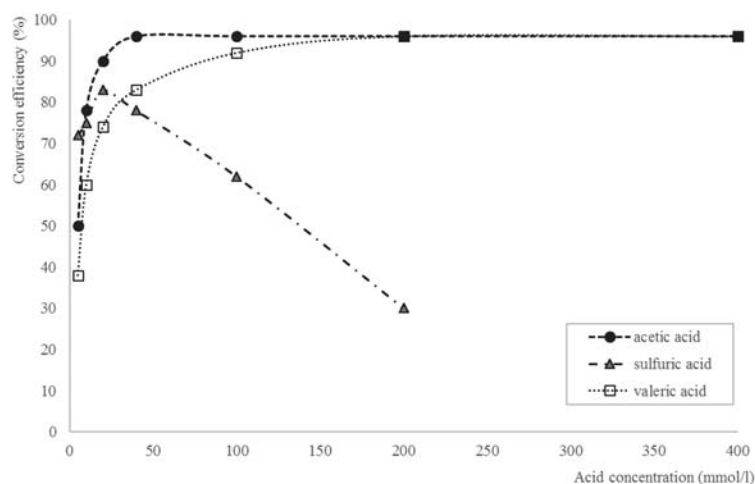
Fig. 2 Dependence of leucine conversion efficiency on acid concentration for different acids (concentration of sodium nitrite 100 mmol/L).

of conversion efficiency was about 85 %. Moreover, higher concentration than 20 mmol/L sulfuric acid significantly decreased the efficiency, what is clear from Fig. 2. The conversion efficiency using acetic, and valeric acid was much better (about 99% for both of them) than in previous case. Optimum concentration of acetic acid was 50 or 100 mmol/L and of valeric acid 200 mmol/L to achieve maximum efficiency about 99%. One of other significant advantages is fact that these acids are also composition of terminating electrolyte.

At laboratory conditions, the kinetic of conversion is very slow. Evidently, yields of this chemical reaction would be very low, which could significantly affect the sensitivity of the final analytical method. Ways how to increase the reaction rate in the favor of corresponding products were tested.

Mixture of leucine solution, 100 mmol/L sodium nitrite, and 100 mmol/L acetic acid was only stirred for different times. After certain time (2, 5, 10, 20, 50, 100, 200 minutes), 5 mL of reaction mixture were twenty times diluted, analysed by isotachophoresis and conversion efficiency was measured.

Moreover, the same experiment was repeated with one exception where the stirring was replaced to ultrasonic bath. As the last combination, stirring of boiled reaction mixture was used. The best conversion efficiency about 99% was achieved by stirring and boiling together for time duration longer than 10 minutes, what is demonstrated in Fig. 3 (next page). Unfortunately, itself stirring or using of ultrasonic bath was not sufficient; efficiency lower than 90%.

3.2 Isotachophoretic separation

Isotachophoretic separation of twenty encoded amino acids after conversion to corresponding hydroxy acids was performed in already mentioned anionic mode.
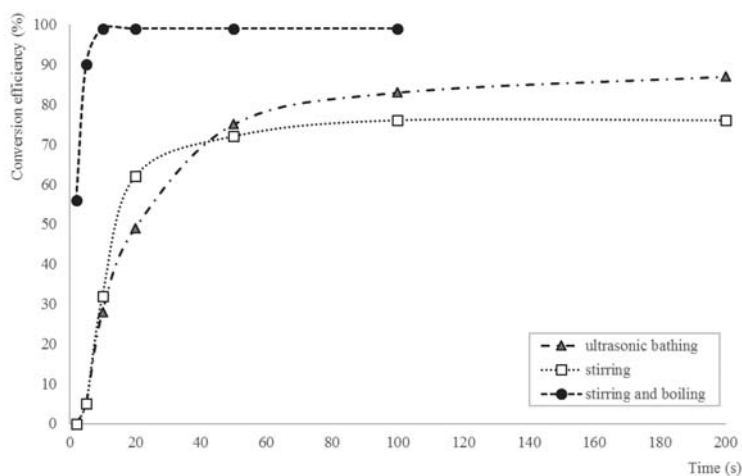
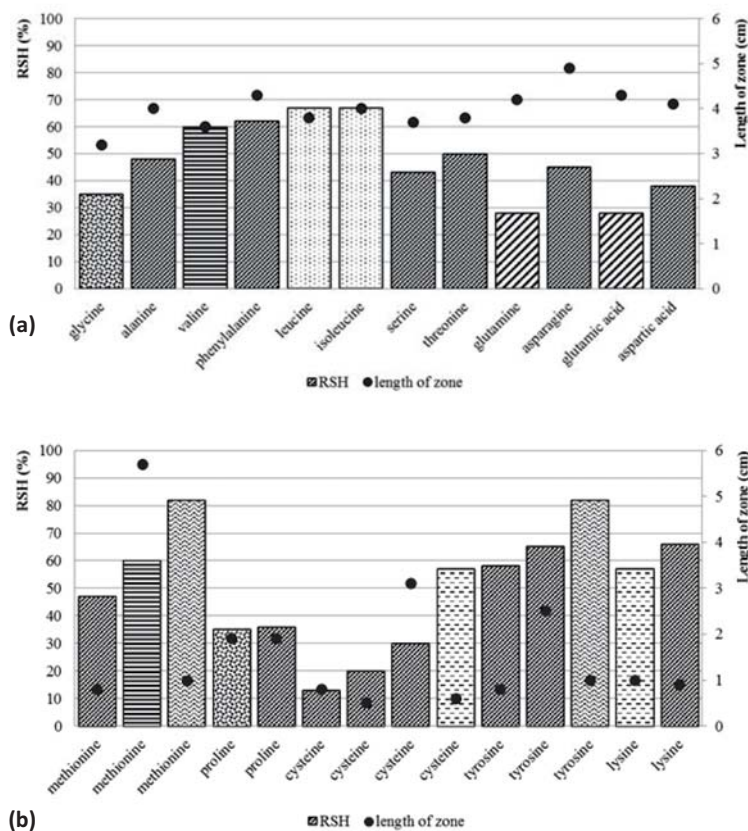**Fig. 3** Relationship between leucine conversion efficiency and time for different reaction supports.

Fig. 4 Relative signal highs (RSHs) and lengths of zones in analytical capillary of (a) amino acids (0.25 mmol/L) with one conversion product, and (b) amino acids (0.25 mmol/L) with the more conversion products (two, three or four).

The most of amino acids provided minimally one isotachophoretically determined product. However, it should be state that reality is more complicated as follow: proline and lysine (two), methionine and tyrosine (three), cysteine (four), and tryptophan, arginine and histidine (no products).

In determination of amino acids providing only one measured product (Fig. 4a), different heights of signal were obtained for each product. 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 after conversion is possible to separate with some addition of α-cyclodextrine to leading electrolyte. Therefore, several electrolyte systems with different concentration of α-cyclodextrine in the leading electrolyte were tested. As the best choice, 10 mmol/L hydrochloric acid, 20 mmol/L α-cyclodextrine, and β-alanine (pH = 3.4) was selected. 20 mmol/L acetic acid was used as terminating electrolyte.

Effect of presence of α-cyclodextrine in the leading electrolyte is evident in the Fig. 5. With the higher concentration of α-cyclodextrine increased the detector response of leucine, isoleucine, and also of terminating electrolyte. Optimum amount of α-cyclodextrine for separation of isoleucine and leucine were chosen based on the maximum distances.

However, it should be mentioned that amino acids having more than one conversation product (Fig. 4b) complicate the analysis because some products create mixed zone (products which create mixed zone have the same pattern in Fig. 4). For that reason, developed method is not suitable for analysis of all encoded amino acids. Fortunately, relatively simple samples, especially dietary supplements, can be analyzed by isotachophoresis.
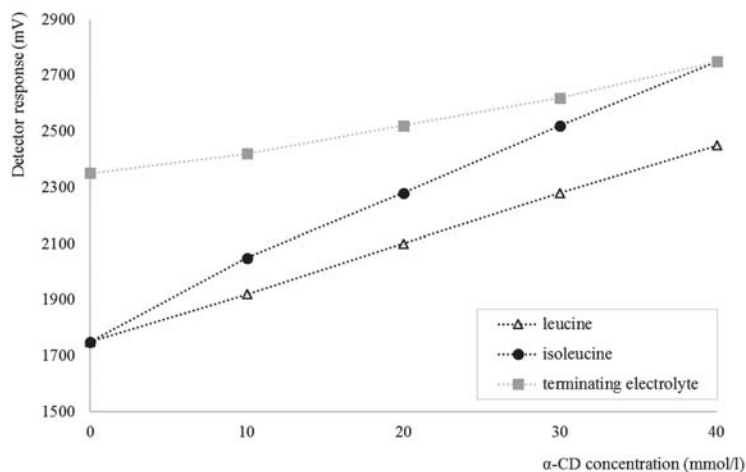
Fig. 5 Dependence of conductivity detector response on α-cyclodextrine concentration in leading electrolyte (pH = 3.4).

4. Conclusions

Isotachophoretic determination of amino acids converted to corresponding hydroxy acids was done. Optimum concentration of sodium nitrite and acetic acid was 0.1 mmol/L. As the more suitable way necessary for amino acids conversion, boiling in water bath for 10 minutes with simultaneous stirring was used. Under these conditions, the conversion efficiency was almost 100 %. Satisfactory separation of hydroxy acids was observed in the 0.01 M HCl, 0.05% hydroxyethyl cellulose 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. To conclude, it can only be said that developed method is not difficult for sample preparation and is completely suitable for determination of amino acids in the various food supplements.

References

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