JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Supercritical Fluid Extraction of Bioavailable Amino Acids from Soils and Their Liquid Chromatographic Determination with Fluorometric Detection

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A new procedure with supercritical CO_2 modified with 0.5 mL of water and 0.75 mL of 0.1 M HCl in situ and 0.75 mL of water on-line at 15 MPa and 50 °C for 45 min was applied for the extraction of bioavailable amino acids from soil samples. Total extraction time was 60 min, but more favorable conditions are even possible for selected groups of amino acids. All analytes were trapped into 20 mL of methanol with satisfactory recovery (94−104%) and determined using high-performance liquid chromatography with fluorometric detection on a Zorbax Eclipse column (4.6 × 75 mm, 3.5 μm) with Na_2HPO_4 and acetonitrile/methanol/water as a mobile phase. Linear calibration curves were obtained ($r > 0.999$ except 0.99823 for Ile) with lower limits of detection (S/N = 3) in the range from 1.54 pg (Gly) to 13.5 pg (Cy2) or from 18.6 fmol (Ser) to 64.8 fmol (Lys). Validation and repeatability data are also given. Comparable results were obtained with a robust, commonly used extraction method (0.5 M ammonium acetate, 60 min in shaker, followed by filtration and lyophilization). Limiting values of artificial release of amino acids were also determined for each soil sample to eliminate any false results to ensure that all extracted amino acids originate from soil solution and exchangeable bound positions of soil samples.

KEYWORDS: Supercritical fluid extraction; ammonium acetate extraction; bioavailable amino acids; real soil sample; high-performance liquid chromatography−fluorometric detection

INTRODUCTION

It has been known that amino acids can be directly taken up by plant roots without previous mineralization (*1*−*3*). These bioavailable (plant-available) amino acids are located in soil solution or are exchangeably adsorbed on soil particles. Generally, it was supposed that plants prefer mineral N forms (NH_4^+ and NO_3^-) against amino acid uptake if these forms are sufficiently released in soil (*4*−*6*). Advances in research showed that such an opinion is not fully legitimate, and that, on the other hand, some plants prefer amino acid nutrition even when mineral nitrogen is sufficiently available or that some plants take up particular nitrogen forms according to their availability in soil (*7*−*10*). From the point of total ecosystem nutrition, the significance of amino acids is high in unfavorable climatic and edaphic conditions (boreal, alpine, and arctic ecosystems), as calculations of mineralization rates in such conditions were not able to explain nitrogen needs for vegetation (*11*−*13*).

According to the soil type and the proximity to rhizosphere, amino acids are available in a concentration range from low micromolar to several millimolar (*14*). Bioavailable amino acids represent only a very small fraction of the total soil amino acid pool (*15*). They are a quite troublesome group of analytes to extract. They should be extracted under conditions not favorable to hydrolysis of peptide bonds and limiting the release of amino acids from inside microbial cells due to osmotic shock and microbial cell lyses. Consequently, it is desirable to limit amino acid degradation in the course of the extraction procedure.

Extraction of bioavailable amino acids from fresh soil samples has to be performed as soon as possible as amino acids' half-lives are very short even at low temperatures. The mean half-life in topsoil at 5 °C was reported to be only 2.9 h (*14*) and generally depends on many factors (*16*). Thus, the ideal extraction method for bioavailable amino acids must be quick and thrifty to soil microorganisms. In addition, it must prevent protein hydrolysis or any other form of amino acid enrichment of the sample, apart from the other demands for modern extraction methods (simple performance, environmental friendliness, low expense, and, most of all, robustness and quantitative recovery).

Nowadays, from a wide range of existing extraction methods, mostly liquid extraction (LE) with demineralized water in a shaker is used (*17*). Water extraction is not efficient to extract basic amino acids strongly adsorbed on the soil colloidal fraction

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10.1021/jf061255k CCC: $33.50
Published on Web 07/26/2006

or neutral ones from strongly acid soils (*18*). Some studies performed with the application of 0.5 M ammonium acetate extraction were more efficient and do not cause hydrolytic effect (*19*, *20*).

For a long time, supercritical fluid extraction (SFE) was considered to be unsuitable for polar analytes. Polar groups (−OH, −COOH) make the extraction difficult, although benzene derivatives with up to three hydroxyl groups are considered to be satisfactorily extractable. Amino acids and polar proteins were considered to be nonextractable. An attempt was made to block polar groups (−COOH, $-NH_2$) via *N*-benzyloxycarbonyl (N-CBZ) derivatization to increase the extractability of amino acids (*21*).

Quantitative extraction of polar molecules requires either long extraction time or very high flow rate to ensure sufficient mass transfer. Also, the polarity of a supercritical fluid must be shifted by means of a modifier addition, because pure CO_2 has a solvation power close to that of hexane and is an excellent solvent for nonpolar compounds. SE-1 extractor adaptation for a high CO_2 flow rate, developed for isoflavone extraction (*22*), providing a gas-phase flow rate of ≈750−950 mL/min with appropriate solvent trapping, is a promising alternative for amino acid extraction.

The aim of this work was (i) to find appropriate extraction conditions for SFE of amino acids from model spiked samples, (ii) to apply these conditions to real soil samples, and (iii) to verify that amino acid enrichment of soil samples cannot occur during the extraction either due to death of microorganisms or because of protein hydrolysis or any other amino acid producing or releasing mechanism. Common validation and reproducibility tests were also made.

MATERIALS AND METHODS

Chemicals. Amino acids standard (AA-S-18) was purchased from Sigma-Aldrich (St. Louis, MO), as well as HPLC grade acetonitrile (ACN), methanol (MeOH), and sodium phosphate. OPA reagent (*o*-phthalaldehyde and 3-mercaptopropionyl acid in borate buffer) was purchased from Agilent Technologies (Waldbronn, Germany). Standards were prepared by dissolution in RO Milli-Q water (Millipore, Bedford, MA) in a concentration range from 1:10 to 1:100. The solutions were stable for at least 1 week when stored in the dark at 4 °C. All solutions were filtered through a 0.45 μm Teflon membrane disk (MetaChem, Torrance, CA) prior to HPLC analysis, unless said otherwise. All other chemicals were of analytical grade.

Soil Samples. Soil samples were taken from a >10 years abandoned meadow (Ah horizon) and in differently managed forest stands (spruce, 99%; fir, 1%; H and Ae horizons) in the Moravian−Silesian Beskids Mountains. The soil type of the abandoned meadow is gleyic luvisol; entic and haplic podzols are present in the forest stands (*23*). Sampling was performed in the period of June−August 2005. Collected samples were sieved through 5 mm mesh and stored in a refrigerator in plastic bags until extraction procedures were started. The concentrations of amino acids determined in the course of this study do not constitute actual concentrations in the field, but are used to compare methodological approaches. Selected properties of soil samples are mentioned in **Table 1**.

Sample Preparation. Model samples were prepared by spiking 25, 30, 50, or 100 μL of standard solution onto clean glass wool. Prior to the standard solution, 0.5 mL of water was added in situ into the extraction cartridge. In some experiments, 0.25−1 mL of 0.1 M HCl was added as in situ modifier. To avoid damage of the stainless steel cartridge and direct influence of HCl to the spiked sample before the extraction start, HCl was added into the middle of a second glass wool lump, placed in the upper part of the cartridge. The lump was big enough not to let HCl soak to cartridge walls. At the extraction start, the upper lump of glass wool was pressed down and HCl was rinsed to the lower H_2O/spiked standard solution system.

Table 1. Selected Physical and Chemical Characteristics of Individual Soil Samples ($n = 6$)

soil property	H horizon	Ae horizon	Ah horizon
clay (%)		11.8	19.9
silt (%)		16.8	27.1
sand (%)		71.4	52.9
total C content (%)	23.0	6.7	5.56
total N content (%)	1.13	0.47	0.56
C/N	20.4	14.3	9.93
pH (H_2O)/pH 0.01 M $CaCl_2$	4.95/4.55	4.64/4.10	4.29/3.83
% of water	68.2	36. 8	31.1

Table 2. Calibration Data, LOD, and LOQ for Amino Acid Determination Using FLD Detection ($n = 10$)

	LOD		LOQ			
	fmol	pg	fmol	pg	calibration eq	correl coeff
Asp	44.3	5.9	147	19.6	$y = 3.426x + 0.092$	0.99996
Glu	55.4	8.2	184	27.2	$y = 3.375x - 0.264$	0.99996
Ser	18.6	2.0	61.9	6.5	$y = 4.997x - 0.420$	0.99995
His	36.5	5.7	121	18.9	$y = 2.322x - 0.157$	0.99979
Gly	20.5	1.5	68.3	5.1	$y = 4.280x + 0.313$	0.99994
Thr	25.3	3.0	84.4	10.1	$y = 3.705x - 0.204$	0.99998
Arg	25.3	4.4	84.3	14.7	$y = 3.482x - 0.096$	0.99986
Ala	24.5	2.2	81.5	7.3	$y = 3.849x - 0.262$	0.99996
Tyr	25.5	4.6	84.8	15.4	$y = 3.684x - 0.468$	0.99990
Cy2	56.0	13.5	186	44.8	$y = 2.327x - 0.075$	0.99901
Val	22.7	2.7	75.7	8.9	$y = 4.065x - 0.075$	0.99995
Met	30.0	4.5	99.8	14.9	$y = 3.626x + 0.115$	0.99991
Phe	24.6	4.1	81.9	13.5	$y = 3.721x - 0.031$	0.99992
Ile	21.8	2.9	72.5	9.5	$y = 4.033x + 1.355$	0.99823
Leu	22.4	2.9	74.5	9.8	$y = 4.038x - 0.055$	0.99991
Lys	64.8	9.5	216	31.6	$y = 1.441x - 0.184$	0.99919

Weights of real samples were 1.25 g of soil (the same as the equivalents from LE). Water and in situ modifier were not added to soil samples that were wet enough and contained the required amount of compounds acting as in situ modifiers. For the LE in the shaker the sample weight was 25 g. Both SFE and LE experiments for each soil sample started at the same time to minimize changes of amino acid content during the time.

Supercritical Fluid Extraction. The AA-S-18 standard solutions of amino acids were diluted with ultrapure water to the ratios of 1:10 and 1:100. In spiked samples, a given volume of amino acid solution was injected into the middle of a matrix bed (glass wool), with modifier added as described above. As for real samples, 1.25 g of soil was weighed into the extraction cell. In situ water was not added to real soil samples, which were wet enough, as the restrictor allows only a limited amount of water in the extraction cell. When the sample was injected or weighed and modifier was added into a 7 mL cartridge, it was immediately sealed and closed in the extraction cell heating block.

The extraction cells were cleaned in an ultrapure H_2O sonication bath and rinsed with MeOH. The inner space of an extraction cell heating block was also washed with 0.1 M HCl, water, and methanol. This eliminated or minimized cross-contamination dragged by the extraction phase modifier during depressurization after the extraction step. Blank samples were prepared by extracting the same amounts of the matrix.

All SFEs were carried out using an SE-l instrument (SEKO-K, Brno, Czech Republic) equipped with a Valco valve (VICI, Schenkon, Switzerland; loop 1 mL) for continuous modifier addition (*24*, *25*). The extraction cell was pressurized at 50−100 °C to 10−40 MPa using SFC/SFE grade CO_2 (Siad, Brno, Czech Republic) with 1−10% v/v of modifier mixture added via Valco valve to the instrument piston pump.

The modified supercritical fluid went through the extraction cartridge filled with the sample for 45 min (dynamic extraction). The extraction was prolonged for 15 min without modifier addition to get water out of the piston micropump. Extraction medium containing analytes was led through a fused silica restrictor (12 cm, 50 m i.d., gas flow rate of

Table 3. SFE: Optimization of Extraction Parameters ($n = 6$)[a]

A. Temperature (Recovery in Percent of Total Spiked Standard)

	40 °C	50 °C	60 °C	75 °C	100 °C	
ASP	85.3	**101.8**	101.6	64.5	41.3	Experiments were not performed at higher temperatures due to corrosive effect of HCl (teflone seals and filters decomposition)
GLU	79.6	**100.7**	100.3	61.2	43.3	
SER	103.6	102.3	**103.4**	101.8	34.6	
HIS	77.2	98.8	**101.6**	53.5	23.2	
GLY	101.2	98.3	100.8	**102.6**	40.8	
THR	102.8	**103.6**	95.4	68.4	31.2	
ARG	94.3	**98.4**	97.7	54.3	34.5	
ALA	**101.9**	97.9	96.6	64.1	34.2	
TYR	**104.1**	101.2	92.1	47.2	28.3	
CY2	90.7	**95.4**	76.4	63.8	19.9	
VAL	101.5	**102.1**	100.3	58.6	32.3	
MET	**103.4**	99.9	97.5	50.9	28.3	
PHE	80.1	**97.6**	91.2	46.3	19.8	
ILE	96.3	98.3	**101.0**	39.8	23.7	
LEU	92.8	94.2	**97.6**	42.3	26.0	
LYS	86. 7	**95.3**	91.1	52.6	16.6	

B. Pressure (Recovery in Percent of Total Spiked Standard)

	10 MPa	15 MPa	20 MPa	25 MPa	30 MPa	35 MPa	40 MPa
ASP	78.2	97.9	97.6	98.4	94.7	**98.1**	96.8
GLU	67.6	**98.9**	93.7	97.1	97.0	96.7	97.6
SER	**102.0**	101.1	95.3	100.2	101.0	98.5	99.0
HIS	52.6	97.1	93.7	96.7	98.2	95.7	**101.6**
GLY	101.5	101.5	101.2	**102.9**	100.1	100.6	97.1
THR	**104.5**	101.0	102.9	94.2	95.4	98.1	96.5
ARG	75.7	96.5	**98.1**	97.2	96.3	94.6	94.6
ALA	93.4	**97.4**	95.8	96.7	94.6	97.1	97.2
TYR	98.4	100.0	101.8	94.7	97.9	**102.8**	101.4
CY2	93.9	97.4	**100.9**	99.5	98.1	97.1	97.6
VAL	100.8	**103.7**	101.8	101.0	98.1	96.1	97.7
MET	100.8	**104.3**	103.4	97.8	103.6	100.4	99.7
PHE	74.7	94.9	96.9	**98.5**	97.3	98.3	96.3
ILE	96.7	97.3	98.2	100.0	97.1	**101.1**	100.7
LEU	88.7	96.4	94.3	97.9	**100.3**	94.1	96.3
LYS	71.6	**98.8**	95.8	95.6	97.5	95.0	94.4

C. In Situ Modifier/Entrainer (Recovery in Percent of Total Spiked Standard)

	1 ml HCl	0.85 ml HCl	0.75 ml HCl	0.65 ml HCl	0.5 ml HCl	0.25 ml HCl
ASP	64.8	72.3	94.2	97.7	**98.8**	53. 5
GLU	65.6	81.4	**97.5**	96.5	92.3	52.9
SER	82.1	94.8	**102.5**	99.1	100.8	77.4
HIS	90.3	94.2	**104.0**	98.9	99.4	59.3
GLY	87.6	93.3	**103.7**	86.2	72.3	76.5
THR	102.8	99.6	**104.9**	101.4	99.8	76.3
ARG	94.2	95.7	92.9	100.3	**103.6**	60.8
ALA	95.1	94.3	**96.7**	95.8	96.2	62.5
TYR	101.3	101.0	**103.0**	97.6	98.9	69.2
CY2	94.0	97.5	**100.7**	79.3	68.7	45.0
VAL	100.3	98.0	100.1	99.3	**103.6**	73.5
MET	101.6	**102.8**	98.9	92.6	85.7	60.8
PHE	99.7	**101.4**	100.5	87.4	71.7	53.8
ILE	**104.8**	97.5	97.0	94.4	90.1	65. 9
LEU	97.4	99.4	**101.9**	97.6	90.7	63.3
LYS	101.3	**102.1**	97.4	89.4	66.5	44.0

Table 3 (Continued)

D. On-Line Modifier/Entrainer (Recovery in Percent of Total Spiked Standard)

	1 ml H_2O	0.85 ml H_2O	0.75 ml H_2O	0.65 ml H_2O	0.5 ml H_2O	0.25 ml H_2O	0.1 ml H_2O
ASP	67.0	85.5	**99.9**	90.0	68.6	57.9	35.2
GLU	90.6	96.1	**99.5**	92.4	67.5	41.3	34.1
SER	85.4	93.1	**102.4**	96.4	89.9	102.0	60.0
HIS	77.8	86.6	97.7	**101.5**	85.0	35.4	22.7
GLY	102.1	**104.1**	99.6	100.5	98.2	100.7	55.7
THR	101.9	100.4	102.3	98.0	**102.6**	55.5	37.1
ARG	80.1	95.5	**96.6**	96.4	83.7	40.3	31.1
ALA	**102.6**	99.2	97.6	90.6	79.3	58.6	32.6
TYR	100.0	97.3	101.5	100.6	**101.7**	35.9	29.2
CY2	82.8	99.1	**100.4**	86.5	74.7	52.0	30.2
VAL	100.4	100.3	101.1	98.6	**102.0**	48.1	33.3
MET	101.7	99.6	**103.2**	100.4	101.3	25.3	16.2
PHE	77.7	86.6	97.5	**98.9**	95.8	27.8	18.2
ILE	95.6	97.6	99.1	**99.4**	98.8	34.1	23.3
LEU	92.3	97.3	98.7	**101.1**	97.3	35.0	25.5
LYS	73.3	81.3	95.4	97.5	**98.3**	28.6	13.4

[a] Shaded entries show the area of good extractability, maximal recoveries for individual amino acids are given in boldface numbers, and selected optimal condition value is highlighted in a bold box.

750−950 mL/min) into a liquid trap. Analytes were quantitatively trapped in 20 mL of a trapping solvent (methanol) at the laboratory temperature. A smaller amount of the methanol would be sufficient, but due to the higher gas flow rate a 50 mL glass flask with a flexible foil seal was used to prevent splashing the solvent out of the trapping vial and to achieve sufficient solvent column height.

Prior to the analyses, the extracts were preconcentrated in a rotary vacuum evaporator IKA RV 05-ST with a water bath HB 4 (all from IKA-Werke, Staufen, Germany), dissolved in 500 μL of 0.1 M HCl, and injected directly into the HPLC-FLD system (spiked samples, some real samples) or filtered through the 0.45 μm filter (MetaChem) before the injection (real samples, spiked real samples) to protect a HPLC column.

Ammonium Acetate Liquid Extraction. Five samples of each soil (weight 25 g) were extracted by 100 mL of 0.5 M ammonium acetate in 250 mL of polyethylene bottles. After 60 min of shaking, soil suspensions were filtered through paper filters at 6 °C. Ten milliliter aliquots of frozen extracts were lyophilized to dryness and dissolved in 500 μL of 0.1 M HCl and filtered through a nylon membrane filter (13 mm, 0.45 μm, Chromatography Research Supplies, Louisville, KY).

High-Performance Liquid Chromatography. The HPLC chromatographic system HP 1100 (Hewlett-Packard, Waldbronn, Germany) was controlled with ChemStation software (rev. A 07.01). The column effluent was monitored with a diode array detector at 338 nm (10 nm bandwidth) and a fluorescence detector at $\lambda_{exc/em}$ 340/450 nm using the OPA reagent for precolumn derivatization. A standard Agilent Technologies procedure [Zorbax Eclipse AAA column, 4.6 × 75 mm, 3.5 μm; mobile phase A, 40 mM Na_2HPO_4 at pH 7.8 (5.5 g of NaH_2PO_4 monohydrate + 1000 mL of H_2O, adjusted to pH 7.8 with 10 M NaOH solution); mobile phase B, ACN/MeOH/water (45:45:10 v/v); gradient, from 0 min 0% B, 9.8 min 57% B, 10 min 100% B, 12.5 min 0% B, to 14 min; flow rate, 2 mL/min; temperature of the column oven, 40 °C] was applied.

Accuracy, Precision, and Recovery. Accuracy, precision, and recovery were evaluated (n = 6−10) with model solutions and soil extracts spiked with the AA-S-18 amino acid standards (concentrations varying from 0.5 to 3 μg/g). Intraday repeatability was verified by analyzing standard solutions and soil extracts during 1 day (spiked with four different concentrations); interday repeatability was verified in a 6-day period with four standard solutions of different concentration (real soil extracts were analyzed in one sequence during a period of <24 h).

The limits of detection (LODs, S/N = 3) were in the range from 1.54 pg (Gly) to 13.5 pg (Cy2) or from 18.6 fmol (Ser) to 64.8 fmol (Lys) for individual amino acids (see **Table 2**). The limits of quantification (LOQs, S/N = 10) were in the range from 5.13 pg (Gly) to 44.8 pg (Cy2) or from 61.9 fmol (Ser) to 215 fmol (Lys). The calibration curves were linear in appropriate concentration ranges with correlation coefficients varying over the range 0.99990−0.99998 except His (0.99979), Arg (0.99986), Cy2 (0.99901), Ile (0.99823), and Lys (0.99919). All data on LODs, LOQs, calibration curves, and correlation coefficients are given in **Table 2**.

Repeatability was determined for 0.5, 1, 2, and 3 μg/g standard solutions and for real soil extracts spiked with the same concentration of analytes (n = 6). For standard solutions, RSDs varied in the ranges 0.6−1.4% (av = 1.05%), 0.5−1.3% (av = 0.78%), 0.2−0.8% (av = 0.60%), and 0.3−0.8% (av = 0.53%) with recoveries of 99.1−100.5% (av = 99.8, 99.7, 99.9, and 99.9%, respectively). For spiked extracts, RSDs varied in the ranges 0.4−1.5% (av = 1.00%), 0.5−1.4% (av = 1.05%), 0.4−1.3% (av = 0.90%), and 0.4−1.1% (av = 0.94%) with recoveries of 98.3−101.4% (av = 100.0, 99.9, 99.9, and 100.1%), respectively.

As for intraday variation, RSDs were in the range 0.4−1.6% (av = 0.88%) and recovery was 98.6−100.9% (av = 99.9%) for standard solutions. For spiked soil extracts, RSDs were in the range 0.5−1.8% (av = 1.14%) and recovery was 98.3−101.7% (av = 100.0%). Interday variation showed RSDs were in the range 0.6−2.1% (av = 1.18%) and recovery was 98.3−101.2% (avg = 99.9%) for standard solution. All validation data are given in **Tables 6** (reproducibility) and **7** and **8** (intraday and interday variation).

RESULTS AND DISCUSSION

Optimization of Supercritical Fluid Extraction Conditions. Several sets of spiked sample experiments were performed to find optimal extraction conditions for amino acids using the high flow rate instrument configuration. Fortunately, these nonvolatile analytes were trapped quantitatively into a foil-sealed vial with methanol as a trapping solvent; no stripping effect was observed for 60 min.

Extraction time was determined for both spiked samples (30 min) and real soil samples (45 min) with on-line modifier addition. Longer time did not improve the results. Then a 15 min extraction without modifier followed to get the water out of the piston micropump and remove completely modifier from the extraction cell to the trapping system. Extraction temperature and pressure were optimized in a cross-linked study (see **Table**

Table 4. Extraction of Amino Acids from Real Soil Samples ($n = 6$)[a]

	SFE (CO_2 with 7.5% H_2O v/v)			LE (0.5 M ammonium acetate)		
	c (nmol)	*c* (μg)	RSD (%)	*c* (nmol)	*c* (μg)	RSD (%)
			H Horizon			
Asp	6.61	0.879	6.28	5.88	0.782	3.36
Glu	10.2	1.50	4.07	10.2	1. 50	2.43
Ser	3.59	0.377	6.52	3.64	0.382	2.82
His	0.65	0.101	3.54	0.66	0.102	4.43
Gly	3.87	0.291	4.91	3.90	0.293	5.07
Thr	3.81	0.453	6.60	3.60	0.429	3.90
Arg	4.91	0.855	4.30	4.93	0.859	3.11
Ala	10.5	0.934	5.10	10.4	0.925	4.64
Tyr	0.61	0.110	5.24	0.61	0.111	7.34
Cy2	4.17	1.00	7.05	3.77	0.906	5.00
Val	3.56	0.417	3.39	3.88	0.454	5.13
Met	8.44	1.26	2.78	8.60	1.28	3.98
Phe	11.2	1.85	4.79	10.7	1.77	4.78
Ile	2.77	0.363	7.27	2.64	0.346	6.98
Leu	6.37	0.835	3.85	6.09	0.799	4.19
Lys	3.44	0.503	4.95	3.41	0.499	5.98
			Ah Horizon			
Asp	12.9	1.71	5.50	12.0	1. 60	5.66
Glu	25.7	3.78	3.64	24.8	3.65	3.40
Ser	8.00	0.841	4.35	8.43	0.886	6.11
His	1.41	0.219	5.54	1.43	0.222	4.45
Gly	10.0	0.751	7.35	10.2	0.767	5.57
Thr	11.1	1.32	6.30	10.8	1.28	4.45
Arg	18.1	3.15	4.76	18.8	3.27	3.70
Ala	28.7	2.56	4.31	26.2	2.34	4.61
Tyr	2.21	0.401	7.06	2.14	0.387	6.20
Cy2	6.17	1.48	6.48	6.22	1. 50	5.45
Val	10.3	1.20	7.21	10.7	1.25	7.52
Met	18.2	2.71	4.60	18.1	2.71	5.99
Phe	6.93	1.15	5.32	7.11	1.17	5.64
Ile	7.73	1.01	5.26	7.54	0.989	5.89
Leu	11.6	1.53	5.82	12.2	1.60	7.22
Lys	4.39	0.642	7.25	4.50	0.659	6.86
			Ae Horizon			
Asp	2.43	0.323	6.38	2.37	0.315	2.60
Glu	13.3	1.96	5.13	13.9	2.05	6.41
Ser	2.94	0.309	2.43	2.80	0.294	5.03
His	4.11	0.638	6.33	4.08	0.633	2.16
Gly	7.29	0.547	3.06	7.32	0.550	6.74
Thr	3.39	0.404	7.49	3.31	0.394	4.81
Arg	4.04	0.705	5.79	4.11	0.716	5.31
Ala	4.34	0.387	6.15	4.31	0.384	6.57
Tyr	0.61	0.111	6.12	0.61	0.110	3.84
Cy2	2.92	0.702	4.09	3.09	0.742	5.46
Val	2.18	0.255	5.01	2.24	0.263	4.25
Met	3.84	0.574	6.01	3.79	0.565	4.53
Phe	4.83	0.798	3.75	4.73	0.781	5.54
Ile	0.74	0.098	4.41	0.76	0.100	3.63
Leu	1.55	0.203	5.85	1.57	0.206	5.95
Lys	1.50	0.219	4.75	1.48	0.216	6.37

[a] Comparison of results from SFE and LE (0.5 M ammonium acetate). Concentration given per 1 g of wet soil.

3) at 20 MPa for different temperatures and at 40 °C for different pressures. Recoveries under best conditions (15 MPa, 50 °C, 7.5% v/v H_2O on-line, 0.75 mL of 0.1 M HCl in situ) are given in **Table 3**.

Most of the analytes are well extractable in a wide range of temperatures and pressures. For Asp, Glu, His, Arg, Phe, and Leu both 50 and 60 °C are good, with highest recovery at 50 °C for Asp, Glu, Arg, and Phe and at 60 °C for His and Leu. Ser and Gly are well extractable from 40 to 75 °C, with maximum recovery at 60 °C for Ser and at 75 °C for Gly. Thr, Ala, Val, Met, and Ile have excellent recoveries in the range from 40 to 60 °C, maximum being at 50 °C for Thr and Val, at 40 °C for Ala and Met, and at 60 °C for Ile. Tyr is well extractable both at 40 °C (best recovery) and at 50 °C; for Cy2 and Lys the only acceptable temperature was 50 °C. The optimum temperature of 50 °C was selected as a compromise with acceptable recoveries of all amino acids.

Table 5. Influence of Human Hand Touch on the Recovery of Amino Acids ($n = 6$)[a]

	1: Gloves c [µg/g]	2: Hand c [µg/g]	Delta c [µg/g]
ASP	0.036	0.037	0.001
GLU	0.036	0.038	0.002
SER	0.065	0.064	-0.001
HIS	0.000	0.000	0.000
GLY	0.045	0.097	0.052
THR	0.017	0.024	0.008
ARG	0.000	0.152	0.152
ALA	0.061	0.030	-0.032
TYR	0.031	0.050	0.019
CY2	0.103	0.171	0.068
VAL	0.038	0.242	0.205
MET	0.053	0.093	0.040
PHE	0.070	0.042	-0.028
ILE	0.015	0.015	0.000
LEU	0.012	0.016	0.004
LYS	0.000	0.090	0.090
Total	0.582	1.161	0.579

[a] Comparison of amino acid content in two blank experiments (0.5 M ammonium acetate extraction): 1, performed completely in surgery gloves; 2, filter paper folded with uncovered hand. Amino acid concentration is given per 1 g of wet soil. Serious enhancement of amino acids content is highlighted. Column 1 shows also the contribution from ultrapure water (data are shown before correction for water volume).

As for the pressure, 10 MPa was optimum for Ser and Thr but was insufficient for many other amino acids, 15 MPa being the best value for Glu, Ala, Val, Met, and Lys. Recovery of all others was quantitative or at least acceptable, except Phe (94.9%). A pressure of 20 MPa is optimum for Arg and Cy2, whereas all higher pressures were found to be the best for one or two amino acids (see **Table 3**). However, all values demonstrated at least one recovery of 93 or 94%. The lowest pressure was selected as the best extraction pressure for the whole group because it was the value with the highest number of maximal or good recoveries and thus the most least damaging to the soil microorganisms.

Ultrapure water and aqueous methanol and ethanol [1−10% (v/v) of each] were tested to find the best modifier composition. Addition of methanol and ethanol unfavorably affected the recovery due to low solubility of amino acids. Ultrapure water was selected as the proper on-line modifier. In the spiked samples, a small amount of HCl was used as either in situ modifier or entrainer.

The concentration of a modifier in the extraction fluid depends on the amount of a liquid modifier added to the extraction phase during every filling of the extractor pump. To find the best volume of the on-line modifier and in situ HCl, up to 1 mL of modifier was added through the Valco valve loop. That is ≈10% of the piston micropump volume. There are large but different intervals of good extractabilities for various analytes and optimum extraction conditions; 7.5% (v/v) of H_2O was selected as a compromise giving the best recovery for Asp, Glu, Ser,

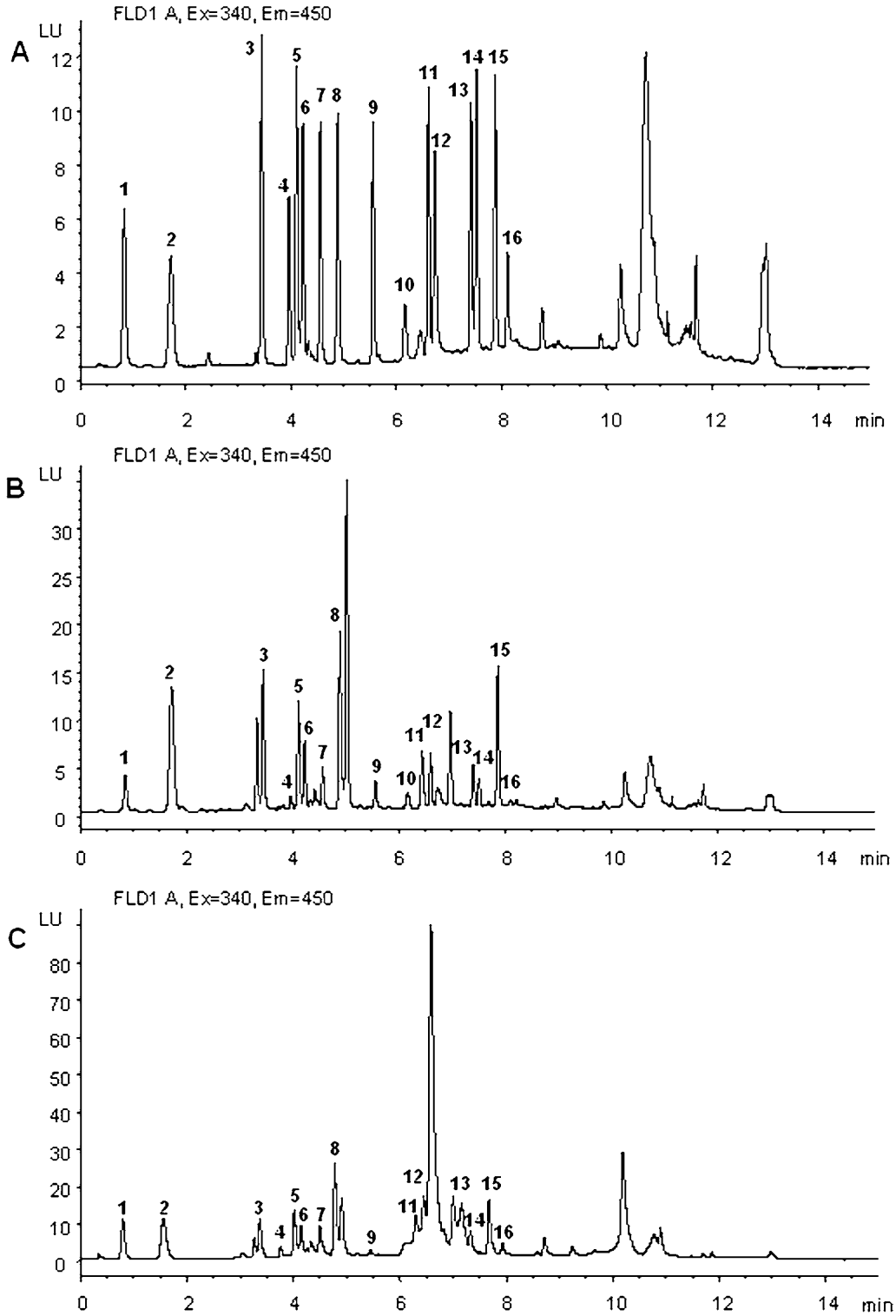

Figure 1. HPLC-FLD chromatogram of standard solution (**A**) and real sample extract (H horizon soil; **B,** SFE; **C**, 0.5 M ammonium acetate extraction). Conditions of both extractions and analysis are given under Materials and Methods. Peaks: 1, Asp; 2, Glu; 3, Ser; 4, His; 5, Gly; 6, Thr; 7, Arg; 8, Ala; 9, Tyr; 10, Cy2; 11, Val; 12, Met; 13, Phe; 14, Ile; 15, Leu; 16, Lys.

Arg, Cy2, and Met and assuring acceptable recoveries of all other amino acids (lowest value was 95.4% for Lys).

As for the in situ HCl addition, 0.75 mL seems to be the most expedient choice (9 of the 16 examined amino acids had a maximum recovery). Although there were two nonquantitative recoveries (94.2% for Asp and 92.9% for Arg), the 0.5 mL variant good for these two amino acids would lead to a substantial decrease of recovery for the other compounds. Thus, the extractability of amino acids from model samples was found to be either excellent or at least acceptable for all examined compounds under selected conditions.

Real Sample Extraction and Method Validation. SFE is known to depend on so-called matrix effect when applied to various real samples. Preliminary experiments were thus performed to verify if the maximum recovery was reached under given conditions. Two small changes were tested: (i) no water was added into the extraction cartridge in the beginning, as the soil samples were wet enough; and (ii) in situ HCl was not added

Table 6. HPLC-FLD Validation: Determination of Amino Acids in Standard Solutions and Ah Horizon Soil Extract Spiked with Known Concentration of Analytes ($n = 6$)

A. Standard Solutions of Known Analyte Concentration

	spiked 0.5			spiked 1			spiked 2			spiked 3		
	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)
Asp	0.502	1.17	100.4	0.995	0.74	99.5	2.002	0.66	100.1	2.990	0.51	99.7
Glu	0.499	1.35	99.7	1.001	0.87	100.1	1.999	0.79	100.0	2.999	0.73	100.0
Ser	0.500	1.32	99.9	1.005	0.60	100.5	1.991	0.60	99.6	2.989	0.56	99.6
His	0.498	0.62	99.7	0.998	0.47	99.8	2.003	0.79	100.1	2.985	0.74	99.5
Gly	0.497	1.25	99.3	0.994	0.80	99.4	1.991	0.21	99.5	3.000	0.47	100.0
Thr	0.500	1.02	99.9	0.997	0.76	99.7	1.997	0.65	99.8	3.005	0.51	100.2
Arg	0.496	1.26	99.1	1.000	1.31	100.0	2.010	0.55	100.5	3.003	0.61	100.1
Ala	0.497	0.85	99.5	0.992	1.08	99.2	2.003	0.55	100.1	2.986	0.34	99.5
Tyr	0.503	1.24	100.5	0.995	0.67	99.5	2.003	0.67	100.2	2.991	0.45	99.7
Cy2	0.498	0.94	99.6	0.998	0.67	99.8	1.993	0.48	99.7	3.005	0.53	100.2
Val	0.498	1.32	99.5	0.991	1.01	99.1	1.986	0.56	99.3	2.990	0.42	99.7
Met	0.502	0.58	100.5	1.000	0.69	100.0	2.002	0.58	100.1	3.001	0.53	100.0
Phe	0.496	1.30	99.3	0.996	0.74	99.6	1.998	0.76	99.9	3.005	0.40	100.2
Ile	0.498	0.58	99.5	0.994	0.85	99.4	1.988	0.64	99.4	3.009	0.69	100.3
Leu	0.501	1.20	100.2	1.003	0.47	100.3	2.001	0.61	100.1	2.994	0.52	99.8
Lys	0.500	0.89	100.1	0.996	0.78	99.6	2.002	0.52	100.1	2.996	0.52	99.9

B. Real Soil Extract Spiked with Known Analyte Concentration

	spiked 0.5			spiked 1			spiked 2			spiked 3		
	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)
Asp	2.225	1.26	100.5	2.715	0.82	100.1	3.684	0.81	99.2	4.739	0.58	100.6
Glu	4.245	1.38	99.1	4.828	0.96	101.0	5.810	0.96	100.5	6.724	0.86	99.1
Ser	1.347	1.10	100.5	1.828	1.27	99.3	2.808	1.16	98.9	3.834	0.87	99.8
His	0.713	1.15	99.2	1.198	1.76	98.3	2.210	0.42	99.6	3.256	1.14	101.2
Gly	1.248	0.80	99.8	1.741	1.13	99.5	2.715	1.32	98.7	3.714	1.00	99.0
Thr	1.830	0.95	100.5	2.335	1.02	100.6	3.334	0.69	100.4	4.363	1.09	101.0
Arg	3.642	0.45	99.7	4.191	0.93	100.9	5.103	1.13	99.1	6.148	0.66	99.9
Ala	3.042	0.87	99.4	3.538	1.08	99.4	4.565	0.71	100.1	5.542	0.58	99.7
Tyr	0.912	1.29	101.2	1.400	0.80	99.9	2.414	0.93	100.6	3.433	0.93	100.9
Cy2	1.968	0.67	99.3	2.471	0.52	99.6	3.457	0.87	99.3	4.454	0.65	99.4
Val	1.711	1.03	100.5	2.228	1.31	101.2	3.199	0.38	99.9	4.239	0.86	100.9
Met	3.219	0.92	100.3	3.690	1.01	99.4	4.749	0.81	100.8	5.746	0.61	100.6
Phe	1.631	1.46	99.2	2.175	1.39	101.4	3.154	1.14	100.3	4.149	0.87	100.1
Ile	1.500	0.52	99.1	1.998	0.84	99.2	2.996	1.17	99.4	4.021	0.36	100.2
Leu	2.032	1.26	100.4	2.495	1.20	98.8	3.515	0.65	99.7	4.510	0.55	99.7
Lys	1.152	0.97	100.9	1.640	0.76	99.9	2.675	1.26	101.3	3.615	0.73	99.3

to the cartridge. During the tests of prospective harm done to soil microbes it was found that the addition of HCl made no further boost to the recovery, because the soil sample was rich enough with other entrainer compounds. All other extraction conditions were confirmed to be optimal.

The efficiency of SFE was compared with validated 0.5 M ammonium acetate extraction using three different types of soil samples (H, Ah, and Ae horizons, see **Table 1**). The results are in the good coincidence with slightly best results for alternately one or another extraction method (see **Table 4**). Results indicate that both methods have acceptable quantitative recoveries and are sufficiently mild not to cause artificial release of amino acids due to hydrolysis or microbial cell destruction, because the coincidence of the same nonextractable part of all 16 analytes for the two very different methods and dissimilar conditions is very unlikely.

However, higher selectivity was achieved with SFE, as the conditions necessary for quantitative extraction of the analytes are mild and many ballast compounds remained in the sample matrix. Because of that, samples can be added right to the chromatographic column without the need of cleanup. However, except for several tests, all extracts were filtered anyway to protect the column.

Anticontamination Precautions. Two possible sources of amino acids were expected: microbes dying in the supercritical fluid environment and hydrolyzed proteins present in real samples [production of amino acids from bovine serum albumin by continuous subcritical water hydrolysis was reported (*26*)]. The albumin hydrolysis tests under various extraction conditions confirmed no amino acid enrichment.

It was known that soil microbes could stand 20 MPa in PSE without problems. However, SFE achieved very mild increase of amino acid content even at this pressure, and evident increase was found between 25 and 35 MPa for all samples. The selected "optimum" pressure, 15 MPa, seems to do no harm to microbes at appropriate mild temperatures. Also, the selected temperature, 50 °C, was found to be mild enough for all soil sample microbes. A considerable increase of amino acid concentrations was seen already at 70 °C; temperatures above 90 °C seem to be lethal for many microbes and completely unusable for extractions of bioavailable amino acids. These findings are in good agreement with common liquid extraction methods, which use very low temperatures.

Several additional relevant sources of contamination were discovered during the extraction procedure. The strongest, but most easily avoidable, source was the touch of a human hand. A single fingerprint (tested for filter holding by a clean unprotected hand during extraction cartridge assembly for ≈3 s) is clearly visible both in the blank and in comparison to two real sample extractions (another example is given in **Table 5**.). Ultrapure water and glass wool were determined as other sources of amino acids contamination.

Table 7. HPLC-FLD Intraday Repeatability Amino Acid Determination in Standard Solution and Spiked Real Sample Extract (Ah Horizon Soil; $n = 6$)

	spiked 0.5			spiked 1			spiked 2			spiked 3		
	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)	c (μg/g)	RSD (%)	recovery (%)
Standard Solutions of Known Analyte Concentration												
Asp	0.503	1.05	100.7	1.003	0.90	100.3	1.998	0.68	99.9	3.001	0.67	100.0
Glu	0.502	1.10	100.4	0.995	0.64	99.5	2.006	0.74	100.3	3.002	0.82	100.1
Ser	0.493	1.33	98.6	1.001	1.03	100.1	2.002	0.83	100.1	2.977	0.96	99.2
His	0.505	1.25	100.9	0.999	0.40	99.9	1.990	0.68	99.5	3.009	0.85	100.3
Gly	0.496	1.52	99.2	1.003	0.86	100.3	2.009	0.92	100.4	2.991	0.43	99.7
Thr	0.501	1.29	100.2	0.999	0.96	99.9	1.994	0.76	99.72	2.998	0.73	99.9
Arg	0.497	1.63	99.5	0.993	0.74	99.3	1.996	0.73	99.8	3.010	0.44	100.3
Ala	0.501	1.51	100.1	0.999	0.97	99.9	1.986	0.84	99.3	3.008	0.83	100.3
Tyr	0.503	1.24	100.5	1.002	0.80	100.2	2.016	0.66	100.8	2.989	0.50	99.6
Cy2	0.495	1.48	99.1	1.001	0.60	100.1	1.996	0.61	99.8	2.981	0.84	99.4
Val	0.496	1.06	99.3	0.997	1.47	99.7	2.005	1.06	100.2	2.987	0.54	99.6
Met	0.499	1.25	99.8	1.005	0.81	100.5	1.985	0.86	99.3	2.979	0.73	99.3
Phe	0.498	0.58	99.7	0.993	1.01	99.3	1.999	0.72	100.0	3.010	0.58	100.3
Ile	0.496	0.72	99.1	1.001	0.72	100.1	2.008	0.98	100.4	2.993	1.11	99.8
Leu	0.504	1.06	100.9	1.008	0.46	100.8	1.999	0.59	99.9	3.006	0.80	100.2
Lys	0.502	0.84	100.3	0.992	1.19	99.2	2.006	0.75	100.3	2.988	0.78	99.6
Real Soil Extract Spiked with Known Analyte Concentration												
Asp	2.199	1.20	99.3	2.712	1.01	99.9	3.688	1.02	99.3	4.761	1.03	101.0
Glu	4.211	1.20	98.3	4.826	0.93	100.9	5.758	0.68	99.7	6.814	0.72	100.5
Ser	1.332	1.10	99.5	1.857	1.43	100.9	2.843	1.25	100.1	3.868	0.90	100.7
His	0.722	1.35	100.5	1.213	1.06	99.6	2.235	0.84	100.7	3.193	1.73	99.2
Gly	1.259	1.19	100.7	1.739	0.73	99.3	2.737	0.92	99.5	3.724	0.73	99.3
Thr	1.841	1.53	101.0	2.316	0.50	99.8	3.320	1.11	100.0	4.300	1.27	99.5
Arg	3.640	0.86	99.7	4.186	1.05	100.8	5.169	0.91	100.3	6.199	0.85	100.8
Ala	3.064	1.39	100.1	3.570	0.89	100.3	4.589	1.24	100.6	5.537	0.80	99.6
Tyr	0.897	1.64	99.6	1.417	1.42	101.3	2.393	1.82	99.7	3.457	1.62	101.8
Cy2	1.962	1.12	99.0	2.467	0.71	99.4	3.487	1.71	100.1	4.483	0.49	100.0
Val	1.700	0.78	99.9	2.194	0.54	99.6	3.231	1.00	100.9	4.188	1.07	99.7
Met	3.220	1.09	100.3	3.702	1.32	99.8	4.709	1.39	100.0	5.762	0.89	100.9
Phe	1.652	1.33	100.4	2.149	1.48	100.2	3.099	1.58	98.6	4.101	1.08	98.9
Ile	1.518	1.53	100.2	2.011	0.94	99.9	3.049	1.31	101.1	3.977	1.46	99.1
Leu	2.001	1.77	98.8	2.531	1.00	100.2	3.553	0.95	100.8	4.506	0.98	99.6
Lys	1.155	1.06	101.1	1.649	1.29	100.4	2.630	1.97	99.6	3.662	1.19	100.6

Table 8. HPLC-FLD Interday Repeatability of Amino Acid Determination in Standard Solution of Known Analyte Concentration ($n = 24$)

	spiked 0.5			spiked 1			spiked 2			spiked 3		
	c (μg/g)	RSD (%)	recovery [%]	c (μg/g)	RSD (%)	recovery [%]	c (μg/g)	RSD (%)	recovery [%]	c (μg/g)	RSD (%)	recovery [%]
Asp	0.498	2.09	99.5	0.991	1.11	99.1	2.008	0.98	100.4	3.012	1.04	100.4
Glu	0.503	1.76	100.5	0.990	1.15	99.0	2.001	1.01	100.1	2.977	0.74	99.2
Ser	0.502	1.50	100.3	1.003	1.30	100.3	1.990	0.82	99.5	3.011	1.22	100.4
His	0.504	1.43	100.8	1.009	1.39	100.9	1.984	0.97	99.2	2.987	1.13	99.6
Gly	0.499	1.16	99.9	0.997	0.67	99.7	2.013	1.15	100.7	2.975	0.96	99.2
Thr	0.499	1.49	99.7	1.011	1.44	101.1	2.006	1.02	100.3	3.022	1.47	100.7
Arg	0.505	1.91	101.1	0.988	1.21	98.8	2.002	0.65	100.1	3.035	1.24	101.2
Ala	0.497	1.16	99.3	1.004	1.23	100.4	1.981	1.11	99.0	2.998	0.85	99.9
Tyr	0.501	1.64	100.1	1.000	0.73	100.0	1.991	1.26	99.6	2.983	0.72	99.4
Cy2	0.502	1.59	100.4	0.999	0.80	99.9	1.983	1.00	99.1	3.014	1.40	100.5
Val	0.493	1.65	98.7	0.999	1.23	99.9	2.009	1.27	100.5	2.975	0.92	99.2
Met	0.501	0.84	100.2	1.007	1.07	100.7	2.003	1.14	100.1	2.972	1.19	99.1
Phe	0.502	1.37	100.5	1.009	1.45	100.9	1.988	1.28	99.4	2.985	1.09	99.5
Ile	0.492	1.54	98.3	0.995	0.82	99.5	1.981	0.91	99.0	3.008	0.65	100.3
Leu	0.498	1.07	99.7	0.995	1.31	99.5	2.004	0.74	100.2	2.993	0.73	99.8
Lys	0.505	1.56	101.1	1.003	1.58	100.3	1.994	1.30	99.7	3.018	1.27	100.6

To avoid as much contamination as possible all experiments were carried out according to standards common in radiochemistry. All glass and metal pieces of equipment were repeatedly rinsed with ethanol after washing, and the inner parts of the extractor were cleaned similarly by weak aqueous HCl followed by MeOH or EtOH. Glass wool was washed in a weak solution of aqueous HCl, washed in MeOH, dried at 70 °C, and kept in a clean sealed glass flask. Removal of amino acids to acceptable level was verified by LE of glass wool (0.5 M HCl, sonic bath). Attention was also paid to keep all equipment untouched by unprotected hands to avoid contamination transfer to surgery gloves. Blank extractions were included periodically to monitor the system cleanliness.

Thanks to the precautions, the only external source of amino acids was ultrapure water. The contribution of amino acids from water was rather small, but it was taken into account anyway (total amount of water in the extract is a known quantity, as the modifier was added via valve loop in a known number of certain volume injections). Of course, the total volume of water depends on the CO_2 flow rate and thus on the extraction pressure (from 9 to 25 mL per 45 min of extraction). Due to anticontamination precautions, RSDs were in the range 2.4−7.5% for

SFE in comparison with the range 2.2−7.5% for LE with 0.5 M ammonium acetate, which is satisfying, especially with a very small sample weight.

Conclusion. All amino acids are extractable quantitatively from both spiked and real soil samples, and very good recoveries can be achieved under common conditions [50 °C, 15 MPa, 7.5% (v/v) of ultrapure water in extraction fluid for 45 min, total extraction time of 60 min]. Negative matrix effects were not observed. Although the content of amino acids varied from one analyte to the other due to both RSDs and nonhomogeneous distribution of analytes in sample materials (especially in SFE, sample weights were very small), the average content of amino acids was in good agreement: 11.7 μg/g (SFE) versus 11.4 μg/g (LE) for the H horizon (SFE recovery = 102.6%), 24.5 μg/g (SFE) versus 24.3 μg/g (LE) for the Ah horizon (SFE recovery = 100.8%), and 8.2 μg/g (SFE) versus 8.3 μg/g (LE) for the Ae horizon (SFE recovery = 99.0%). Considering the small sample weights and matrix complexity, these results are more than satisfying.

The advantage of SFE is in the lower sample consumption (suitable, i.e., for continual monitoring of smaller laboratory or clima-box experiments), and results are available sooner. In both methods, extraction time is ≈60 min, but as for further treatment, rotary vacuum evaporation went much more quickly then filtration and lyophilization. On the other hand, multiple samples can be processed on the shaker at the same time, whereas SFE requires successive processing of individual samples and adequate sample storage between experiments to minimize changes of analyte content in soils or application of a multiposition SFE apparatus.

Thus, both SFE and 0.5 M ammonium acetate extraction are viable methods with very good repeatability that can be used alongside or selected according to accessible instrumentation, available amount of a sample, and number of experiments to be performed at the same time. However, amino acids proved to be well extractable by water-modified supercritical CO_2 at high flow rates.

ABBREVIATIONS USED

Ala, L-alanine; Arg, L-arginine; Asp, L-aspartic acid; Cy2, L-cystine; Glu, L-glutamic acid; Gly, glycine; His, L-histidine; Ile, L-isoleucine; Leu, L-leucine; Lys, L-lysine; Met, L-methionine; Phe, L-phenylalanine; Ser, L-serine; Thr, L-threonine; Tyr, L-tyrosine; Val, L-valine.

LITERATURE CITED

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Received for review May 4, 2006. Revised manuscript received June 15, 2006. Accepted June 22, 2006. This work was supported by the Grant Agency of the Czech Republic (Postdoctoral Project 526/03/D058).

JF061255K