

A reprint from

**JOURNAL
OF
LIQUID
CHROMATOGRAPHY**

JOURNAL OF LIQUID CHROMATOGRAPHY

Editor: DR. JACK CAZES **Editorial Secretary: ELEANOR CAZES**
P. O. Box 1218
Sharon Hill, Pennsylvania 19079

Associate Editors:

DR. HALEEM J. ISSAQ
NCI-Frederick Cancer Research Facility
Frederick, Maryland

DR. STEVEN H. WONG
University of Connecticut
School of Medicine
Drug Analysis Division
Farmington, Connecticut

For subscription information write to:

Promotion Department
Marcel Dekker, Inc.
270 Madison Avenue
New York, N.Y. 10016

PULSED AMPEROMETRIC DETECTION OF AMINO ACIDS SEPARATED BY ANION EXCHANGE CHROMATOGRAPHY

D. A. MARTENS AND W. T. FRANKENBERGER, JR.

*Department of Soil and Environmental Sciences
University of California
Riverside, California 92521*

ABSTRACT

Previous work has shown that the analysis of amino acids requires derivatization for spectrophotometric (UV-Vis) and fluorescence detection schemes. In this study, direct determination of both primary and secondary amino acids with pulsed amperometric detection (PAD) was achieved under alkaline conditions by oxidation of the amine and hydroxyl functional groups with a gold working electrode. PAD was found to be more sensitive (0.01 to 1.2 nM mL⁻¹) than ninhydrin derivatization (4.5 to 55.0 nM mL⁻¹) with UV-Vis detection. Detection of amino acids by PAD was in good agreement with previous published work on the amino acid composition for the enzyme, ribonuclease A. High performance anion-exchange chromatography (HPAC) with PAD for amino acid analysis requires little to no sample preparation and was not subjected to matrix interferences previously noted with reverse phase separation and OPA or ninhydrin detection. Application of the developed methodology indicated that several wheat (*Triticum aestivum* L.) varieties grown in sterile nutrient-sand media had a wide range in amino acid composition in their respective root mass and root exudates.

INTRODUCTION

Amino acids are the basic structural units of proteins and peptides. Separation of free and/or hydrolyzed amino acids and their derivatives with liquid chromatography is easily achieved by use of reverse-phase or anion or cation exchange stationary phases. However, consistent reverse phase separations are not possible without extensive sample pretreatment. Inorganic ions such as phosphates or organic compounds including carbohydrates, organic acids, flavinoids or liminoids in complex biological materials will influence elution of amino acids with reverse-phase separation. The activity and therefore the retention of any analyte by the separator column will be influenced by the concentration of other solutes present in the matrix (1). In contrast, the retention mechanism in ion exchange separation provides chromatography that is nearly matrix-insensitive. This becomes important when separations must utilize gradient elution for complex biological matrices.

Numerous amino acid detection procedures have been developed involving the production of amino acid derivatives subject to fluorescent, visible or ultraviolet detection. Without derivatization, amino acids do not fluoresce and only tyrosine, phenylalanine and tryptophan respond to UV-Vis detection. *o*-Phthaldialdehyde (OPA), a fluorescent derivatizing agent, offers excellent sensitivity with subpicomole detection limits (2,3), but many derivatives of OPA are not stable. OPA only reacts with primary amines and double peaks have often been reported for some OPA derivatives (3,4). Spectrophotometric detection upon precolumn derivatization with DANSYL-Cl (dimethylaminonaphthalenesulphonyl), DABSYL (dimethylaminoazobenzenesulphonyl), PTH (phenylthiohydantoin) and PTC (phenylthiocarbonyl) have been used, but lengthy derivatization procedures and production of extraneous peaks has limited their use (5). Ninhydrin (6) and phenyl isothiocyanate (7, 8) are used to form amino acid derivatives for UV-Vis detection, but their sensitivity and reactivity with other sample components has limited their use with complex matrices.

Separation and detection procedures for amino acids that does not require derivatization would be the preferred methodology due to low

cost, convenience and simplicity. The use of laser-based optical activity (9) and refractive index detection have been used for detection of non-derivatized amino acids, however, poor sensitivity, selectivity, and reproducibility has limited the use of these detection methods. Electrochemical detection of amino acids has received little attention due to previous research showing that amino acids except for tryptophan, histidine and tyrosine were not electrochemically-active with carbon, gold, platinum or silver electrodes (11) when separated by cation-exchange methods (10). However, Hui and Huber (12) and Krafil and Huber (13) reported the direct anodic detection of amino acids with a constant potential at an oxide-covered Ni electrode in an alkaline medium. Polta and Johnson (14) reported that amino acids would become electrocatalyzed at a Pt electrode in an alkaline environment due to the formation of PtOH. The alkaline media was found to be important in the formation of the hydroxide form of the metal used as the anode in detection of amino acids by amperometry (15).

Electrochemical detection of organic compounds with HPLC separation has been limited due to electrochemical reactions deposited on the working electrode that can alter surface characteristics. Alternating cycles of positive and negative voltage (pulsed amperometry) have been reported to maintain the working electrode in a reactive state in the detection of oxidizable organic compounds (16). PAD equipped with a gold electrode is selective only to compounds containing oxidizable functional groups such as hydroxyl, amine and sulfide groups. Carboxylic acids and inorganic species do not interfere with detection by PAD. Martens and Frankenberger (17-19) reported that monosaccharides, aminosaccharides and glycuronic acids can be detected with PAD with low limits of detection (μM range) and the methodology was applicable to a wide range of biological samples.

The present investigation was to evaluate HPAC-PAD for quantification and direct detection of amino acids. PAD parameters such as applied potentials and durations were determined to optimize sensitivity for amino acid detection. HPAC-PAD was utilized to determine the amino acid composition of a ribonuclease A enzyme and of root exudates and root mass of several aseptically-grown wheat (*Triticum aestivum* L.) varieties.

MATERIALS AND METHODS

Reagents

Standard amino acids, amino acid standard solution for protein hydrolysates (0.1M HCl), ribonuclease A enzyme (EC 3.1.27.5) and ninhydrin solution were obtained from Sigma Chemical Company (St. Louis, MO). Methanesulfonic acid was obtained from Kodak Chemical Company (Rochester, NY).

Chromatographic Instrumentation

The HPAC-PAD analysis was performed on a Dionex (Sunnyvale, CA) LC gradient pump module and Model PAD2 detector equipped with a solvent-compatible electrode. Sample injection was via a Dionex autosampler equipped with a 220 μ L sample loop. Amino acids were separated on an AminoPac PA1 pellicular anion-exchange resin (250 x 4 mm) and an AminoPac PA1 guard column (25 x 3 mm) (Dionex, Sunnyvale, CA) at the flow rate of 0.8 mL min⁻¹ at ambient temperature. The mobile phase consisted of a sodium hydroxide, sodium borate and sodium acetate gradient system. Separation of the amino acid was achieved with four solutions as follows: A was 23 mM sodium hydroxide with 7 mM sodium borate; B was 80 mM sodium hydroxide with 23 mM sodium borate; C was 0.65 M sodium acetate, and D was 1M sodium hydroxide with 0.3 M boric acid. These were combined in the following proportions:

Time	Eluant			
	(A. %)	(B. %)	(C. %)	(D. %)
0-10 min	100	0	0	0
10-16.5 min	100 - 0	0 - 100	0	0
16.5-20 min	0	100	0	0

	Eluant			
20-32 min	0	100→0	0→100	0
32-50 min	0	0	100	0
50-60 min	0	0	0	100
60-70 min	100	0	0	0

The mobile phase was prepared with degased 18-M Ω water and filtered through a 0.22 μ m GS Millipore membrane filter (Bedford, MA). Sodium hydroxide (0.3 M) was used as a post column addition to reduce baseline shifts that occurred with the sodium hydroxide gradient and to increase electrode sensitivity. Detection was by triple-pulsed amperometry with a gold-working electrode (16). The following working pulse potentials and durations were used for detection of amino acids: E₁ = 0.15 V (t₁ = 300 ms); E₂ = 0.85 V (t₂ = 120 ms); E₃ = -0.60 V (t₃ = 60 ms). The CHOH and NH₃ groups are oxidized at E₁, E₂ removes the reaction products, while E₃ cleans the electrode at a negative potential. Hydrodynamic voltammetry was used to choose the applied potentials. The response time of the PAD was set to 1 sec and output range was set at 1000 nA. Chromatographic data were collected and plotted using the Dionex Autolon 300 software.

Ninhydrin detection of amino acids (10) was monitored with a Beckman Model 165 UV-Vis spectrophotometer (570 nm) after separation of amino acids on an AminoPac PA1 column (Dionex, Sunnyvale, CA). Ninhydrin was added at a rate of 0.7 mL min⁻¹ and mixed in a beaded mixing coil at 130°C.

The ribonuclease A enzyme (2 mg) was hydrolyzed in a heat-sealed acid washed (3:1 sulfuric acid; nitric acid) 20 ml ampoule (Kontes Scientific Glassware, Vineland, New Jersey) with 0.5 mL 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 120°C for 22 h (20). The extract was partially neutralized with 0.5 mL 3.5N sodium hydroxide, diluted with 0.023 M NaOH (Eluant A), filtered through

a 0.22 μm GS Millipore membrane filter and injected for HPAC-PAD analysis.

The two wheat varieties (Yolo and Altar 84; Foundation Seed, University of California, Davis, CA) used in this study were surface sterilized with 70% ethanol (5 min) prior to a 10 min rinse with sodium hypochlorite-Tween 20 solution (0.5 ml Tween 20 to 100 mL sodium hypochlorite). Under sterile conditions, the disinfectant solution was decanted and rinsed with several 100 mL aliquots of sterile water. The disinfected seeds were then placed in sterile germination pouches and germinated at 28°C. After germination they were placed in sterile 250 mL Erlenmeyer flasks (triplicate) containing acid-washed sand and 1N Hoagland's nutrient solution (21) and placed into a previously sterilized [95% ethanol and UVB radiation (24 h)] growth chamber (100 W m⁻²) at ambient temperatures with a photoperiod of 12h. After 17 days, the plant and root materials were removed from the sand and the sand was rinsed with 5 x 10-mL aliquots of 20% ethanol to extract the free amino acids (22). The ethanol wash was concentrated on a hot plate (50°C) under a stream of N₂ gas to approximately 1 mL and then diluted to 5 ml with 0.23 mM sodium hydroxide (Eluant A), filtered through a 0.22 GS μm Millipore membrane filter, diluted with Eluant A and injected. The root material was air-dried and hydrolyzed in 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole for 18 h at 120°C (20). The root extracts were partially neutralized with 1 ml 3.5N sodium hydroxide, filtered through a 0.22 μm GS Millipore membrane filter, diluted with Eluant A and injected.

RESULTS AND DISCUSSION

Extraction

Most protein amino acid analyses are performed upon hydrolysates prepared in hot mineral acid media. The acid of choice is often constant boiling 6M hydrochloric acid. However, of the twenty

common protein amino acids, serine, threonine, tyrosine, and tryptophan can be partially or completely degraded by the use of hydrochloric acid hydrolysis (23). Simpson et al. (20) reported the quantitative recovery of protein amino acids with 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole as a catalyst for hydrolysis. The methanesulfonic acid method was performed on samples in this study to allow quantitative recovery of tryptophan, serine, threonine and tyrosine and because of the limited sample preparation required for the extracts when analyzed with ion-exchange separation.

Detection

An ideal LC method for amino acid separation and detection should be free from matrix interferences, quantitative, simple, rapid, sensitive, reproducible and exhibit good resolution (24).

In general, ion-exchange separation of amino acids have had longer elution times when compared with reverse-phase chromatography (5). The chromatographic run time of 50 min for the HPAC-PAD analyses reported here compare with the elution times reported for precolumn derivatization by DANSYL (30 min), DABSYL (25 min), PTC (45 min), PTH (25 min) and OPA (35 min), with reverse-phase separation (5).

The use of PAD resulted in less interference problems for the direct injection of the biological samples when compared with ninhydrin-UV-VIS detection (570 nm). Evaluation of strong cation (3-propylsulfonic acid, H⁺; Supelco, Bellefonte, PA) or strong anion exchange (3-quaternary propylammonium, Cl, Supelco, Bellefonte, PA) solid phase extraction resins for selective recovery of amino acids in the biological samples for use with ninhydrin detection resulted in incomplete recovery of the 20 applied protein amino acids when compared with direct injection and PAD.

PAD potentials employed in the detection of amino acids were found to respond more to the duration of the sampling potential (E₁) than to the sampling potential voltage. Sensitivity more than doubled when the E₁ duration was less 400 ms. A duration time of 300 ms provided the lowest S/N ratio compared with shorter or longer durations.

Chromatographic parameters including retention time (t_R), column capacity factor (k'), precision (RSD%) and limits of detection (LOD) for 30 amino acids with HPAC-PAD are listed in Table 1. Retention times for the selected amino acids showed that incomplete resolution was noted for asparagine, alanine and threonine, but good separation was achieved for the remaining 17 protein amino acids as noted in Fig. 1. Table 1 shows that PAD can detect many nonprotein as well as protein amino acids. The possible coelution of the nonprotein and protein amino acids was not found to be a problem in the analyses reported here. Modification of the eluant program and an increase in chromatography time would reduce this coelution problem. The time of analysis as specified here for detection of the 20 protein amino acids was <50 min.

The precision of HPAC-PAD was determined by ten 220 μ l injections of combined standards. The relative standard deviations for detection of amino acids ranged from 1.4% to 3.5%. The LODs were determined by spiking a methanesulfonic acid digestion blank with known levels of the protein amino acids. LODs ranged from 1200 nM to 10 nM (268 pM to 2.2 pM injection⁻¹) with HPAC-PAD (220 μ l injection), based on a 3-fold signal-to-noise ratio for the baseline ($S/N=3$). Figure 2 compares the detection of an amino acid test solution (Sigma, 2.5 μ M/mL; diluted 50x) by HPAC-PAD and HPAC-UV-Vis (ninhydrin derivatization; detection at 570 nm). Overall, PAD was much more sensitive than ninhydrin detection with HPAC.

The HPAC-PAD response was linear over an order of magnitude from the LOD for each of the protein amino acids. The HPAC-PAD was approximately 4x less sensitive than UV-Vis detection with DABSYL, DANSYL, PTH and PTC derivatives and 10x less sensitive than OPA derivatives (5). Secondary amines can be determined with OPA after addition of sodium hypochlorite, but this addition reduces the sensitivity for the remaining amino acids (25). LODs for PAD were comparable with LODs for OPA with sodium hypochlorite addition. Ammonium was not detected by PAD. Limited sample preparation, good sensitivity, and direct detection of both primary and secondary amino acids makes HPAC-PAD the ideal method for determination of the amino acid composition of protein hydrolysates.

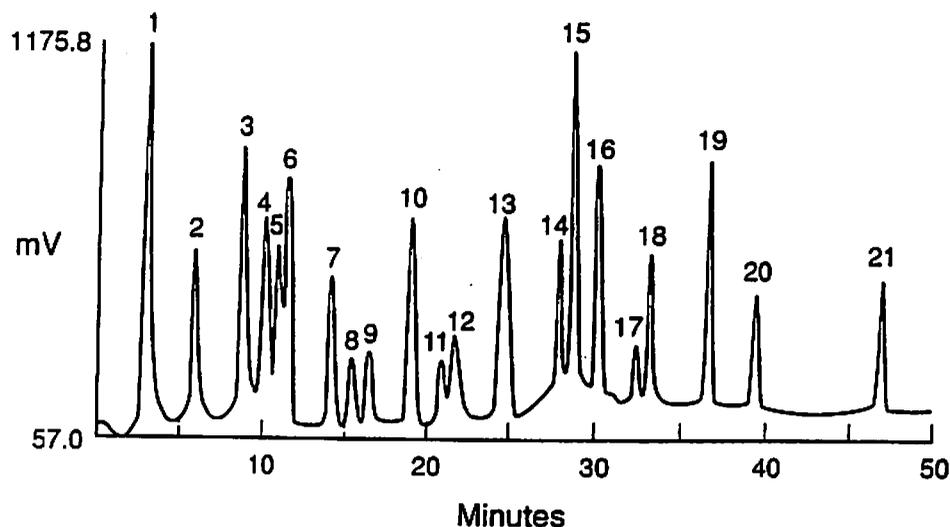


Figure 1. Chromatogram of protein amino acids detected by HPAC-PAD ($nM\ ml^{-1}$) 1 = Arg (22); 2 = Lys (86); 3 = Gln (26); 4 = Asn (19); 5 = Thr (10); 6 = Ala (70); 7 = Gly (83); 8 = Ser (12); 9 = Val (53); 10 = Pro (33); 11 = Ile (48); 12 = Leu (48); 13 = Met (25); 14 = System peak; 15 = His (16); 16 = Phe (23); 17 = Glu (43); 18 = Asp (47); 19 = Cys + Cyx (21); 20 = Try (21); 21 = Trp (18).

Detection of Amino Acids in Biological Matrices

HPAC-PAD determination of the methanesulfonic acid hydrolyzed ribonuclease A enzyme resulted in near agreement for the reported amino acid composition (20, 26) (Fig. 3; Table 2). The lower recoveries for Ile, Leu and Val are due to the use of a 22h digestion period which is insufficient for complete recovery of these amino acids (20). The 22h digestion period, however, does give satisfactory recovery of the remaining amino acids, including serine, tyrosine and threonine.

Colonization of germinating plant seeds and roots of seedlings by microorganisms depend on the extent of nutrition released by the growing seedlings as exudates. Root exudates are low molecular weight compounds that have been found to increase the population of soil

TABLE 1
Chromatographic Parameters in Detection of Protein and Nonprotein
Amino Acids by HPAC-PAD.

Amino acid	IUPAC Abbreviation	$t_{R(\min)}$	k'^a	LOD ^b	RSD ^c
Arginine	Arg	2.67	0.26	40	2.5
Homoarginine		2.85	0.36	50	2.6
Betaine		2.95	0.41	75	2.1
Ornithine		5.30	1.52	70	1.6
Methionine sulfoxide		5.35	1.54	60	1.4
Lycine	Lys	5.50	1.62	570	3.2
Glutamine	Gln	8.55	3.07	10	1.8
Asparagine	Asn	9.99	3.76	190	2.0
Threonine	Thr	10.79	4.14	150	3.2
Citrulline		10.46	4.00	130	2.1
Alanine	Ala	11.32	4.39	480	3.5
Glycine	Gly	14.04	5.69	830	3.0
Hydroxyproline		14.14	5.73	120	1.8
α -aminobutyric acid		14.30	5.80	250	2.6
Serine	Ser	15.24	6.26	270	1.5
Valine	Val	16.25	6.74	510	3.1
Proline	Pro	18.70	7.90	190	1.8
Isoleucine	Ile	20.84	8.92	1000	3.3
Leucine	Leu	21.80	9.38	1000	3.4
Norleucine		23.50	10.19	900	2.3
Methionine	Met	23.86	10.36	40	2.3
Ethionine		28.00	12.33	150	2.8
Histidine	His	28.67	12.65	35	2.7
Phenylalanine	Phe	29.15	12.88	55	2.3
Glutamic acid	Glu	32.12	14.30	1200	3.2
Aspartic acid	Asp	32.89	14.66	420	3.4
Cysteine	Cys	36.40	16.33	320	3.1
Cystine	Cyx	36.80	16.52	330	2.9
Tyrosine	Tyr	39.04	17.60	300	2.1
Tryptophan	Trp	46.10	20.95	140	3.3

^a $k' = (t_R - t_M)/t_M$, where t_R = retention time of solute and t_M = retention time of solvent front

^bLOD, limits of detection (nM). Assumed to be three times the signal-to-noise at baseline (S/N)=3.

^cRSD, relative standard deviation (%). Based on ten injections of standard amino acid at levels listed in Fig. 1.

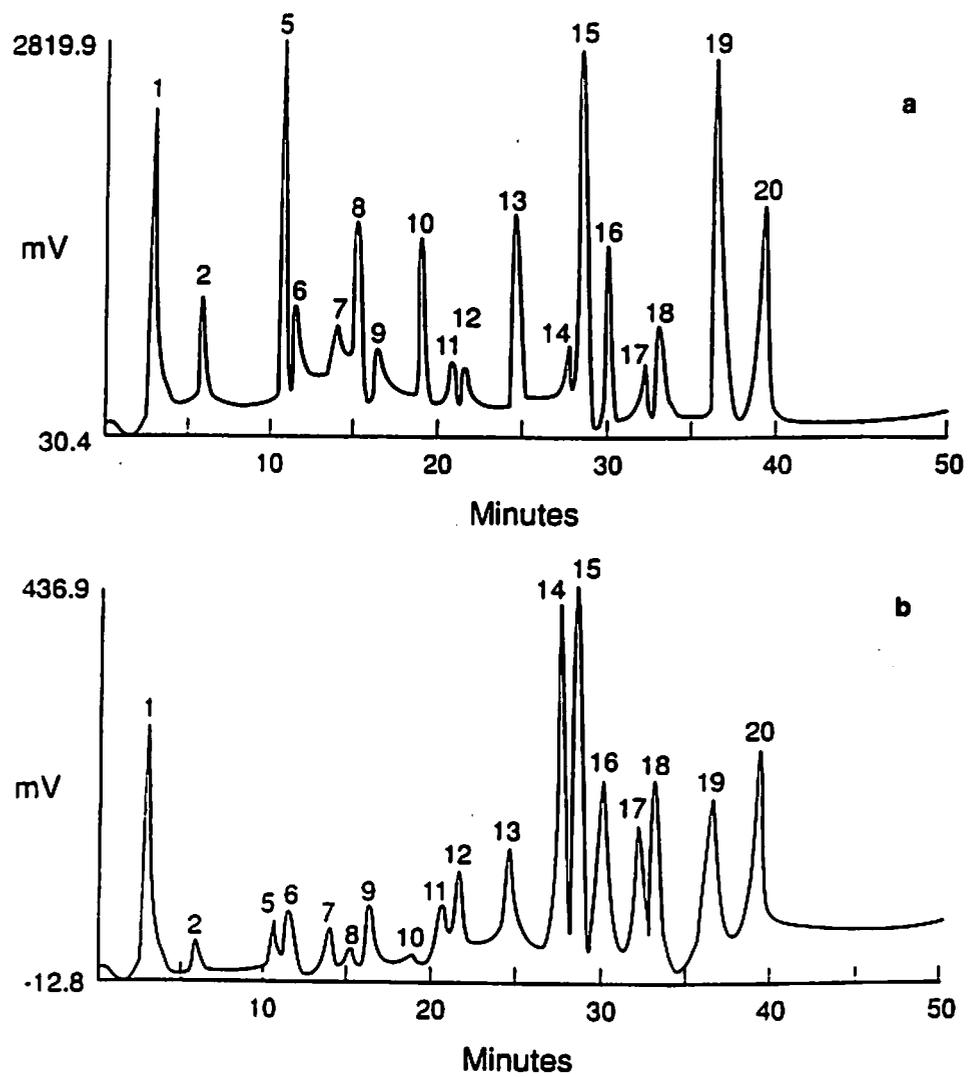


Figure 2. Chromatograms of amino acids (50 nM amino acid mL^{-1}) detected by a) HPAC-PAD and b) HPAC-UV-Vis. Peak identities listed in Fig. 1.

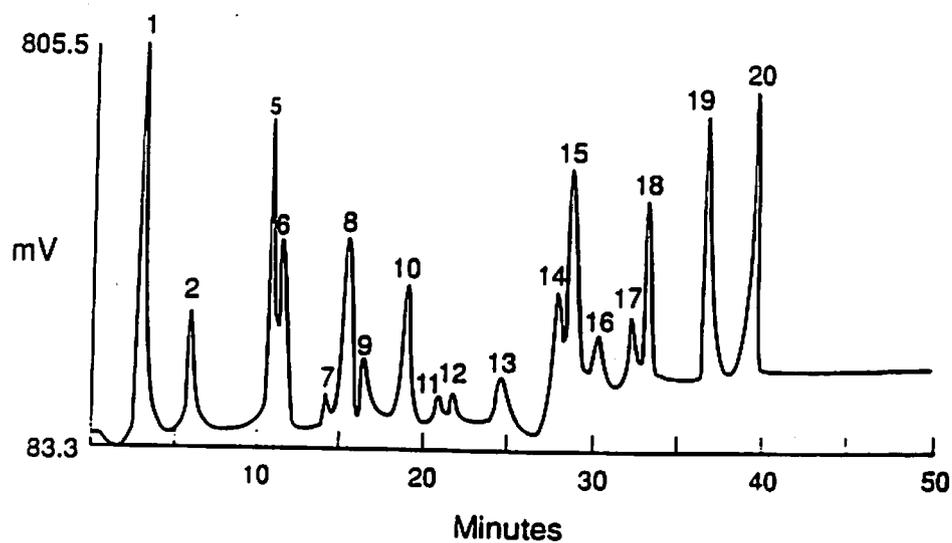


Figure 3. Chromatogram of hydrolyzed amino acids from ribonuclease A detected by HPAC-PAD. Peak identities listed in Fig. 1.

TABLE 2
Amino Acid Composition of Ribonuclease A as Detected by HPAC-PAD.^a

Amino Acid	Smyth et al. (26)	HPAC-PAD
Arg	4	3.5
Lys	10	9.8
Thr	10	10.1
Ala	12	11.9
Gly	3	2.8
Ser	15	14.8
Val	9	8.4
Pro	4	4.0
Ile	3	2.3
Leu	2	1.8
Met	4	4.0
His	4	3.9
Phe	3	2.7
Glu	12	11.8
Asp	15	14.9
Cys-Cyx	8	7.8
Tyr	6	5.8
Trp	0	0

^aData are expressed as the number of residues per molecule.

TABLE 3
Amino Acid Composition of the Root Mass and Collected Exudates of
Two Wheat Varieties^a

Amino Acid	Wheat Variety			
	Yolo		Altar 84	
	Exudate	Root	Exudate	Root
	nM plant ⁻¹	nM g ⁻¹	nM plant ⁻¹	nM g ⁻¹
Arg	1.3	21.3	1.0	5.8
Lys	1.0	154.3	0.4	298.3
Gln	ND ^b	ND	0.1	ND
Asn	ND	ND	0.4	ND
Thr	0.3	24.5	0.1	12.0
Ala	1.5	81.7	0.2	63.6
Gly	ND	213.7	0.2	62.2
Ser	0.1	28.8	0.1	16.4
Val	0.6	28.5	0.3	24.5
Pro	ND	37.3	0.3	27.5
Ile	0.2	23.2	0.2	209.7
Leu	0.2	23.9	0.3	336.7
Met	ND	0.8	0.1	31.8
His	0.3	0.2	ND	8.6
Phe	ND	7.2	ND	12.3
Glu	ND	31.7	ND	304.7
Asp	ND	48.1	ND	49.8
Cys-Cyx	0.2	2.6	ND	6.7
Try	ND	5.0	ND	25.9
Trp	ND	22.8	ND	23.5
Total amino acids	5.7	755.6	3.7	1520.0

^aThree wheat plants of each variety were aseptically grown for 17 days. Exudates were extracted with 20% ethanol for amino acid analysis. The root mass was air-dried and hydrolyzed with 4N methanesulfonic acid for 22 h at 120°C.

^bNot detected.

microbes in contact with the roots (rhizosphere) compared with the nonrhizosphere soil. Amino acids are one class of compounds that have been detected in plant root exudates (27). Microbial metabolism of the released amino acids has been shown to affect plant development. The biosynthesis of methionine into ethylene (28) and tryptophan into indole-3-acetic acid (29) by soil microorganisms has led researchers to suggest that amino acids released into the rhizosphere may significantly influence plant growth and vigor.

The aseptically-grown wheat varieties had a wide range in amino acids present in their root mass (Table 3). The use of HPAC-PAD indicated that the amino acid composition of the root was not the same as the amino acid composition of their respective root exudates (Table 3). Methionine was detected in the exudate of the Altar 84 variety, but not in the Yolo variety. Tryptophan was not detected in either exudate.

This study reveals that HPAC-PAD can be used for direct detection of both primary and secondary protein and nonprotein amino acids. Minimal sample preparation is needed for the HPAC-PAD analysis. HPAC-PAD was much more sensitive than ninhydrin detection and comparable with precolumn derivatization procedures with less interferences in determination of amino acids in biological matrices.

REFERENCES

1. Pickering, M. V., Ion-exchange Chromatography of Free Amino Acids, LC-GC. *Z*, 484-490, 1989.
2. Jones, B. N., and Gilligan, J. P., o-Phthaldialdehyde Precolumn Derivatization and Reversed-Phase High-Performance Liquid Chromatography of Polypeptide Hydrolysates and Physiological Fluids, *J. Chromatog.*, **266**, 471-482, 1983.
3. Gardner, W. S., and Miller, W. H., III., Reverse-Phase Liquid Chromatographic Analysis of Amino Acids After Reaction with o-Phthalaldehyde, *Anal. Biochem.*, **101**, 61-65, 1980.
4. Lindroth, P., and Mopper, K., High Performance Liquid Chromatographic Determination of Subpicomole Amounts of Amino Acids by Precolumn Fluorescence Derivatization with o-Phthaldialdehyde, *Anal. Chem.*, **51**, 1667-1674, 1979.

5. McClung, G., and Frankenberger, W. T., Jr., Comparison of Reverse-Phase High Performance Liquid Chromatographic Methods for Pre-column Derivatized Amino Acids, *J. Liq. Chromatog.*, 11, 613-646, 1988.
6. Hamilton, P., Ion Exchange Chromatography of Amino Acids, A Single Column High Resolving, Fully Automatic Procedure, *Anal. Chem.*, 35, 2055-2064 1963.
7. Tarr, G., Rapid Separation of Amino Acid Phenylthiohydantoins by Isocratic High-Performance Chromatography, *Anal. Biochem.*, 111, 27-32 1981.
8. Black, S., and Coon, M., Simple, Rapid and Highly Efficient Separation of Amino Acid Phenylthiohydantoins by Reverse-Phase High Performance Liquid Chromatography, *Anal. Biochem.*, 121, 281-285, 1982.
9. Reitsma, B. Ph.D. Dissertation, Iowa State University, Ames, IA, 1987.
10. Moore, S., Spackman, D. H., and Stein, W. H., Chromatography of Amino Acids on Sulfonated Polystyrene Resins, *Anal. Chem.*, 30, 1185-1190, 1958.
11. Maljoy, M., and Reynaud, J. A., Electrochemical Investigations of Amino Acids at Solid Electrodes. Part II. Amino Acids Containing no Sulfur Atoms: Tryptophan, Tyrosine, Histidine and Derivatives, *J. Electroanal. Chem.*, 114, 213-223, 1980.
12. Hiu, B. S., and Huber, C. O., Amperometric Detection of Amines and Amino Acids in Flow Injection Systems with a Nickel Oxide Electrode, *Anal. Chim. Acta.*, 134, 211-218, 1982.
13. Krafil, J. B., and Huber, C. O., Flow Injection Sample Processing of Amino Acids Separated by Ion-Exchange Chromatography. *Anal. Chim. Acta.*, 139, 347-352, 1982.
14. Polta, J. A., and Johnson, D. C. The Direct Electrochemical Detection of Amino Acids at a Platinum Electrode in an Alkaline Chromatographic Effluent. *J. Liq. Chromatog.*, 6, 1727-1743, 1983.
15. Fleischman, M., Korinek, K., and Pletcher, D. J., The Kinetics and Mechanism of the Oxidation of Amines and Alcohols at Oxide-Covered Nickel, Silver, Copper, and Cobalt Electrodes. *Chem. Soc. Perkin.*, 11, 1396-1403, 1972.

16. Hughes, S., and Johnson, D. C., High Performance Liquid Chromatographic Separation with Triple-pulse Amperometric Detection of Carbohydrates in Beverages. *Anal. Chim. Acta.*, 132, 11-22, 1983.
17. Martens, D. A., and Frankenberg, W. T., Jr., Determination of Saccharides by High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection *Chromatographia*, 29, 7-12, 1990.
18. Martens, D. A., and Frankenberg, W. T., Jr., Determination of Aminosaccharides by High Performance Anion-exchange Chromatography with Pulsed Amperometric Detection. *Talanta*, 38, 245-251, 1991.
19. Martens, D. A., and Frankenberg, W. T., Jr., Determination of Glycuronic Acids by High Performance Anion Chromatography with Pulsed Amperometric detection, *Chromatographia*, 30, 651-656, 1990.
20. Simpson, R. J., Neuberger, M. R., and Liu, T. Y., Complete Amino Acid Analysis of Proteins From a Single Hydrolysate, *J. Biol. Chem.*, 251, 1936-1940, 1976.
21. Hoagland, D. R., and Arnon, D. I., The Water Culture Method for Growing Plants without Soil, *California Agr. Expt. Sta. Circ.*, 347, 1950.
22. Gilbert, R. G., and Altman, J., Ethanol Extraction of Free Amino Acids from Soil, *Plant Soil*, 24, 229-238, 1966.
23. Hunt, S. *Chemistry and Biochemistry of the Amino Acids*. Barrett, G. C., Ed., Chapman and Hall, New York, 1985, p. 376-398, 1985.
24. Heinrikson, R. L., and Meredith, S. C., Amino Acid Analysis by Reverse Phase High Performance Liquid Chromatography: Precolumn Derivatization with Phenylisothiocyanate, *Anal. Biochem.*, 136, 65-74, 1984.
25. Jones, B. N., Pääbo, S., and Stein, S., Amino Acid Analysis and Enzymatic Sequence Determination of Peptides by an Improved o-Phthaldialdehyde Precolumn Labeling Procedure, *J. Liq. Chromatog.*, 4, 565-586, 1981.
26. Smyth, D. G., Stein, W. H., and Moore, S. J., The Sequence of Amino Acid Residues in Bovine Pancreatic Ribonuclease: Revisions and Confirmations, *Biol. Chem.*, 238, 227-234, 1963.

27. Rovira, A. D., Plant Root Exudates, Bot. Rev., 35, 35-57, 1969.
28. Arshad, M., and Frankenberger, W. T., Jr., Production and Stability of Ethylene in Soil, Biol. Fertil. Soils., 10, 29-34, 1990.
29. Frankenberger, W. T., Jr., Chang, A. C., and Arshad, M., Response of *Raphanus sativus* to the Auxin Precursor, L-Tryptophan Applied to Soil, Plant Soil., 129, 235-241, 1990.