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Identification of α-amino acids by hydrophilic interaction chromatography-tandem mass spectrometry in fertilizers

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Hydrophilic interaction chromatography with a triple quadrupole mass spectrometric detector (MS/MS) operating in a multiple reaction monitoring mode was applied for identification of 21 free α-amino acids in fertilizer samples. The electrospray ionization source (ESI) was set to positive ionization. ESI-MS/MS parameters were optimized individually for each analyte under direct standards infusion. Among three hydrophilic interaction chromatography stationary phases studied (Atlantis HILIC, Acquity BEH HILIC, and Acquity BEH Amide), the acquity BEH Amide phase showed the best performance. Complete separation of all α-amino acids was achieved in 15 min by gradient elution with a water / acetonitrile mobile phase containing 3 mmol/L ammonium acetate, 3 mmol/L ammonium formate and 20 mmol/L formic acid additives. The developed HILIC-MS/MS method was applied for identification of free α-amino acids in four biological fertilizer products of natural origin.

Key words: hydrophilic interaction chromatography, tandem mass spectrometry, amino acids, fertilizer

INTRODUCTION

Growing demand of food all over the world requires not only effective mineral fertilizers containing micro and macro nutrients, but also physiologically active growth stimulants [1]. In past decades natural physiologically active compounds such as citric acid, humates, amino acids and other were started to use in agriculture [1]. The use of amino acids during plant growth is a well-known method to increase crop yield and quality, beside that amino acids may form chelates with trace elements and rear earth minerals which can kill bacteria and insects and decrease an amount of residual pesticides [2]. Usually amino acid based fertilizers are recommended to use under critical growth periods: after transplantation, during the flowering period and at climatic stresses. They are particularly effective when used in combination with microelements due to their chelation properties as chelated nutrients are more plant available than complexed or uncomplexed ones [1].

Up to this date there have been several qualitative and quantitative approaches for amino acids analysis. By far, the most popular ones are chromatographic [3–11] and electromigration [12] methods. However, most of them include pre- or post-column derivatization due to weak response to conventional detectors and high analytes polarities. Thus derivatization reagents such as ethyl chloroformate were used for gas chromatographic analysis followed by mass spectrometry (MS) [9] or flame ionization [11] detection. Jia et al. [4] suggested the ultra performance liquid chromatography (UPLC) method for the separation of 23 amino acids and 7 biogenic amines on reversed phase (RP) sorbent with pre-column derivatization with dansyl-chloride. Other RP-LC method using online derivatization with o-pthaldialdehyde followed by fluorescence detection was developed by Zhao and co-workers [3]. For the separation of free amino acids in the RP-LC mode an ion-pairing reagent is needed due to their

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weak retention on the hydrophobic stationary phase [5, 7]. A complicated mobile phase containing pentafluoroheptanoic, trifluoroacetic and formic acids was applied to separate 16 amino acids by Samy et al. [5]. Alarcon-Flores and co-workers [7] separated 19 underivatized essential amino acids on the RP stationary phase by adding pentadecafluorooctanoic acid to the mobile phase as an ion-pairing reagent.

While an additional derivatization step might be time-consuming and an ion-pairing agent in the mobile phase would affect MS detectability, hydrophilic interaction chromatography (HILIC) is an attractive alternative for separation of underivatized small polar analytes [13, 14]. In HILIC retention is based on strong hydrophilic interaction between the hydrophilic stationary phase and polar analytes, which makes this mode an ideal option for amino acids analysis. Moreover, higher organic content in the mobile phase is favoured by an electrospray ionization (ESI) source for better sensitivity [6, 8]. In recent years few articles have been published on amino acids separation in the HILIC mode. Xu and co-workers [8] on the TSK-GEL Amide stationary phase separated 15 amino acids from rat serum. Yao [6] quantified 20 amino acids in *Ginkgo Biloba* leaves employing a novel UPLC system coupled with tandem mass spectrometric (MS/MS) detection.

This paper provides separation and identification of 21 free α-amino acids using HILIC coupled with tandem mass spectrometry. As far as we know, this is the first HILIC-MS/MS approach for identification of all essential α-amino acids in fertilizers samples.

EXPERIMENTAL

All separations were performed on the Agilent 1290 Infinity UPLC system followed by Agilent 6410 Triple Quad (Agilent Technologies, USA) tandem mass spectrometric detection. The UPLC system comprised a binary pump, automatic degasser, column heater and autosampler. The MS/MS system was equipped with an ESI source operating in the positive ion mode. Three columns obtained from waters were tested for amino acids separation performance: Acquity BEH HILIC (100 × 2.1 mm, I. D., 1.7 μm), Acuity BEH Amide (100 × 2.1 mm, I. D., 1.7 μm), and Atlantis HILIC (100 × 2.1 mm, I. D., 3.0 μm). The injection volume was 2 μL. Data collection and processing was performed with a MassHunter 5.0 (Agilent). A mechanical KdScientific (Holliston, MA, USA) syringe was used for direct MS analyses.

L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, glycine, L-glutamine, L-glutamic acid, L-histidine, L-hydroxyproline, L-leucine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L- tyrosine, L-valine (Fig. 1) were purchased from Sigma Aldrich (St. Louis, MO, USA) as the L-amino acid

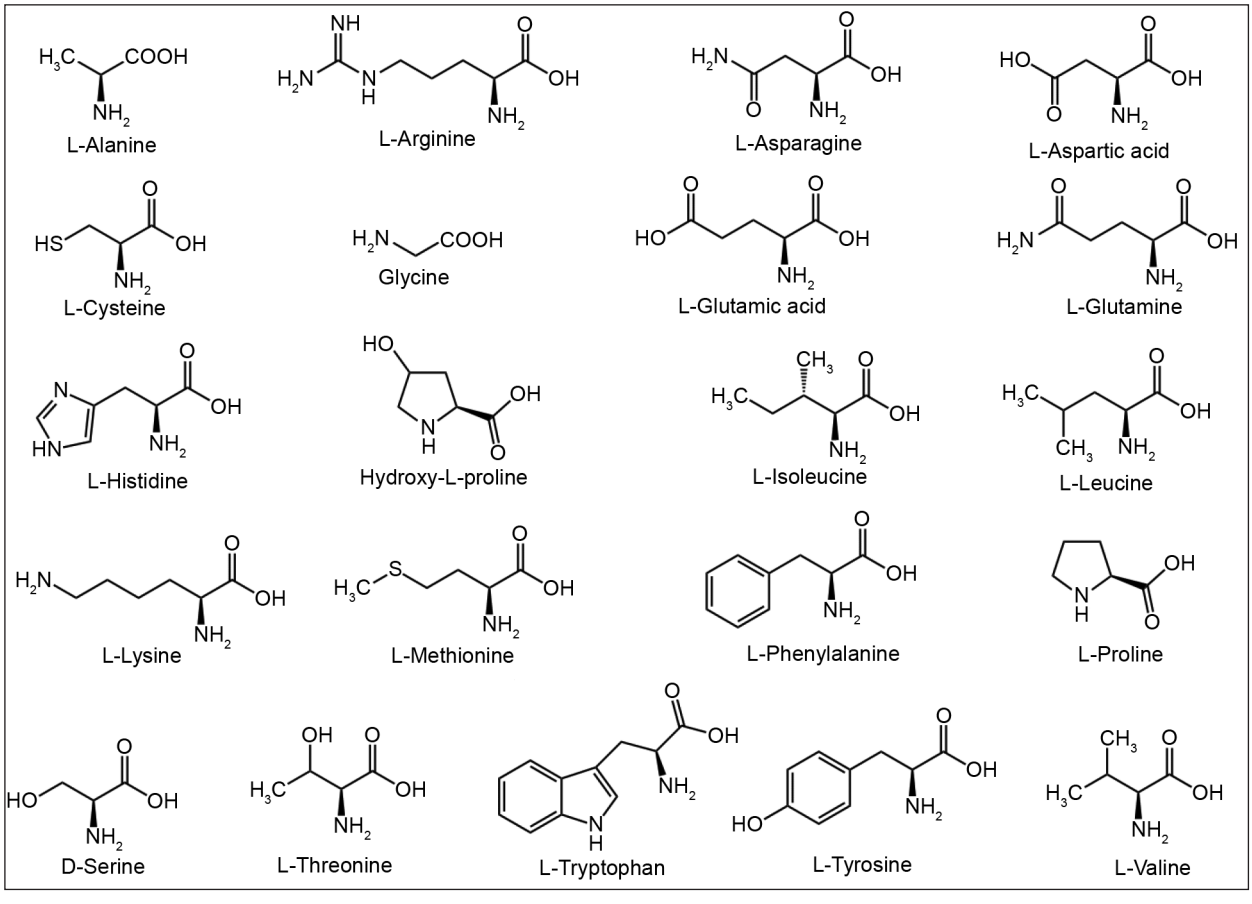

Fig. 1. Chemical structures of the investigated α-amino acids

Table 1. **Optimized MRM transition parameters for the analyzed amino acids. Two most intense MRM transitions are presented for each analyte**

Amino acid	Abbreviation	Precursor ion	MRM transition	Fragmentor voltage, V	Collision energy, eV
Alanine	Ala	90	**90 → 44**[a]	70	10
Arginine	Arg	175	**175 → 70**	90	25
			175 → 116		11
Asparagine	Asn	133	**133 → 74**	70	13
			133 → 87		4
Aspartic acid	Asp	134	**134 → 74**	70	12
			134 → 88		5
Cysteine	Cys	122	**122 → 76**	70	11
			122 → 87		9
Glutamic acid	Glu	148	**148 → 84**	70	14
			148 → 130		4
Glutamine	Gln	147	**147 → 84**	70	16
			147 → 130		5
Glycine	Gly	76	**76 → 30**	70	4
			76 → 48		3
Histidine	His	156	**156 → 110**	100	12
			156 → 95		15
Hydroxyproline	Hyp	132	**132 → 68**	100	22
			132 → 86		10
Isoleucine	ILe	132	**132 → 69**	90	16
			132 → 86		5
Leucine	Leu	132	**132 → 86**	90	6
			132 → 69		18
Lysine	Lys	147	**147 → 84**	90	16
			147 → 130		5
Methionine	Met	150	**150 → 104**	80	6
			150 → 133		4
Phenylalanine	Phe	166	**166 → 120**	90	10
			166 → 103		22
Proline	Pro	116	**116 → 70**	60	14
Serine	Ser	106	**106 → 60**	70	8
			106 → 42		18
Threonine	Thr	120	**120 → 74**	70	7
			120 → 102		3
Tryptophan	Trp	205	**205 → 188**	75	2
			205 → 146		2
Tyrosine	Tyr	182	**182 → 136**	60	8
			182 → 165		3
Valine	Val	118	**118 → 72**	50	7
			118 → 55		23

[a] – The selected identification transitions are in bold.

standard kit. Ultra LC-MS acetonitrile was purchased from Carl Roth GmbH & Co (Karlsruhe, Germany), ammonium acetate, formic acid, acetic acid and methanol (all LC-MS grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

A mass analyzer was operating in a multiple reaction monitoring (MRM) mode and both quadrupoles were set at unit mass resolution. ESI parameters were set as follows: capillary voltage 3 kV, gas temperature 300 °C, gas flow 7 L/min, nebulizer 30 psi. MRM parameters were set as presented in Table 1, cell accelerator voltage and dwell time were kept constant for all analytes and were set at 6 eV and 20 ms, respectively.

Stock solutions were prepared at 1 mg/L concentration in an aqueous 1 mol/L formic acid solution; tyrosine was additionally acidified with 0.1 mol/L HCl for better solubility and stored at 4 °C temperature, protected from light. Working standard solutions were prepared daily by diluting stock solutions in the mobile phase.

RESULTS AND DISCUSSION

MS/MS parameters

The ionization source and MS parameters were optimized manually via a direct infusion of the standard solution of 10 mg/L of each amino acid at 180 μL/h flow rate. The

working standard solutions were prepared by diluting stock solutions in a water / acetonitrile (1:1, v/v) solution prior to the analysis. Full scan MS spectra showed the most abundant protonated $[M+H]^+$ ions for all amino acids. The first quadrupole was set to a single ion monitoring mode for the selected protonated precursor ion and the fragmentor voltages (from 50 V to 150 V) were tuned individually to each amino acid. Then product ion MS experiments were acquired and from the collision induced dissociation (CID) spectra most abundant transition ions were selected. Only one transition was monitored for alanine and proline due to their low molecular weights. The rest of amino acids produced at least two MRM transitions each. Most of the amino acids gave MRM transitions corresponding to loss of formic acid $[M\text{-}HCOOH+H]^+$ or formic acid and ammonia $[M\text{-}HCOOH\text{-}NH_3+H]^+$, whereas Arg and Trp led to formation of $[M\text{-}104+H]^+$ and $[M\text{-}NH_3+H]^+$ ions, respectively. The collision energies were also tuned manually by monitoring product ion intensity in a MRM mode in the range from 2 to 30 eV. All optimal transitions as well as fragmentor voltages and collision energies for the investigated amino acids are presented in Table 1.

HILIC separation

In order to achieve the optimum separation three different HILIC columns were tested. As expected, the bare silica Atlantis HILIC (3 μm particle size) stationary phase showed the strongest retention and lowest efficiency. Even with higher elution strength mobile phases basic analytes (pI ≥ 7.6) were relatively strongly retained and a significant peak tailing was observed, which made this stationary phase inconvenient for further optimization. In both, BEH HILIC and BEH Amide stationary phases considerably better overall separation performance was observed. This is not surprising because new generation Waters UPLC BEH type columns contain smaller particles (1.7 μm). In addition, in this type silica based stationary phases the bridging ethylene groups are embedded into the silica matrix and nearly one third of the surface silanols is removed [15]. BEH Amide column was selected for further optimization due to slightly better resolution and lower peak tailing obtained in this phase.

Next, various mobile phase additives, namely acetic and formic acids, ammonium acetate and ammonium formate, were tested in terms of retention, resolution and MS signal response. The results showed that none of the individual mobile phase additive can provide acceptable resolution of all acids. This is illustrated in Fig. 2 for the separation of the isobaric Ile/Leu amino acid pair. Only combination of three mobile phase additives (formic acid, ammonium formate and ammonium acetate) together with the gradient elution resulted in suitable resolution of all acids. The final mobile phase composition was as follows: A – aqueous solution containing 20 mmol/L formic acid, 3 mmol/L ammonium formate and 3 mmol/L ammonium acetate; B – 20 mmol/L formic acid, 3 mmol/L ammonium formate, 3 mmol/L ammonium acetate in water / acetonitrile 10/90 (v/v). The gradient elution program: 0–7 min 95% B to 90% B; 7–15 min 90% B to 70%; within the next minute returns to the initial mobile phase composition and equilibrates for 4 min prior to next injection. The representative chromatogram of all 21 analytes is shown in Fig. 3. Despite the fact that even under optimized conditions some acids (e. g., Asp and His) exhibit

Table 2. **Analysis results of commercially available biological fertilizer products of natural origin. + is indicated if the analyte was identified, n. d. – not detected**

Amino acid	Sample 1	Sample 2	Sample 3	Sample 4
Ala	+	+	+	+
Arg	+	+	+	+
Asn	+	n. d.	+	n. d.
Asp	+	+	n. d.	n. d.
Cys	n. d.	n. d.	n. d.	n. d.
Gly	+	+	+	n. d.
Gln	+	n. d.	n. d.	n. d.
Glu	+	+	n. d.	n. d.
His	+	+	+	+
Hyp	+	+	n. d.	n. d.
Ile	+	+	+	+
Leu	+	+	+	+
Lys	+	+	+	+
Met	+	+	n. d.	n. d.
Phe	+	+	+	+
Pro	+	+	+	+
Ser	+	+	+	+
Thr	+	+	+	+
Tyr	+	+	+	+
Trp	+	+	+	+
Val	+	+	+	+

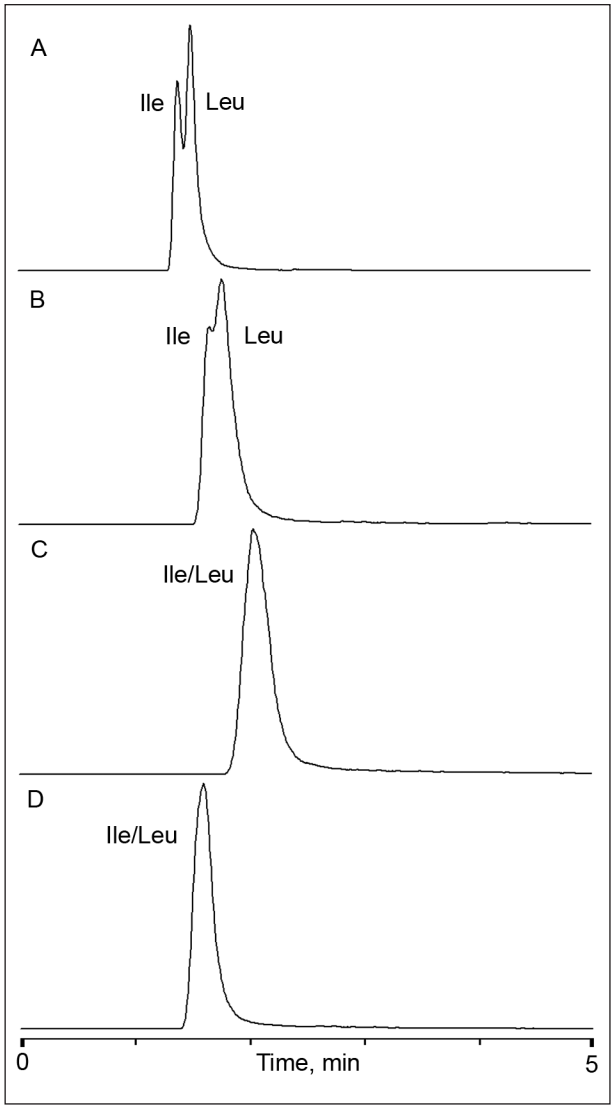

Fig. 2. Separation of isobaric amino acids with different mobile phase modifiers. Isocratic elution with the 20:80 (v/v) H_2O/CH_3CN mobile phase containing: A – 10 mmol/L ammonium acetate, B – 10 mmol/L ammonium formate, C – 10 mmol/L acetic acid, D – 10 mmol/L formic acid

broad and tailing peaks, the obtained overall performance is adequate for identification purposes.

Sample analysis

Most of the protocols employed for the extraction of amino acids from natural sources are based on the extraction with acidified aqueous or aqueous / organic solutions [5–7]. In this study three acids (HCOOH, CH_3COOH and HCl) as additives to aqueous / acetonitrile 1:5 (v/v) extractant were briefly compared for the best extraction properties. The results showed that for the same samples acetic acid provided slightly higher peak areas for most of the analyzed amino acids. The optimized extraction protocol was as follows: 0.50 g of the crude fertilizers sample was extracted with 10 mL of an aqueous / acetonitrile (1:5, v/v) solution containing 1 mol/L CH_3COOH in an ultrasonic bath for 10 min, then 1:1

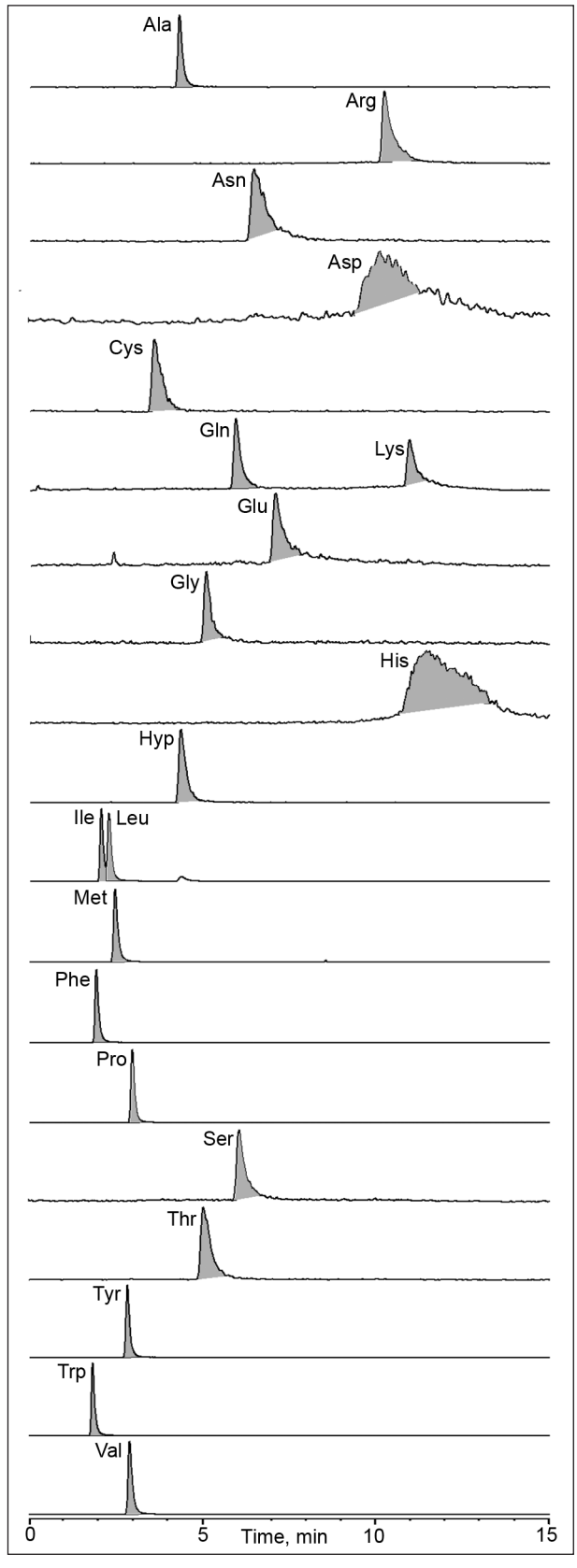

Fig. 3. Optimized HILIC-MS/MS MRM chromatograms of 21 α-amino acids on the BEH Amide stationary phase. Mobile phase: A – aqueous solution containing 20 mmol/L HCOOH, 3 mmol/L $HCOONH_4$, and 3 mmol/L CH_3COONH_4; B – 20 mmol/L HCOOH, 3 mmol/L $HCOONH_4$, 3 mmol/L CH_3COONH_4 in H_2O/CH_3CN 10/90 (v/v). Gradient elution: 0–7 min 95% B to 90% B; 7–15 min 90% B to 70%

(v/v) diluted with acetonitrile and centrifuged for 2 min at 10 000 rpm. 1 mL of the supernatant was filtered through a 0.2 μm nylon syringe filter and analyzed.

The developed method was applied to identify free α-amino acids in biological fertilizer products of natural origin. The results obtained are presented in Table 2.

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AMINORŪGŠČIŲ IDENTIFIKAVIMAS TRĄŠOSE HIDROFILINĖS SĄVEIKOS CHROMATOGRAFIJOS-TANDEMINĖS MASIŲ SPEKTROMETRIJOS METODU

S a n t r a u k a

Hidrofilinės sąveikos chromatografija su tandeminiu trigubo kvadrupolio masių spektrometriniu detektavimu daugialypių reakcijų stebėjimo režime pritaikyta 21-os alfa aminorūgšties identifikavimui komercinėse trąšose. Detektavimas buvo atliekamas teigiamos elektropurkštuvinės jonizacijos sąlygomis. Jonizacijos šaltinio ir masių analizatoriaus parametrai kiekvienai analizei buvo optimizuoti tiesioginio įleidimo būdu. Palyginus aminorūgščių atskyrimą trimis skirtingomis hidrofilinės sąveikos kolonėlėmis (Atlantis HILIC, BEH HILIC ir BEH Amide), nustatyta, kad BEH Amide kolonėlė pasižymi geriausiu bendru efektyvumu. Visos rūgštys visiškai atskiriamos per 15 min. naudojant gradientinę eliuciją vandens / acetonitrilo judria faze su 3 mmol/L amonio acetato, 3 mmol/L amonio formiato ir 20 mmol/L skruzdžių rūgšties priedu. Sukurtas metodas pritaikytas aminorūgščių identifikavimui keturių skirtingų gamintojų natūralios kilmės biologinėse trąšose.