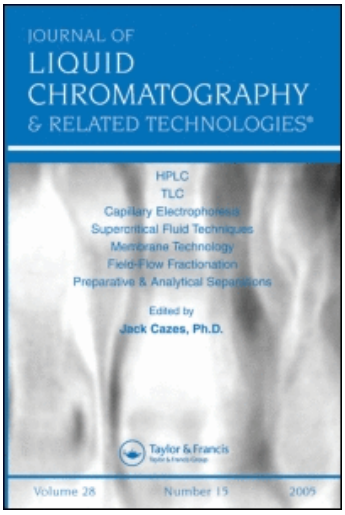

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THE USE OF REVERSE-PHASE HPLC FOR SOIL AMINO-N ANALYSIS

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ABSTRACT

Soil organic matter was extracted from field soils using mild pretreatment and a Chelex 100 resin bed. The extracts were separated by molecular weight using a Sephadex G-25 gel permeation chromatography column. Reverse-phase high performance liquid chromatography and o-phthalialdehyde precolumn derivatization were used to rapidly determine the primary amino compounds present in the 6 N HCl-soluble fractions. Twenty amino-N compounds were quantitatively identified in the hydrolysates.

INTRODUCTION

Classical fractionation of soil organic matter is based on the solubility of humic substances in strong base and strong acid, producing humin, fluvic and humic acids (1,2). Many researchers, however, have characterized soil organic matter fractions on the basis of molecular weight, using the technique of gel permeation chromatography (2). Soil fractions, varying in molecular weight, have then been hydrolysed and analysed for amino-N compounds.

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Approximately 98% of the N found in soils is organically combined; 20-40% of the total acid hydrolyzable N is in the form of amino acids (2,3,4). The first modern study of soil amino acids was conducted by Bremner in 1950 (5); he detected 19 amino acids by paper partition chromatography. Using ion exchange and gas chromatography, researchers have identified many other amino acids in soils, including some compounds which are not normal constituents of proteins. The quantitative and qualitative analysis of soil amino-N compounds has been the subject of a number of recent reviews (2,3,4).

Amino sugars account for about 5-10% of the total soil N (2,3,4). Amino sugars have been isolated from acid soil hydrolysates by paper partition chromatography and ion-exchange chromatography (2). Although D-glucosamine and D-galactosamine are the two most common amino sugars isolated, other amino sugars have been identified in soils (2).

HPLC analysis of soil constituents is in an infant stage of development. HPLC has been used for the determination of metals (6), pesticides (7), and naturally occurring phenolic acids in soils (8). There are no published papers, however, describing the use of reversed phase HPLC for soil amino-N analysis. This paper, therefore, describes the use of HPLC for characterization of amino acids and amino sugars in acid hydrolysates of molecular weight fractions of soils.

MATERIALS AND METHODS

Apparatus

The chromatography system used consisted of a Beckman Model 334 Gradient Liquid Chromatograph comprised of a Model 421 microprocessor system controller, two Model 110A high-performance pumps, a Model 210 sample injector valve and a Model 157 fluorescence detector with a 9 µl flow cell, a 305-395 nm excitation filter, a 420-650 nm emission filter and a RFU range setting of 0.05. An Ultrasphere-ODS 15 cm x 4.6 mm I.D., 5 µm particle size column

was used for the separation. This was fitted with a guard column (30 mm x 4.6 mm I.D.) packed with Vyopac reverse phase column packing. Chromatographic peaks were integrated using Chromatochart software (Interactive Microware, Inc.) developed for the Apple IIe microcomputer. A Hewlett Packard 7133A recorder was used to generate the chromatograms.

The gel permeation chromatography was carried out on an LKB 75 x 2.6 cm column packed with Sephadex G-25-80. The fractions were collected using a Buchler LC200 linear fraction collector after passage through a flow-through cell of a Perkin-Elmer Coleman Model 124 double beam U.V. Spectrophotometer set at 260 nm.

Reagents

HPLC grade methanol was obtained from Anachemia (Lachine, Quebec), o-phthaladehyde, 2 mercaptoethanol and tetrahydrofuran were purchased from Caledon Laboratories Ltd., (Georgetown, Ontario) and sodium dodecyl sulfate was purchased from Chromatographic Specialties Ltd. (Brockville, Ontario). High purity water was obtained with a Gelman Sciences Water I system (Montreal, Quebec). Individual amino acids, amines, and Sephadex G-25-80 (20-80 μ particle size) were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Chelex 100 (50-100 mesh, Na form) was obtained from Bio-Rad Laboratories Ltd. (Mississauga, Ontario). All other chemicals were reagent grade. All solutions were filtered through a 0.22μm cellulose acetate membrane filter before using on the HPLC.

Preparation of Chemical Solutions

The preparation of the OPA derivatizing solution followed the method of Jones et al. (9).

The OPA derivatives were prepared as follows: A 10-20 μl standard or unknown or water blank was mixed with 10 μl 2% sodium dodecyl sulfate (SDS) in 0.4 M sodium borate (pH 9.5) and 10 μl OPA derivatizing solution. This was mixed using the Vortex-Genie for 1 minute. Then 40-100 μl of 0.1 M potassium phosphate (pH 4.0) was added, the solution mixed (10-15 sec) and 10 μl injected onto the reverse phase column.

Soil Extraction and Fractionation

The soils used for extraction came from Ormstown, Quebec. The soil, a Franklin stoney loamy sand, had a pH of 6.2, 5.1% organic matter and a total N of 5.4 mg N/g soil. Field moist soil was pretreated with a HF-HCl solution to dissolve silicate minerals and then the procedure continued as per Figure 1 (Soil extraction flow diagram). The use of the Chelex 100 for removing metals from the soil solution evolved from the work of Thomas & Bowman (10). The crude extract was fractionated by molecular weight (Sephadex G-25-80 GPC column) using H_2O as the elutant at a flow rate of 2.5 ml/min. Figure 2 shows a typical UV scan (260 nm) of the molecular weight fractionation of a crude extract into a high and low molecular weight fraction. Following fractionation, the peak fractions were pooled, flash evaporated at 30°C and taken up to a 25 ml volume. An aliquot (2-5 ml) of the sample was acid hydrolyzed (6 N HCl) for 16 hours, flash evaporated and taken up in HPLC grade water. Chloride-free samples were made up to a 25 ml volume and passed through a 0.22 µm Millipore filter. The sample was then analyzed by HPLC for amino acids, amines, and amino sugars.

HPLC Gradient System

The solvent and gradient system was adopted from Umagat et al. (11). Solvent A was tetrahydrofuran: 0.05 M sodium acetate (pH 6.6) 1:99 and Solvent B was methanol. The flow rate was set at 1.2 ml/min and the gradient program was as follows: 15% B for 2 min for the beginning of the program, linear step to 28% B for 1 min, isocratic elution step at 28% B for 10 min, linear step to 44% B for 5 min, linear step to 56% B in 1 min, isocratic elution step at 56% B for 10 min, linear step to 80% B in 1 min and isocratic development at 80% B for 7 min.

RESULTS

Figure 3 represents a chromatogram from the HPLC analysis of a HCl-hydrolysed standard of 22 amino-N compounds. Each peak

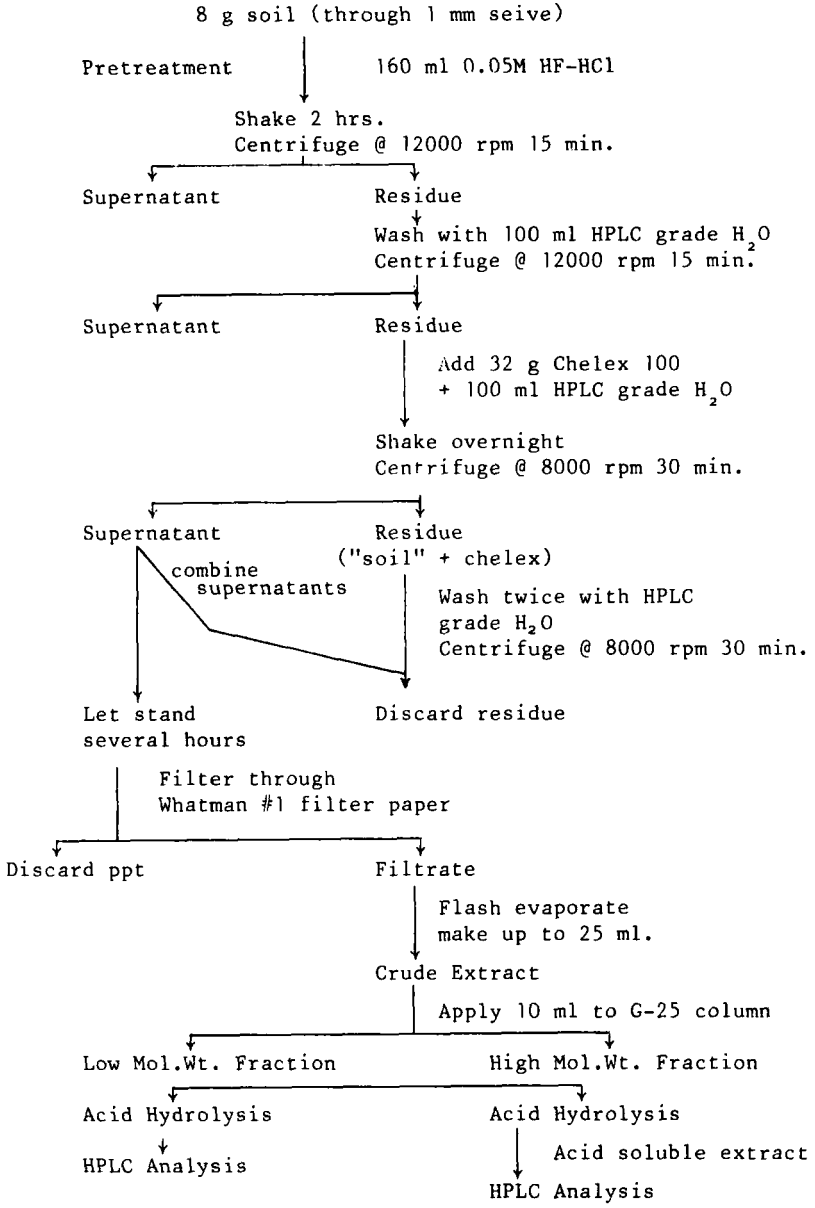

Figure 1. Soil Extraction Flow Diagram

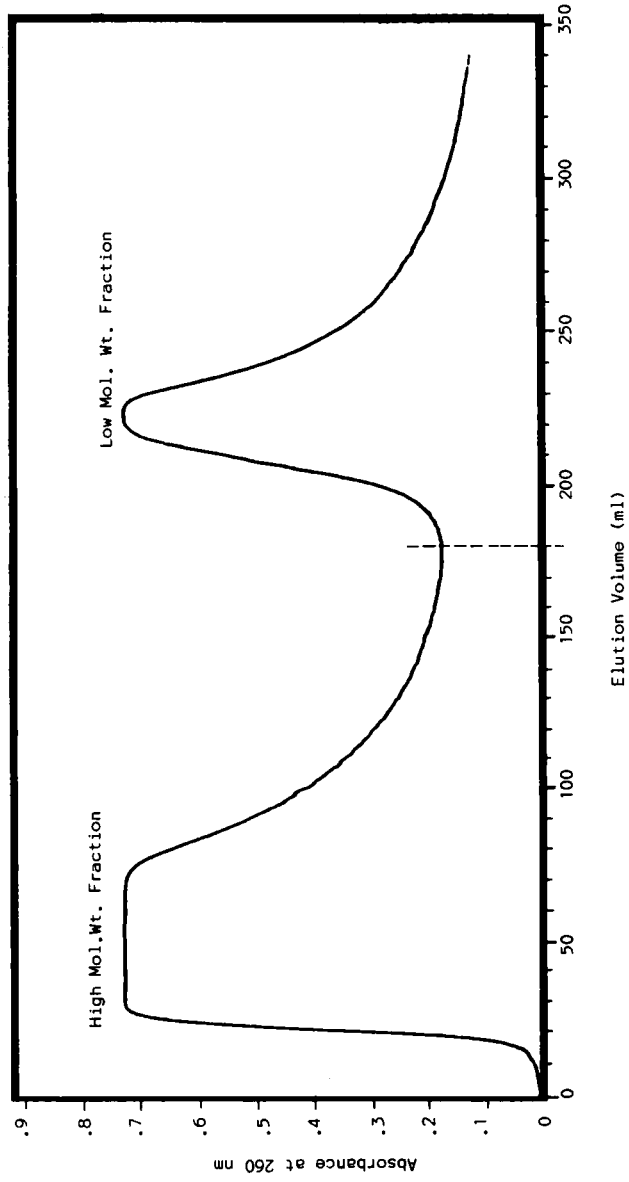

Figure 2. Elution fractionation pattern of a crude soil extract off a 2.6 x 75 cm column of Sephadex G-25-80.

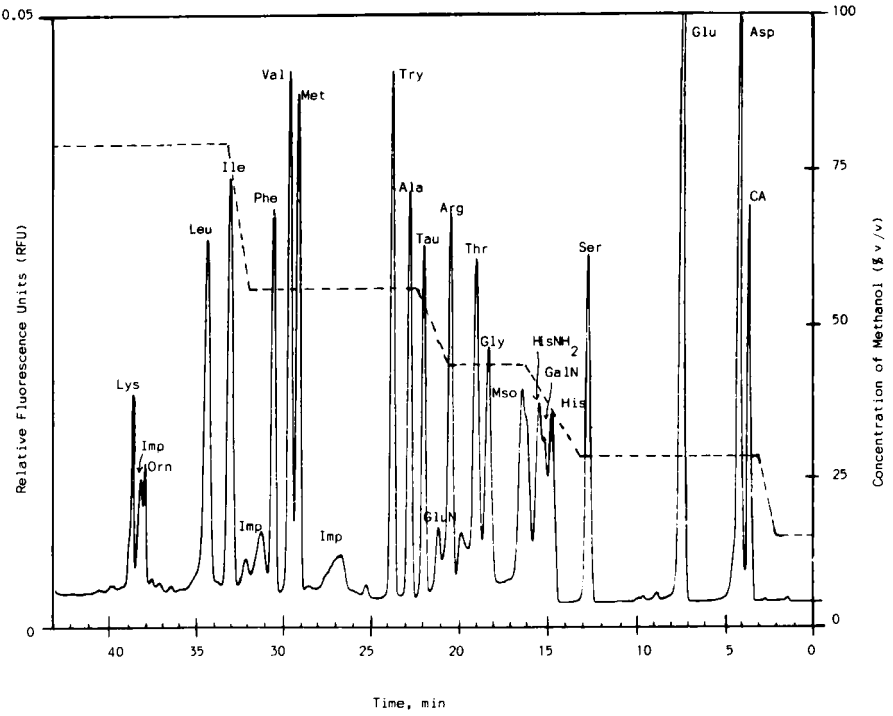

Figure 3. HPLC chromatogram of amino-N standards with each peak representing 20 pmoles. Non-standard abbreviations used: Gal-N, galactosamine; $HisNH_2$, histamine; Mso, methionine sulfoxide; Glu-N, glycosamine; Tau, taurine; Imp, impurity.

of the standard is equivalent to 20 pmols. Figure 4 is a chromatogram of a typical acid soluble crude extract; a chromatogram from a high molecular weight (HMW) fraction was not illustrated because it would be similar to a crude extract. Figure 5 shows a chromatogram of a typical low molecular weight (LMW) fraction. The LMW fraction showed the most variation between samples of the three extracts. Present evidence would indicate that the amino acids determined in the LMW fraction are quantitatively equivalent to an un-hydrolysed LMW sample; apparently the amino acids found in the LMW fraction exist unbound by peptide linkages. Furthermore, in order to differentiate the amino-N compounds from the blank,

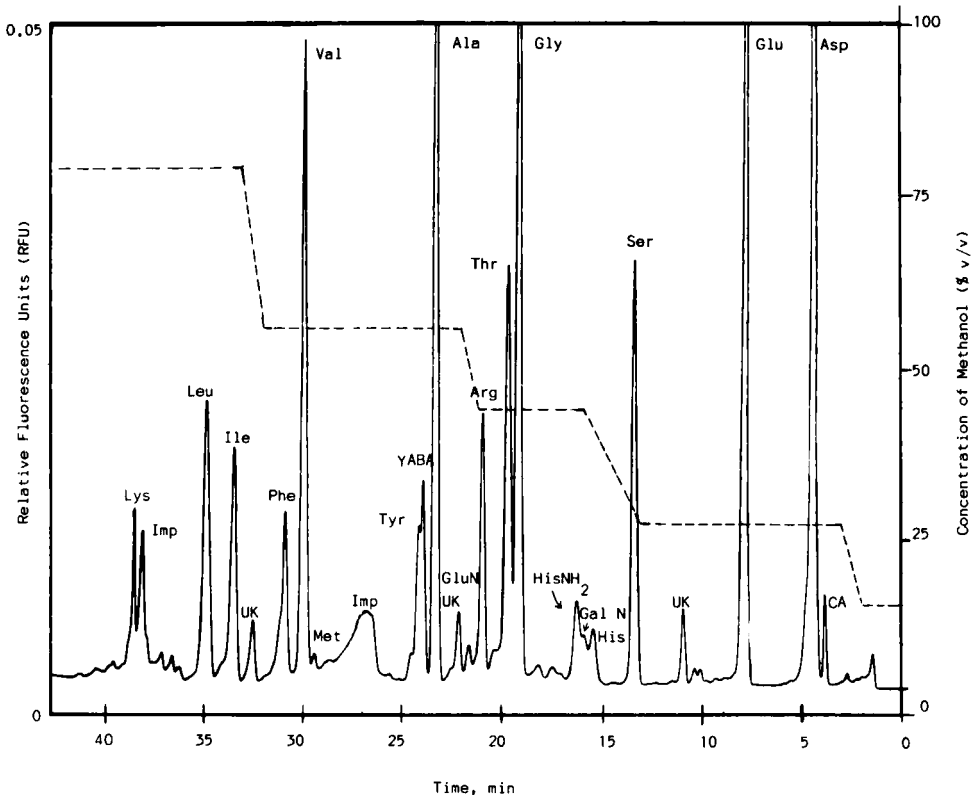

Figure 4. HPLC chromatogram of a crude soil organic matter extract. Non-standard abbreviations used: UK, unknown; Gal-N, galactosamine; $HisNH_2$, histamine; GluN, glucosamine; YABA, 4-aminobutyric acid; Imp, impurity.

the concentration of LMW derivatized sample was increased in comparison to a corresponding crude or HMW extract. The mean quantities of amino-N compounds (μmol) determined in the crude extract and the two molecular weight fractions is indicated in Table 1. The crude and HMW results are the means of seven different extracts from different soil fertility plots, the LMW results are the mean of four different soil extract. The variation in fertility treatments may account for the relatively large standard deviation found for many of the amino-N compounds.

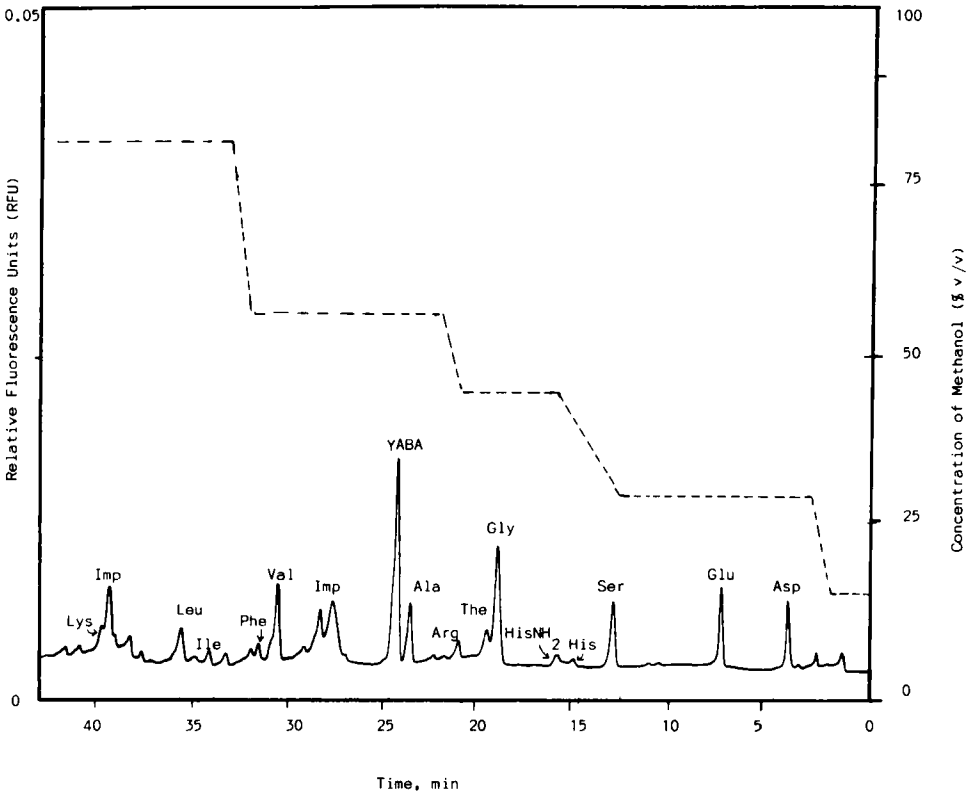

Figure 5. HPLC chromatogram of a low molecular weight soil organic matter fraction. Non-standard abbreviations used: $HisNH_2$, histamine; YABA, 4-aminobutyric acid; Imp, impurity.

The results of the HPLC analysis of the crude and HMW fractions are similar to the published studies (2,4) and to unpublished research conducted by the senior author using the molecular weight fractionation technique (12). In both the crude and HMW extracts (Table 1), aspartic acid, glutamic acid, glycine, and alanine occur in the greatest quantity. These results are similar to the published work, except for arginine, which constitutes a much lower proportion of the total amount of amino-N in our study than is indicated in the literature. In addition, proline

and hydroxyproline are not identified because the secondary amino acids are not detected by OPA.

The results of Table 1 show that the glucosamine:galactosamine ratio was greater than 1 for the crude and HMW extracts, but less than the 1.8:1 ratio determined by other workers (4). The detection of these two amino sugars, along with the amino acids, in an HCl hydrolysate was a positive step towards the use of HPLC. Other amino sugars may be present in soil fractions extracted by a less rigorous technique than acid hydrolysis.

It was noted that the quantity of histamine exceeded that of histidine in all extracts. There are few published reports in the soil science literature which mention the identification of histamine or any of the other amines (2).

TABLE 1

The Mean Amino-N Content (μmol) of Soil Organic Matter Extracts

	Crude Extract	High Mol.Wt. Fract.	Low Mol.Wt. Fract.
CA	0.45 ± 0.18	0.30 ± 0.16	0.05 ± 0.06
Asp	7.89 ± 3.60	3.82 ± 0.83	0.05 ± 0.04
Glu	6.39 ± 2.06	3.36 ± 0.86	0.07 ± 0.05
Ser	2.77 ± 1.06	1.46 ± 0.45	0.02 ± 0.02
His	0.52 ± 0.47	0.17 ± 0.13	N.D.
*Gal-N	1.53 ± 1.08	0.83 ± 0.63	N.D.
*$HisNH_2$	1.48 ± 1.45	1.10 ± 0.52	0.02 ± 0.02
Gly	6.98 ± 2.57	3.01 ± 0.49	0.08 ± 0.02
Thr	2.89 ± 1.35	1.55 ± 0.32	0.07 ± 0.07
Arg	1.88 ± 0.76	0.99 ± 0.21	0.03 ± 0.01
*Glu-N	1.70 ± 2.10	1.33 ± 1.00	N.D.
Ala	5.27 ± 1.59	2.71 ± 0.54	0.05 ± 0.02
Tyr	1.03 ± 1.01	0.82 ± 0.83	0.05 ± 0.03
Met	0.07 ± 0.07	N.D.	N.D.
Val	2.75 ± 0.95	1.27 ± 0.29	0.01 ± 0.01
Phe	0.97 ± 0.46	0.50 ± 0.20	0.02 ± 0.01
Ile	1.48 ± 0.67	0.64 ± 0.20	0.03 ± 0.01
Leu	1.39 ± 1.00	0.63 ± 0.26	0.04 ± 0.06
Lys	2.08 ± 1.22	1.25 ± 0.55	0.08 ± 0.06

N.D. = not detectable

* = Non standard abbreviations used: Gal-N, Galactosamine; $HisNH_2$, Histamine; Glu-N, Glucosamine

DISCUSSION

The advantages of reversed phase HPLC over the other systems previously used to determine soil amino-N compounds is precision, rapidity and reproducibility. With the addition of the Chromatochart software and the Apple IIe microcomputer, the HPLC analysis becomes easily quantitative. The disadvantages of the described system are the inability to derivatize secondary amino acids by OPA and to resolve methionine from tryptophan. The latter, however, is destroyed in the acid hydrolysis. We found that the Ultrasphere-ODS column performed better than the Waters Resolve™ column because we could not adequately separate glycine and threonine or quantitatively determine histidine with the Resolve column.

The authors are presently conducting enzyme hydrolysis of the same crude and HMW fraction extracts. We expect the less-rigorous hydrolysis procedure will provide many other unknown peaks of various amino-N compounds. Future efforts, furthermore, will attempt to reconcile the differences in amino-N compounds between the different fertility treatments.

ACKNOWLEDGEMENTS

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