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Determination of Free Proteinogenic Amino Acids in Soil Solutions by HPLC with Phenyl Isothiocyanate Derivatization

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A high-performance liquid chromatography method for the determination of seventeen free proteinogenic amino acids (FPAA) in soil solutions is described. Sample preparation involves obtaining soil solution and *pre-column derivatization of free amino acids using phenyl isothiocyanate. Phenyl thiocarbamyl derivatives are separated on a reversed-phase column by using linear gradient elution with 140 mM sodium acetate buffer solution (pH 6.0) contained 0.05% triethylamine as mobile phase A and acetonitrile-water (60:40,v/v) as mobile phase B. The described method showed adequate linearity, recovery values, limits of detection, and suitable for the determination of FPAA in soil solutions.*

Keywords: amino acid derivatization, soil solution, free amino acids, amino acids in soil, phenyl isothiocyanate, HPLC.

Определение свободных протеиногенных аминокислот почвенного раствора методом ВЭЖХ с предколоночной дериватизацией фенилизотиоцианатом

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Описан способ определения семнадцати свободных протеиногенных аминокислот почвенного раствора методом высокоэффективной жидкостной хроматографии. Пробоподготовка

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включает в себя получение почвенного раствора и предколоночную дериватизацию свободных аминокислот фенилизотиоцианатом. Хроматографическое разделение производных аминокислот проводили на обратнофазной колонке в линейном градиенте при использовании системы, состоящей из буфера, приготовленного добавлением 0,5 мл/л триэтиламина к 0,14 М ацетату натрия (pH 6,0) и смеси ацетонитрил-вода (60:40). Описанный способ имеет удовлетворительные аналитические характеристики и приемлем для определения концентраций свободных протеиногенных аминокислот в почвенном растворе.

Ключевые слова: дериватизация свободных аминокислот, почвенный раствор, свободные аминокислоты.

Introduction

Concentrations of free proteinogenic amino acids (FPAA) in soil solutions are important factors to determine [1, 2]. For this purpose the HPLC method is used, which is a powerful tool in the analysis of many components in soil solution (organic acids, sugars, amino acids, etc.) [1, 3-6].

However, the determination of free amino acids (FAA) can be complicated by the influence of the complex sample matrixes of the sample [7, 8]. The soil solution is also a complex system and has a large number of organic and mineral components [9, 10], which can affect the results of the quantification. Besides, in order to effectively detect amino acids with ultraviolet or *fluorescence detectors*, they are usually requires a chemical modification (derivatization) step [11]. Phenyl isothiocyanate (PITC) is one of the most widely used reagents for the derivatization of FAA in biological fluids (blood plasma, root exudates et al.) [7, 12, 13]. It reacts with primary and secondary amino acids and gives the consistent phenyl thiocarbamyl (PTC) derivatives that can be detected by the UV-detector on wavelength of 254 nm [14].

In this paper, we describe a method for qualitative and quantitative determination of FPAA in soil solutions by HPLC with pre-column PITC derivatization.

Experimental

Chemicals

L-Amino acids, phenyl isothiocyanate (PICT) and triethylamine purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were from Component Reactive (Moscow, Russian). Reagent-grade sodium acetate, sodium phosphate, *glacial* acetic acid and hydrochloric acid were from Ecopharm (Kazan, Russian). Water was obtained using a Milli-Q water system from Millipore (Bedford, MA, USA).

Soil Sampling, Soil Solution Obtaining and Preparation of Standards

Soil samples were obtained from A horizons of Haplic Luvisol (*classified* according to WRB soil classification system) [15] in the Tytushsky region of the Republic of Tatarstan, Russian (55°68′ N, 49°13′ E). Air dried and sieved finer than 1 mm samples (20 g) were placed into a plastic tube (11 cm diameter and 7 cm high) and saturated with 6 mL of Milli-Q water. After 72 hour of incubation at room

temperature soil solutions were obtained by vacuum through a filter paper. Extracted solutions were filtered by a 0.22-μm pore membrane.

A stock solution containing a mixture of 18 amino acid standards was prepared in 0.1 M HCl at an individual concentration of 2.5 mmol L^{-1}. Calibration solutions at different concentrations (1.5, 7.5, 15, 30, 120, 300, 600 μmol L^{-1}) were prepared by serially diluting appropriate amounts of the stock solution with 0.1 M HCl.

Derivatization

The pre-column derivatization procedure used was a modified version of the reported Refs.[11,14]. A standard solution (20 μL) or 400 μL of soil solution was dried under vacuum at 65°C in small plastic tube (10 × 50 mm). The dry residue was neutralized by adding 30 μL of a 2:2:1 mixture of methanol-triethylamine-water (v/v), mixing well and drying under vacuum. Derivatization was performed by adding 30 μL of a mixture of 7.5:1:1:0.5 methanol- triethylamine-water-PITC (v/v). The tubes were mixed well and allowed to stand at room temperature for 20 min. After derivatization, the tubes were vacuum-dried at 45°C. Prior to injection, the residue was resuspended with 300 μL of 5 mM sodium phosphate buffer (pH 7.4) contained 5% acetonitrile and filtered through 0.22-μm pore membrane.

Instrumentation and Chromatographic Conditions

The HPLC analysis was carried out on a Flexar HPLC system (Perkin Elmer, USA) which consisted of a Flexar Binary LC Pump and a Flexar UV/Vis detector. Separation of amino acid derivatives was performed on a *Brownlee Analytical* C_{18} *column*, 150 mm × 4.6 mm, 5 μm (Perkin Elmer, USA). PTC derivatives of amino acids are separated on a reversed-phase column by using linear gradient elution with 140 mM sodium acetate buffer solution (*adjusted* to pH 6.0 with *glacial acetic acid*) contained 0.05% triethylamine as mobile phase A and acetonitrile-water (60:40,v/v) as mobile phase B. The gradient elution program is shown in Table I. Wavelength of UV/Vis-detector was 254 nm, and the injection volume was 20 μL.

Results and discussion

The overall time required for the analysis of one sample of soil solution is 72 min, of which 35 min is spent on the derivatization step and 37 min - on the chromatographic separation. A typical calibration standard chromatogram containing 7.5 μmol L^{-1} of each amino acid (each peak corresponds to 10 pmol

Table 1. Gradient elution program for the separation of PTC derivatives of amino acids

Time (min)	% Eluent A	% Eluent B
0	90	10
14.0	70	30
24.0	52	48
30.0	0	100
34.0	0	100
37.0	90	10

Table 2. Precision of measurement, recoveries and detection limits obtained for each amino acid (n=6)

Amino acid	Precision of measurement CV [a] (%)	Recovery (%)	Detection limit (pmol amino acid injected to column)
Asp ac	0.47	76.0	0.33
Glu ac	0.63	90.3	0.42
Asn	1.05	110.1	0.25
Ser	1.94	101.7	0.33
Gln	1.11	94.9	0.32
His	0.98	97.7	0.46
Arg	0.45	99.5	0.48
Thr	0.55	97.2	0.35
Ala	0.99	107.9	0.48
Pro	1.23	102.1	0.43
Tyr	1.11	107.1	0.39
Val	1.15	109.7	0.25
I-leu	0.86	105.7	0.38
Leu	0.90	102.2	0.35
Phe	1.54	106.7	0.40
Trp	1.90	107.3	0.22
Lis	1.87	93.1	0.32

[a] Coefficient of variation (calculated from the ratios of peak area)

Table 3. Concentrations of free proteinogenic amino acids in soil solution

Amino acid	Concentrations[a] (µmol L^{-1}, n = 6)
Asp ac	3,10±0.06
Glu ac	8.15±0.37
Asn	0,71±0.17
Ser	8.61±3.63
Gln	BQL[b]
His	4.03±0.090
Arg	8.78±0.05
Thr	10.68±0.09
Ala	15.21±0.29
Pro	6.02±0.63
Tyr	24.75±0.74
Val	11.56±0.16
I-leu	3.20±0.34
Leu	4.27±0.09
Phe	2.25±0.14
Trp	0.66±0.14
Lys	0.51±0.018
TPFAA[c]	112,46±7.09

[a] The concentrations are presented as mean ± standard deviation
[b] below the limit of quantification
[c] Total proteinogenic free amino acids

of amino acid) appears in Fig. 1a. The column gradient elution program described in [11] has been optimized for the separation of amino acids on the *Brownlee Analytical* C_{18} *column* (150 mm × 4.6 mm, 5 μm) and provided in Table 1.

The validation of analytical procedure was performed according to EURACHEM [16]. Linearity of the peak areas for different concentrations, ranging from 2 - 800 picomoles of individual amino acids (calibration solutions containing 1.5 - 600 μmol L^{-1} of each amino acid) were determined. Correlation coefficients for these data exceeded 0.987. Recoveries of amino acids from soil solutions were evaluated by measuring the concentration of each amino acid in soil solution spiked with three

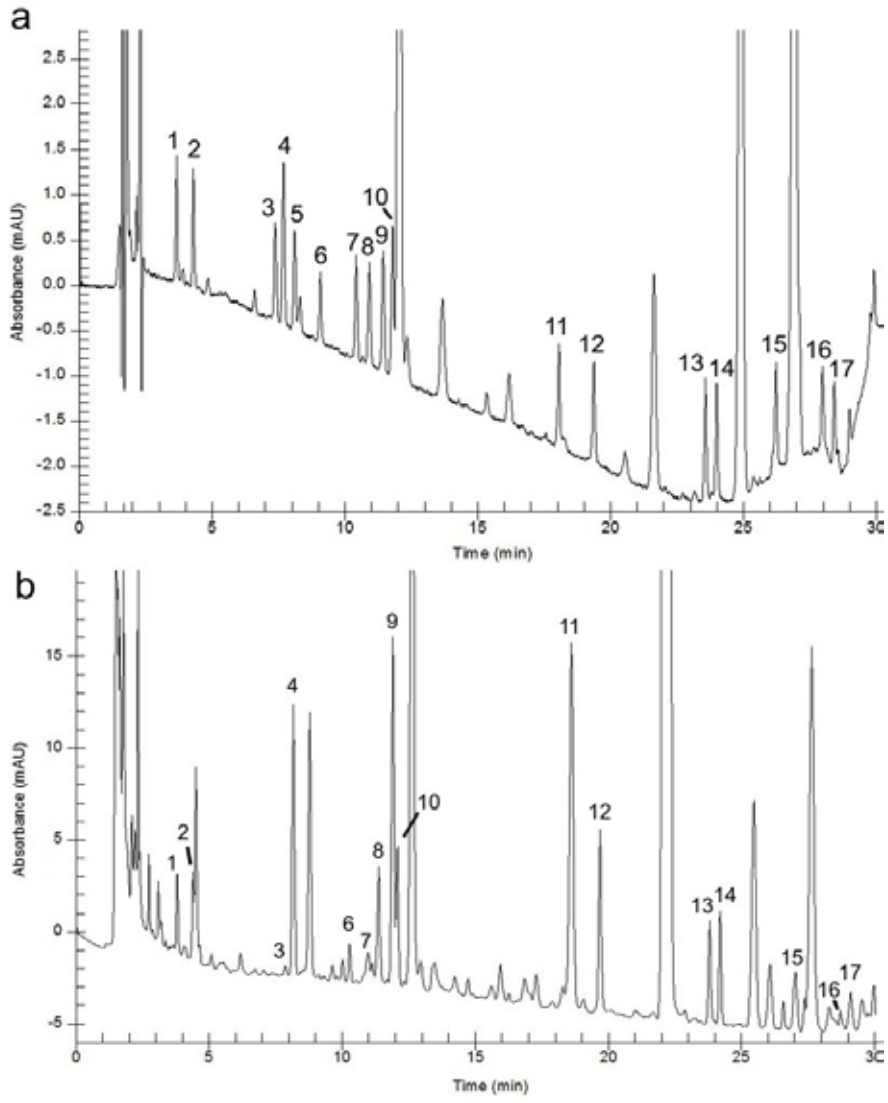

Fig. 1. Chromatograms of (a) PTC derivatives of amino acids in a standard solution containing 7.5 μmol L^{-1} of each amino acid; (b) soil solution. Peak identification (1) aspartic acid (Asp ac), (2) glutamic acid (Glu ac), (3) asparagine (Asn), (4) serine (Ser), (5) glutamine (Gln), (6) histidine (His), (7) arginine (Arg), (8) threonine (Thr), (9) alanine (Ala), (10) proline (Pro), (11) tyrosine (Tyr), (12) valine (Val), (13) isoleucine (I-leu), (14) leucine (Leu), (15) phenylalanine (Phe), (16) tryptophan (Trp), (17) lysine (Lys)

different levels (7.5, 30, 300 μmol L^{-1}) of standard solution. The recoveries were ranged from 90.3% to 109.7% for most of amino acids (Table 2). For aspartic acid it was around *76*%. In order to receive much higher recoveries of aspartic acid, suggested to use a nitrogen stream rather than heating under vacuum to drive off the excess derivatization reagent [11]. The high recoveries of most amino acids shows, that matrices of obtained soil solutions did not have a significant effect on the derivatization step, but it is possible, the PITC method can be affected by high concentrations of salts in solutions [8], obtained, for example, from *saline soils*. The detection limit values for free amino acids were low, between 0.22 and 0.48 pmol of amino acid injected to column (14.6-32 nmol L^{-1} of soil solution). The average recoveries (n=6), the detection limits (signal-to-noise ratio=3) and the precision of measurements (for 30 μmol L^{-1} of standard solution) of 17 amino acids are shown in Table 2. Intraday (n=6) and interday (n=3) precision values not exceeded 9%. (n =6).

In soil solutions except proteinogenic amino acids there are a lot of other organic components (aminosugars, nonproteinogenic amino acids), which can react with PITC [7, 17], giving the derivatives that can be detected by the UV-detector and appearing on the chromatogram as peaks. Thus, despite a large number of unknown peaks the column gradient elution program allowed to *determine* all 17 amino acids in educated soil solutions (Fig.1b). However, the content of glutamine has been below the determination limit. The total free proteinogenic amino acid (TFPAA) concentrations in soil solutions was 112.4±7.09 μmol L^{-1}, which is consistent with the studies [1, 18]. The major amino acids were tyrosine and alanine (22.0 and 13.5% respectively from TFPAA).

Conclusion

The method described in this paper was suitable for determining free proteinogenic amino acids in soil solutions. It includes *soil solution obtaining***,** pre-column derivatization of free amino acids with PITC, and determination PTC derivatives of amino acid with HPLC. Matrices of educated soil solutions did not have a significant effect on the quantitative determination results.

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References

1. Jones D.L., Shannon D., Junvee-Fortune T., Farrar, J.F. Plant capture of free amino acids is maximized under high soil amino acid concentrations. *Soil Biol. Biochem. 2005*. Vol. 37. P. 179–181.

2. Owen A.G., Jones D.L. Competition for amino acids between wheat roots and rhizosphere microorganisms and the role of amino acids in plant n acquisition. *Soil Biol. Biochem. 2001*. Vol. 33. P. 651–657.

3. Yu Z., Zhang Q., Kraus T.E.C., Danhlgren R.A., Anastasio C., Zasoski R.J. Contribution of amino compounds to dissolved organic nitrogen in forest soils. *Biogeochemistry 2002*. Vol. 61. P. 173–198.

4. Patrick A.W. van Hees, Jones D.L., Jentschke G., Godbold D.L. Organic acid concentrations in soil solution: effects of young coniferous trees and ectomycorrhizal fungi. *Soil Biol. Biochem. 2005*. Vol. 37. P. 771–776.

5. Martens D.A., Frankenberger W. T., Jr. Soil saccharide extraction and detection. *Plant Soil 1993*. Vol. 149. P. 145–147.

6. Kögel I. Reversed-phase high-performance liquid chromatography of naturally occurring phenolic acids in soils. *J. Plant Nutr. Soil Sci. 1983.* Vol. 146. P. 525–530.

7. Fernandez-Figares I., Rodriguez L.C., Gonzalez-Casado A. Effect of different matrices on physiological amino acids analysis by liquid chromatography: evaluation and correction of the matrix effect. *J. Chromatogr. 2004.* Vol. 799. P. 73–79.

8. Lai F., Mayer A., Sheehan T. Matrix effects in the derivatization of amino acids with 9-fluorenylmethyl chloroformate and phenylisothiocyanate. Biotech. 1991. Vol. 11. P. 236–44.

9. Roman R., Caballero R., Bustos A. Variability of soil solution ions in fallowland fields in central spain. *Edafologia 2002.* Vol. 9. P. 161–172.

10. Takeda A., Tsukada H., Takaku Y., Hisamatsu S. Fractionation of metal complexes with dissolved organic matter in a rhizosphere soil solution of a humus-rich andosol using size exclusion chromatography with inductively coupled plasma-mass spectrometry. *Soil Sci. Plant Nutr. 2009.* Vol. 55. P. 349–357.

11. Gonzalez-Castro M.J., Lopez-Hernandez J., Simal-Lozano J., Oruna-Concha M.J. Determination of amino acids in green beans by derivatization with phenylisothiocianate and high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. Sci. 1997.* Vol. 35. P. 181–185.

12. Marco S., Hjalmar P.P., Letty A.W., Carel A.W., Ben J.J.L. Amino acid synthesis is necessary for tomato root colonization by pseudomonas fluorescens strain WCS365. *Mol. Plant Microbe In. 1997.* Vol. 10. P. 102–106.

13. Hariharan M., Naga S., Van Noord T. Systematic approach to the development of plasma amino acid analysis by high-performance liquid chromatography with ultraviolet detection with precolumn derivatization using phenyl isothiocyanate. *J. Chromatogr. 1993*. Vol. 621. P. 15–22.

14. Fierabracci V., Masiello P., Novelli M., Bergamini E. Application of amino acid analysis by high-performance liquid chromatography with phenyl isothiocyanate derivatization to the rapid determination of free amino acids in biological samples. *J. Chromatogr. 1991.* Vol. 570. P. 285–91.

15. IUSS Working Group WRB. *World Reference Base for Soil Resources. World Soil Resources Report*; FAO, Rome, 2006; No. 103.

16. *EURACHEM* Working Group. *Analytical Methods: A Laboratory Guide to Method Validation and Related Topics;* United Kingdom, 1998.

17. Anumula K.R., Taylor P.B. Quantitative determination of phenyl isothiocyanate-derivatized amino sugars and amino sugar alcohols by high-performance liquid chromatography. Anal. Biochem. 1991. Vol. 197. P. 113–120.

18. Raab T.K., Lipson D.A., Monson R.K. Soil amino acid utilization among species of the cyperaceae: plant and soil processes. *Ecology 1999.* Vol. 80. P. 2408–2419.