A Desalting Technique for Amino Acid Analysis of Use in Soil and Geochemistry

Glenn E. Pollock* and Audrey K. Miyamoto

A desalting method has been developed for use in the preparation and analysis of amino acids from soil or ground rock samples. The method uses acid hydrolysis followed by precipitation of most cations as fluorides, using hydrofluoric acid. Adjustment of the filtrate to pH 8 to 8.5 causes substantial removal of the last remaining multivalent cation, ferric ion. Sodium chloride is removed by saturating the solution with HCl. The filtrate from the sodium ion removal is evaporated to dryness, the residue dissolved in water and passed through a short, strong cation exchange column. Operations are simple, processing volumes have been reduced over conventional systems, and amino acid recoveries are reasonably high.

Soil scientists and geochemists frequently wish to investigate the amino acid composition of soils or rocks for a variety of reasons. To do this it is usually necessary to process large samples to obtain sufficient material for study. The method normally used for amino acid isolation involves hydrolysis in 6*N* HCl followed by evaporation to dryness and desalting (Bremner, 1965; Harris *et al.*, 1961). Generally, strong cation exchangers (*e.g.*, AG50W x $8H^+$) are the most frequent and effective choice for desalting rock or soil hydrolyzates. These hydrolyzates, however, require large columns of 200 to 500 ml of resin for 50- to 100-g samples and large volumes for elution of the amino acids. Long and cumbersome evaporations, losses, and contamination can occur and frequently aluminum salts contaminate the eluates. Electrolytic desalting has been used (Carson, 1966), but this procedure was found to give large losses of dicarboxylic acids, and for large samples, similar problems just mentioned also occur.

Our work required a desalting method which would contain few steps, be operationally simple, give high amino acid recoveries, and reduce the volumes of liquid to be evaporated. It was found early in our search that the basic problem in desalting was removal of the multivalent cations of iron, aluminum, calcium, and magnesium. Sodium and potassium ions are easily reduced in concentration by saturating an aqueous solution of amino acids with HCl gas. By common ion effect, NaCl and KCl solubility is so reduced that they can be filtered off. The filtrate, containing the amino acids, is evaporated and is suitable in most cases for Spackman-Stein-Moore ion exchange or paper chromatographic analysis. Unfortunately, the multivalent cations previously mentioned are not as easily removed or reduced in concentration. In the course of our studies, however, a method was developed which may be of interest to soil scientists and geochemists. In our method, all four multivalent cations are reduced to noninterfering levels for all samples studied.

MATERIALS AND METHODS

Sample Description. Two soils of different organic composition were used in this study. Salinas loam was a soil of mixed alluvial formation with a calcareous subsoil. The sample used was a mixture of the first 8 in. of soil. Waukena was a soil from acid igneous rock, alluvial in formation with saline and alkali salt composition. Since this soil was not under cultivation, it can be stated that the sample was a homogenous mixture of the A and approximately 20% of the B horizon.

Ames Research Center, NASA, Moffett Field, Calif. 94035

Two geochemical samples were chosen which were of different inorganic and amino acid composition. The Saanich inlet sample was a rich marine sediment from a stagnant fjord of Puget Sound on the coast of British Columbia. The volcanic ash was igneous rock collected about 1.5 km north of the Crater of Paricutin, Mexico, and of very low amino acid composition. The ash sample was collected about 2 years after ash fall.

The model igneous rock salt mixture was chosen and made up to conform generally to the analysis by Clarke and Washington (1924; Parker, 1967).

Model System. A salt mixture corresponding to the content of cations in igneous rock was prepared to test the desalting technique. Titanium, SiO_2, and trace elements present in igneous rock were not included in the mixture. To the mixture of salts (Table I) was added 660 μg of total amino acids (in equimolar concentration) in 1 ml. The sample was processed according to the desalting procedure described later.

EQUIPMENT AND REAGENTS

In the desalting method described, the following specialized equipment was used: polypropylene test tubes (50–100 ml), polypropylene Erlenmeyer flasks (50–100 ml), polypropylene pipets (1, 5 and 10 ml), Nalgene Buchner funnels, a Fisher filtrator, Teflon stirring bars and rods, Millipore 10 μ Teflon filter discs, and Whatman GF/A filter discs.

The polypropylene test tubes were used for evaporations under vacuum since they retain their form at elevated temperatures (30–40° C) while the Erlenmeyer flasks do not.

Reagents used were of ACS analytical grade.

Cation Analysis. Cation analysis was carried out using a Perkin-Elmer Model 303 atomic absorption spectrophotometer and the methodology described in Perkin-Elmer's supplement book (Perkin-Elmer Corp., 1966). Since a mixture of cations was present in most analyses, special precautions were taken to eliminate interferences when necessary.

Sodium and potassium were analyzed on samples with no special modifications and using an air:acetylene flame. Iron was analyzed by adding 10,000 ppm of La^{3+} as $LaCl_3$ and using an air:acetylene flame. Calcium and aluminum were analyzed by adding 1000 ppm of K^+ as KCl, and using a nitrous oxide:acetylene flame. Magnesium was analyzed by adding 1500 ppm of Sr^{2+} as $SrCl_2$, and using a nitrous oxide:acetylene flame.

Amino Acid Analysis. Amino acids were quantitatively determined by the gradient elution technique of Spackman *et al.* (1958) with the exception that $SnCl_2$ was left out of the ninhydrin and NaCN put in the buffers according to Rosen *et al.* (1962). BioRad A4 and A5 resins were used for sepa-

Table I. Model System Composition

Stock Solutions	G/200 M1	Mg Cation/M1	M1 Stock Solution To Give 660 Mg Total Cations
$AlCl_3 \cdot 6H_2O$	100	55.875	4.33
$FeCl_3$	50	86.075	1.76
$CaCl_2$	50	90.250	1.20
$MgCl_2 \cdot 6H_2O$	100	59.790	1.05
NaCl	50	98.370	0.51
KCl	50	131.10	0.34
			(3.64 g of total salt) (9.18 ml of total volume)

rations. Soil samples were hydrolyzed, filtered, and evaporated to dryness and then taken up in water and diluted to a given volume. The hydrolyzate sample was divided into two equal parts. To one part was added an equimolar mixture of amino acids. Both samples were processed through the complete desalting procedure and amino acid recoveries were calculated from values obtained.

Desalting Method. A soil or pulverized rock sample was mixed with 6*N* HCl (one part of soil to three parts of HCl) and hydrolyzed under reflux according to Bremner (1965) for 12 hr. After cooling, the hydrolyzate was filtered through Whatman GF/A paper on a Nalgene Buchner funnel, and the insoluble residue washed with water. The filtrate was evaporated to dryness to remove the HCl. The mixture was dissolved in water and made up to a known volume. To add an adequate amount of aqueous HF, it was necessary to take a small aliquot of the hydrolyzate solution and titrate it to pH 8 with 0.5*N* NaOH. The mequiv necessary for neutralization of the whole sample were calculated and the small aliquot was returned to the parent sample. The calculated mequiv for the whole sample gave an approximate measure of the mequiv of HF necessary to be added to the whole sample. Experience indicated that the addition of twice the number of mequiv of HF as required to neutralize the sample to pH 8 with NaOH. gave sufficient fluoride ion to precipitate substantially the cations, Na^+, K^+, Ca^{2+}, Mg^{2+}, and Al^{3+}. The sample was cooled in an ice bath and water was added so the final fluoride ion concentration would not exceed 3*N* after the addition of the aqueous 10*N* HF solution. After HF addition was completed, the sample was stirred for 5 min at room temperature in the polypropylene flask with a Teflon stirring bar. The slurry was filtered through a 10-μ, Millipore, Teflon filter disk on a Nalgene Buchner funnel into a polypropylene Erlenmeyer flask using a Fisher filtrator. The precipitate was washed with a small known volume of 3*N* HF. The volume of filtrate was measured or roughly calculated from the original volumes, added reagents, and rinses. It was assumed that approximately the same number of mequiv of NaOH would be needed to neutralize the filtrate to pH 8 as were added as HF. The approximate number of ml of 4*N* NaOH needed were calculated. At pH 8, the sodium ion concentration should be around 1.5*N*. If it is much greater than this, large amino acid losses may occur. From the calculations made, water was added to the HF filtrate, so that with the added 4*N* NaOH, the dilution of the suspension at pH 8 gave the necessary concentration of sodium ion. The 4*N* NaOH was added with cooling and stirring till pH 8 to 8.5 was reached. The pH is not stationary, but decreases with time. It was necessary to stir and adjust the pH about every 15 min for 1.5 hr. The

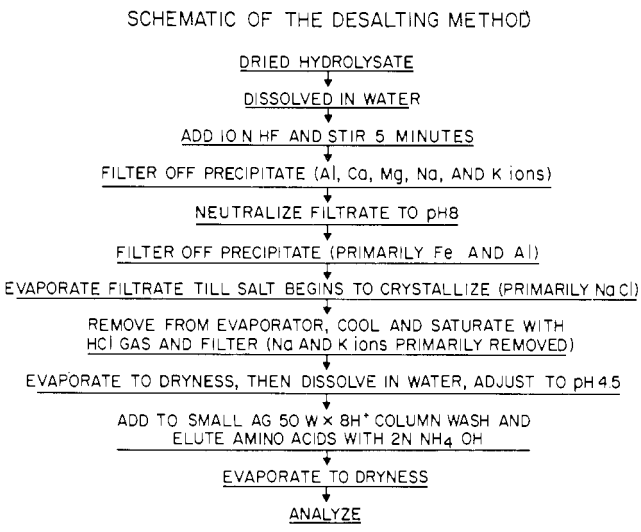

Figure 1. Schematic of the desalting method

precipitate which formed under these conditions was mainly an iron precipitate. It was not pure ferric hydroxide, however, but probably mixed ferric fluorohydroxides. The suspension was filtered with suction through a fine porosity sintered glass funnel and rinsed with a small volume of water adjusted to pH 8. The filtrates were adjusted to pH 1 to 2 and concentrated on a rotary evaporator until salt crystals began to form. The flask was removed from the evaporator and placed in an ice bath, and the solution was saturated with HCl gas. Approximate saturation was calculated from the estimated or measured liquid volume. After saturation with HCl gas, the salt precipitate was filtered off through the Teflon Millipore filter disk (10 μ). The salts were washed with a small volume of HCl saturated water, and before concentrating the filtrate to dryness, it was stirred in the polypropylene Erlenmeyer flask for about 1 hr at 30° to 40° C to remove some of the HCl to prevent bumping. Soil samples or nitrogen rich marine samples contain very little salt of any kind and could be analyzed by paper chromatography or an amino acid analyzer without further processing. Geological rock samples which require processing 50 to 100 g may have 1 to 2 g of salt residue at this point. Most of this residue is NaCl but some iron is also present. Since amino acid concentrations in these samples may be quite low and require most of the sample for analysis, removal of the residual salt is necessary. This was accomplished with a small cation exchange column (*e.g.*, sieved AG50W x $8H^+$, 100- to 200-mesh). Its size varied with the amount of residual salt, but was generally between 10 to 25 ml. The sample was dissolved in water and adjusted between pH 4.5 to 6 and placed on the column, which was rinsed with water to wash out neutrals and anions. The amino acids were eluted with 3-bed volumes of 2*N* NH_4OH. The sample was completely free of cations and was evaporated to dryness and analyzed for amino acids. Figure 1 summarizes the desalting method schematically.

Our experience indicates that using the small cation exchange column in the final step is necessary for all samples if one intends to analyze the amino acids by gas chromatographic methods. The salt does not necessarily interfere with derivatization or analysis but some neutral or anionic compounds are present at the end of the HCl step. These cause interference in gas chromatographic analysis whereas they do not react with ninhydrin and cause no problem with the Spackman-Stein-Moore method.

Table II. Removal of Cations by Precipitation Milligrams of Cation in Total Sample

SAMPLE	Al	% REMOVED	Ca	% REMOVED	Mg	% REMOVED	Fe	% REMOVED	Na	% REMOVED	K*	% REMOVED
WAUKENA												
TOTAL HYDROLYSATE (10 g)	138.0	0	129.0	0	70.5	0	154.0	0	163.0	0	45.7	0
HF FILTRATE	11.0	92.0	1.53	98.8	1.41	98.0	152.0	1.30	5.86	96.4	6.45	85.9
HF/pH 8 FILTRATE	1.94	98.6	0.102	99.9	0.006	99.9	2.33	98.5	—	—	—	—
HF/pH 8/HCl FILTRATE	1.00	99.9	0.005	99.9	0.002	99.9	3.05	98.0	1.89	98.8	4.55	90.0
IGNEOUS ROCK MIX												
INITIAL (0.66 g)	242.0	0	108.0	0	62.5	0	152.0	0	50.6	0	45.0	0
HF FILTRATE	54.0	77.7	0.325	99.7	0.367	99.4	104.0	31.6	0.226	99.6	7.63	83.1
HF/pH 8 FILTRATE	2.35	99.0	0.051	99.9	0.209	99.7	4.72	96.9	—	—	—	—
HF/pH/HCl FILTRATE	2.24	99.1	0.016	99.9	0.003	99.9	4.67	96.9	3.20	93.7	4.83	89.3
SALINAS												
TOTAL HYDROLYSATE (5 g)	156.0	0	121.5	0	61.0	0	157.5	0	28.4	0	43.40	0
HF/pH 8/HCl	2.00	99.9	0.027	99.9	0.035	99.9	3.64	97.7	2.19	92.3	0.433	99.1
SAANICH INLET												
TOTAL HYDROLYSATE (1 g)	25.0	0	2.21	0	1.32	0	20.0	0	17.4	0	6.27	0
HF/pH 8/HCl	0.400	99.9	0.130	94.1	0.006	99.9	2.03	89.9	2.64	84.8	3.0	99.9
VOLCANIC ASH												
TOTAL HYDROLYSATE (50 g)	2,030.0	0	1,020.0	0	651.0	0	655.0	0	1,210.0	0	38.4	0
HF/pH 8/HCl	14.0	99.9	0.665	99.9	0.081	99.9	103.0	84.3	320.0	73.6	5.72	85.1

*Values of K may be somewhat high since no correction was made for Na concentration.

RESULTS AND DISCUSSION

The desalting method was evaluated primarily by following the removal of the cations of interest and determining the recovery of amino acids added to samples of soil, ground rock, and a mixture of salts. Table II shows the type of removal of cations obtained. Attention is called to the results obtained with Waukena and the synthetic igneous rock sample because in these samples most cations were determined after each step of the desalting procedure. The HF treatment very significantly affects the concentration of five of the six cations of interest, *i.e.*, aluminum, calcium, magnesium, potassium, and sodium. Iron is removed also, depending upon the sample, but to a much smaller extent than the other cations. It was necessary to adjust to pH 8 to remove iron. The other ions, especially aluminum, are also significantly reduced by this pH adjustment. The nature of the precipitate is undoubtedly very heterogeneous and dependent upon the soil or rock sample. Since iron is the only cation remaining in high concentration after the HF treatment, it will naturally be the major constituent in the precipitate and will exist at least initially as ferric fluoride. The pH, when adjusted to 8, drifts; hence, a change in the nature of the precipitate undoubtedly occurs since ferric hydroxide is much less soluble than ferric fluoride. The precipitate is not allowed to convert completely to hydroxides since our experience has shown that ferric and aluminum hydroxides can cause high losses of certain amino acids (Pollock *et al.*, 1970). It has been found, however, that stirring 1.5 hr with periodic readjustment to pH 8 removes the iron to a great extent and still allows high amino acid recoveries. The heterogeneous precipitate is probably made up of fluorohydroxides which are fairly insoluble yet have a low exchange or adsorptive capacity for amino acids. The other samples gave results similar to Waukena, although calcium and iron are somewhat higher in the final volcanic ash and Saanich Inlet samples. In all samples reported in Table II, the total desalting procedure was used including the small AG50W x $8H^+$ column (10 ml). With all of these samples except the volcanic ash, it was unnecessary to use this column, but we standardized on this size for ease in packing, since we found that when smaller columns were used, *e.g.*, 2 ml, channeling could be disastrous. With the volcanic ash sample, a 10-ml column was not adequate. Calculations after the HCl precipitation step indicated that a 12- to 13-ml column would be sufficient, but a 25-ml column was used as a safety factor. With this methodology, we did not exceed a total volume of 400 ml in the processing of the 50 g of volcanic ash sample. Conventional cation column technology alone would probably fail to process this particular sample since the concentration of aluminum and iron is so high.

While we have used the complete procedure for the evaluation of these samples, cation column treatment is unnecessary for the soil samples which we examined. They can be diluted and placed on an amino acid analyzer after the HCl precipitation and evaporation steps.

Amino acid recoveries obtained on these samples using the complete processing procedure described are shown in Table III. Amino acid recoveries from these samples in which the amino acid contents varied greatly generally exceeded 80%.

Table III. Percent Recovery of Added Amino Acids

AMINO ACIDS	WAUKENA (10g) %	SALINAS (5g) %	SAANICH INLET (1g) %	VOLCANIC ASH (50g) %	IGNEOUS ROCK MIXTURE 1 PART AMINO ACID 1000 PARTS CATIONS %
ASPARTIC ACID	80.6	100.0	82.8	75	90.2
THREONINE	87.0	93.3	86.1	105	87.3
SERINE	83.6	93.6	86.8	50	85.1
GLUTAMIC ACID	74.4	82.7	81.7	100	74.9
PROLINE	98.9	93.8	85.4	TOO LOW FOR ACCURACY	93.3
GLYCINE	82.3	71.0	84.7	TOO HIGH (OFF SCALE)	97.1
ALANINE	87.9	96.0	85.4	78	96.7
α-NH_2-n-BUTYRIC	96.8	95.3	86.2	90	91.6
VALINE	92.8	90.6	90.2	82	92.2
α, ϵ-DIAMINOPIMELIC	61.5	87.7	86.0	42	71.8
ISOLEUCINE	92.6	93.6	87.6	90	90.2
LEUCINE	89.4	95.0	87.6	115	90.1
TYROSINE	82.3	82.8	82.9	TOO LOW FOR ACCURATE MEASUREMENT	—
PHENYLALANINE	87.9	99.0	82.1	80	86.3
LYSINE	80.3	84.2	101.0	40	89.6
HISTIDINE	64.3	84.4	80.0	*	50.5
ARGININE AND γ-NH_2-n-BUTYRIC	83.3	—	90.1	*	70.2
NATIVE AMINO ACID CONTENT (TOTAL)	1.0 mg/ gm SOIL	3.6 mg/ gm SOIL	11 mg/gm SEDIMENT (DSB)	0.006 mg/ gm ROCK	0.28 mg/gm OF SOLUBLE SALT

* UNKNOWN OVERLAPPING NINHYDRIN POSITIVE SUBSTANCE

The volcanic ash sample in which amino acid concentrations are low gave some organic interferences in the basic amino acid class and a low recovery for lysine.

Our initial aims in working on desalting procedures for amino acid analysis were stated earlier, and while we have not been completely successful in this endeavor, *e.g.*, the method does contain several steps; nevertheless, it is operationally simple, gives reasonably high amino acid recoveries, and liquid volumes have been reduced over conventional ion exchange desalting methods. As our study neared completion, a paper by Sowden (1969) was published; his amino acid recoveries were high and iron and aluminum were removed by a liquid-liquid extraction technique. While we have not completely evaluated his approach, we have determined that it requires large volumes of the organic extractant for small soil samples and hence would be rather unwieldly for geological samples or large soil samples. However, a combination of our method and some modification of a liquid-liquid extraction technique might prove fruitful. We verified his findings that Biogel P-2 (Schwartz and Zobin, 1966), ion-retardation resin (Rollins *et al.*, 1962) and Dowex $50H^+$ resin alone (Young and Mortenson, 1958) are inadequate for desalting soil and rock hydrolyzates. We are continuing to work in this field and hope to simplify and improve on this method in the near future.

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