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PURIFICATION OF PLANT EXTRACTS FOR ION-EXCHANGE CHROMATOGRAPHY OF FREE AMINO ACIDS

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SUMMARY

Quantitative aspects of the ion-exchange method for purifying free amino acids from plant extracts were examined. Individual amino acids were well recovered provided excessive washing of the adsorbed amino acids with water was avoided (dilute acid could be used instead) and sufficient eluant was used. Sulphur amino acids were partly oxidised, but the loss of cystine might be acceptable and the methionine value could be corrected. Tests on extracts of Italian ryegrass (*Lolium multiflorum*) S22, Sitka spruce (*Pinus sitchensis*) and Scots pine (*Pinus sylvestris* L.) gave generally satisfactory results although with 0.2 *N* sodium hydroxide eluant, some hydrolysis of glutamine was observed.

INTRODUCTION

When free amino acids are extracted from plant materials, sugars, salts, lipids and pigments are co-extracted and can interfere with their chromatographic separation. The interference is especially severe in paper and thin-layer chromatography, where purification is essential. Moore and Stein[1] stated that inorganic salts did not affect the analytical performance of ion-exchange columns. However, the repeated application of crude plant extracts to a column might slowly reduce the separation efficiency of the resin due to build-up of polyvalent inorganic cations, lipids and strongly adsorbed pigments. Therefore a purification step should be considered.

A common method of purification is to adsorb the amino acids on a short column of cation-exchange resin in the acid form. Anions and neutral substances, including organic acids and sugars, are washed out with water or dilute acid[2] and the amino acids are eluted with a dilute base solution. Metal cations are not displaced because of their strong affinity for the resin exchange sites, while some pigments and lipids are also retained by the resin matrix. For eluting the amino acids, volatile bases have been used as they are removable by distillation. Dilute ammonium hydroxide has been used most often[3–7]. Because of its colour reaction with ninhydrin the ammonia must be completely removed from the eluted amino acids. A further disadvantage of ammonia was the ease with which it could contaminate the buffer solutions used in the chromatographic analysis, necessitating the use of separate laboratories for the purification and analysis stages. Harris *et al.*[2] therefore

used 2 *N* triethylammonium hydroxide, which gives only a slight colour with ninhydrin.

Nowakowski and Byers[8] reported incomplete recovery of basic amino acids using Dowex 50-X8 (H^+) resin and eluting with 2 *N* ammonium hydroxide or using Dowex 50-X4 both in H^+ or NH_4^+ form[2]. They also obtained unreliable results by eluting with 1 *N* and 6 *N* hydrochloric acid[9]. Eluting with hydrochloric acid could undoubtedly cause some hydrolysis of both glutamine and asparagine as well as releasing some inorganic cations[10].

This paper describes an investigation into the recoveries of amino acids after preliminary ion-exchange purification and the effect of the latter on their subsequent chromatographic resolution. In view of Moore and Stein's observation dilute sodium hydroxide was used as an eluant in the preliminary purification, because it could be more convenient and effective than any of the volatile bases or strong hydrochloric acid.

EXPERIMENTAL

The low recoveries of basic amino acids obtained by Nowakowski and Byers[8] were possibly due to using an insufficient quantity of eluent relative to the amount of resin, as it is known that the basic amino acids are difficult to elute. If so, the volume of eluant could be increased, or the amount of resin reduced. The first alternative would be more time consuming and the subsequent prolonged evaporation might increase the risk of losing amino acids and hydrolysing the amides; the second was therefore chosen.

Harris *et al.*[2] showed that a column of 350 mg of moist ion-exchange resin was ample to adsorb 100 μmoles of amino acids plus all metal cations in 5 ml of blood serum, with a large safety margin. A total of about 20 μmoles of amino acid is needed for ion-exchange chromatography, so the above quantity of resin seemed reasonable. To give a greater safety margin 2.0 g of moist resin was used.

Apparatus and material

Purification was carried out on Bio-Rad analytical-grade cation-exchange resin (AG 50W-X4 200–400 mesh) contained in glass tubes (200 × 12 mm I.D.) with fused-in porosity 2 sintered-glass support discs and taps. Amino acids were determined on a Technicon NC-1 single-column amino acid analyser.

BDH chromatographically pure amino acids were dissolved in 0.01 *N* hydrochloric acid to make a solution approximately 0.1 m*M* in each compound. The solution was standardised against a standard amino acid mixture supplied by Technicon.

Preparation of column

2.0 g of moist resin (drained with suction on a No. 2 sintered glass crucible) was slurried with water and poured into a 200 × 12 mm glass tube and allowed to form a column about 2 cm long with an even horizontal surface. In all further manipulations, care was taken to avoid disturbing the surface of the resin. The resin was washed successively at a rate of flow of about 1 ml/min with 10 ml of 1 *N* hydro-

chloric acid, 40 ml of 0.2 *N* sodium hydroxide and 10 ml of 1 *N* hydrochloric acid. In between washes and after the second hydrochloric acid wash the resin was rinsed with water until free of electrolyte.

Determination of eluent volume

A 10-ml 0.1 m*M* mixed amino acid solution (in 0.01 *N* HCl) was passed through a column containing 2.0 g of resin. The resin was washed with 10 ml of water and then eluted with 0.2 *N* sodium hydroxide. The effluent was collected in 2.0-ml fractions which were tested for amino acids with ninhydrin reagent. 50 ml of alkali were required for complete elution. When, before alkali elution, the adsorbed amino acids were washed on the column with 50 ml of water (to simulate removal of impurities) 60% of the aspartic acid was lost. There was no loss if the washing was done with 45 ml of 0.01 *N* HCl followed by 5 ml of water or if the amount of resin was doubled. In the second case the volume of eluant had to be increased to 70 ml to get complete elution. For 2 *N* ammonium hydroxide eluant the corresponding volumes were 30 and 35 ml for columns of 2.0 and 4.0 g resin, respectively.

Extraction of plant material

1.00 g of dried material was extracted by refluxing for 1 h with 120 ml of either water or 80% (v/v) aqueous ethanol. The hot extract was filtered through a No. 3 sintered glass crucible and the insoluble matter washed twice with 10 ml of hot solvent. The combined extract and washings were reduced in volume at $<40°$ under reduced pressure in a rotary evaporator and made up to 100 ml in a volumetric flask with water or 80% (v/v) ethanol.

Purification of amino acids

A measured volume of solution, containing about 20 μmoles of amino acids (*e.g.* 1 μmole each of twenty amino acids) adjusted to pH 1–2, was passed through the column; the rate of flow was maintained at 1.0 ml/min. Alcoholic solutions were diluted with twice their volume of water before adding to the column. The resin was washed with 45 ml of 0.01 *N* hydrochloric acid followed by 5 ml of water. The amino acids were eluted with either 35 ml of 2 *N* ammonium hydroxide or 50 ml of 0.2 *N* sodium hydroxide. The ammonia eluates were evaporated to dryness in a rotary evaporator ($<40°$); 5-ml portions of water were added and re-evaporated until all ammonia had been removed. Sodium hydroxide eluates were neutralised to methyl red with 1 *N* HCl before evaporating to dryness ($<40°$). The residues were redissolved in 10 ml of 0.01 *N* HCl containing 1.0 μmole of norleucine as internal standard.

Ion-exchange chromatography

An aliquot of the solution (containing *ca.* 0.1 μmole of each amino acid) was applied to the column of the Technicon amino acid analyser. A modified buffer gradient was used to get satisfactory separation between ethanolamine, γ-aminobutyric acid and ammonia (see Tables I and II).

RESULTS

Chromatography of pure amino acids after preliminary ion-exchange treatment

The results of chromatographic analyses after adsorption and re-elution

TABLE I

BUFFER COMPOSITIONS

Titrate each buffer to nominal pH with 6 *N* hydrochloric acid using an expanded scale pH meter. Make up to 1 l with water and readjust pH.

	pH		
	2.875	*4.20*	*5.00*
Trisodium citrate dihydrate, g	14.71	14.71	14.71
2.00 *N* sodium hydroxide, ml	25.0	25.0	25.0
Thiodiglycol, ml	5.0	5.0	nil
BRIJ 35 solution (100 g+300 ml water), ml	10	10	10
Distilled water, ml	900	900	900
Sodium chloride, g	nil	nil	35.07

TABLE II

BUFFER GRADIENT

Autograd Chamber No.	*Buffer pH*	*Amount of buffer (ml)*	*Methanol (ml)*
1	2.875	70	5
2	2.875	72	3
3	2.875	75	–
4	2.875	75	–
5	4.20	75	–
6	5.00	75	–
7	5.00	75	–
8	5.00	75	–
9	5.00	75	–

with 0.2 *N* sodium hydroxide are compared in Table III with those obtained on the untreated mixture. The recoveries were generally satisfactory and there were no losses of basic amino acids. Some oxidation of the sulphur amino acids occurred. The oxidation product of cystine could not be detected and therefore no correction factor could be applied. The loss was about 5–15%. With methionine about 20–30% was converted to the sulphoxides but these appeared on the chromatograms and were measured. The sum of methionine and methionine sulphoxide showed satisfactory recovery of methionine. The ammonia figure was unreliable due to the combined effects of traces of ammonia in the reagents which concentrate in the final solution analysed and possible losses during drying of the eluate. It would, however, normally be determined on a separate subsample, *e.g.*, by the method of Bremner and Keeney[11].

Table IV gives the results of the parallel experiments in which 2 *N* ammonium hydroxide was used as eluant. The recoveries including those of the basic amino acids are again acceptable.

Similar results were obtained by Nguyen and Paquin[12], who used a 20×1 cm

TABLE III

DETERMINATION OF AMINO ACIDS* IN AQUEOUS SOLUTIONS DIRECTLY AND AFTER ION-EXCHANGE TREATMENT

Acids adsorbed on column of 2.0 g of resin, eluted with 0.2 *N* NaOH.

Amino acid	*Amount (μmoles per litre)*		
	*Direct determination (Experiment 1**)*	*After ion-exchange treatment*	
		*Experiment 2****	*Experiment 3****
Aspartic acid	95.0	95.9	98.9
Threonine	94.1	96.4	96.1
Serine	96.0	100.1	100.0
Glutamic acid	98.3	100.9	95.8
Proline	96.4	99.6	103.3
Glycine	96.9	99.6	99.2
Alanine	92.7	95.6	93.7
Valine	95.5	96.2	99.5
Cystine	95.1	91.1	81.9
Methionine§	95.3	98.1	96.0
Isoleucine	99.2	99.5	102.0
Leucine	92.3	92.7	92.4
Tyrosine	94.5	96.4	96.6
Phenylalanine	94.9	97.8	99.2
Ethanolamine	102.1	–	91.0
γ-Aminobutyric acid	94.8	91.5	88.5
Ammonium	72.3	130.6	126.7
Ornithine	102.3	100.7	98.5
Lysine	99.0	98.5	97.8
Histidine	87.9	87.5	87.3
Arginine	97.7	94.3	95.7
Standard error	±2.25	±2.74	±2.69

* Plus ethanolamine and ammonium.
** Mean of three analyses.
*** Mean of two analyses.
§ Sum of methionine and methionine sulphoxides.

purification column and eluted with 100 ml of 2 *N* ammonium hydroxide followed by 100 ml of 4 *N* ammonium hydroxide.

Chromatography of amino acids from plant extracts after preliminary ion-exchange treatment

Free amino acids were determined on leaves of Italian ryegrass (*Lolium multiflorum*) S22 and fresh shoots of Sitka spruce (*Pinus sitchensis*) and Scots pine (*Pinus sylvestris* L.) from various nutrition experiments.

Where enough material was available comparative analyses were carried out on portions of the extract given a chloroform treatment as described by Nowakowski and Byers[8]. Generally, after ion-exchange treatment the solutions were clear and light yellow, while the chloroform-extracted solutions were turbid and olive green.

TABLE IV

DETERMINATION OF AMINO ACIDS* IN AQUEOUS SOLUTIONS DIRECTLY AND AFTER ION-EXCHANGE TREATMENT

Acids adsorbed on columns of 2.0 or 4.0 g of resin, eluted with 2.0 *N* NH_4OH.

Amino acid	*Amount (μmoles per litre)*		
	*Direct determination***	*After ion-exchange treatment*	
		*2.0 g resin****	*4.0 g resin****
Aspartic acid	101.1	100.9	97.6
Threonine	106.3	104.2	103.6
Serine	108.3	107.2	110.3
Glutamic acid	103.0	103.3	102.0
Proline	108.5	105.3	106.5
Glycine	107.4	106.1	106.7
Alanine	105.6	102.8	103.4
Valine	98.9	100.3	98.8
Cystine	103.2	98.0	95.3
Methionine§	103.5	99.5	98.4
Isoleucine	102.6	100.7	101.7
Leucine	101.2	102.3	101.5
Tyrosine	102.2	102.8	101.6
Phenylalanine	101.1	99.6	100.8
Ethanolamine	94.0	100.3	91.8
γ-Aminobutyric acid	101.1	110.2	101.4
Ammonium	146.4	–	141.2
Ornithine	105.9	101.3	99.6
Lysine	104.7	103.2	98.2
Histidine	108.6	102.2	98.7
Arginine	105.1	110.4	101.2
Standard error	±3.48	±3.82	±3.42

* Plus ethanolamine and ammonium.
** Mean of three analyses.
*** Mean of two analyses.
§ Sum of methionine and methionine sulphoxides.

However, there was no obvious difference between the chromatograms obtained after either treatment.

Results are shown in Tables V, VI and VII. No figures for threonine were obtained because it was not separated from asparagine with the chromatographic system used.

The tables show that the basic amino acids were completely recovered from plant extracts also. There were no systematic deviations attributable to the amount of resin used or the type of eluant (0.2 *N* NaOH or 2 *N* NH_4OH). The values for amino acids after ion-exchange treatment generally agreed well with those obtained for the extracts which were chloroform-treated. An exception can be seen in Table VII. The conifer extracts showed consistent increases in glutamic acid after ion-exchange purification. This was due to hydrolysis of glutamine, possibly by the 0.2 *N* sodium hydroxide used for elution. An analogous increase was not seen for aspartic acid

TABLE V

DETERMINATION OF FREE AMINO ACIDS IN ITALIAN RYEGRASS EXTRACT DIRECTLY* OR AFTER ION-EXCHANGE TREATMENT (ELUTION WITH 2 *N* NH_4OH)

Grass extracted with 80% (v/v) ethanol.

Amino acid	*Amount (μmoles per gram of dry matter)***						*Standard error*
	Sample I			*Sample II*			
	Directly analysed	*Ion-exchange treatment*		*Directly analysed*	*Ion-exchange treatment*		
		2.0 g of resin	*4.0 g of resin*		*2.0 g of resin*	*4.0 g of resin*	
Aspartic acid	9.70	10.1	10.0	6.60	6.60	6.60	±0.19
Serine	2.41	2.53	2.51	2.46	2.54	2.55	±0.066
Glutamic acid	4.47	4.32	4.36	4.50	4.41	4.37	±0.054
Proline	3.72	3.61	3.62	4.17	3.98	3.95	±0.14
Glycine	nil	nil	nil	nil	nil	nil	
Alanine	8.02	7.69	7.61	6.90	6.70	6.75	±0.075
Valine	2.13	2.07	2.05	2.26	2.20	2.20	±0.015
Cystine	nil	nil	nil	nil	nil	nil	
Methionine	nil	nil	nil	nil	nil	nil	
Isoleucine	0.75	0.75	0.76	0.85	0.85	0.89	±0.0076
Leucine	0.79	0.80	0.80	1.08	1.12	1.14	±0.017
Tyrosine	0.70	0.71	0.69	0.83	0.88	0.84	±0.020
Phenylalanine	0.63	0.61	0.63	0.85	0.84	0.89	±0.0058
Ethanolamine	trace	trace	trace	trace	trace	trace	
γ-Aminobutyric acid	5.47	5.44	5.61	5.59	6.27	6.00	±0.15
Ammonium	15.09	40.7	38.1	14.4	30.9	37.1	±0.70
Ornithine	trace	trace	trace	trace	trace	trace	
Lysine	0.65	0.59	0.61	0.77	0.81	0.82	±0.023
Histidine	trace	trace	trace	trace	trace	trace	
Arginine	0.65	0.65	0.69	0.76	0.71	0.80	±0.019

* Chloroform-soluble substances removed.

** Means of two chromatographic analyses.

TABLE VI

DETERMINATION OF FREE AMINO ACIDS IN ITALIAN RYEGRASS EXTRACTS AFTER ION-EXCHANGE TREATMENT (ELUTION WITH 0.2 *N* NaOH)

Amino acid	*Amount (μmoles per gram of dry matter)* *				
	Water extraction		*80% (v/v) ethanol extraction*		*Standard error*
	Experiment 1	*Experiment 2*	*Experiment 1*	*Experiment 2*	
Aspartic acid	3.01	3.07	2.62	2.70	±0.034
Serine	4.58	4.72	4.50	4.33	±0.063
Glutamic acid	3.49	3.77	3.81	4.34	±0.12
Proline	2.06	2.06	1.95	2.10	±0.084
Glycine	0.75	0.86	0.70	0.72	±0.022
Alanine	4.49	4.44	4.32	4.18	±0.053
Valine	2.90	2.88	2.72	2.68	±0.047
Cystine	nil	nil	nil	nil	
Methionine	nil	nil	nil	nil	
Isoleucine	1.27	1.28	1.24	1.22	±0.010
Leucine	1.28	1.33	1.27	1.25	±0.016
Tyrosine	0.48	0.46	0.44	0.46	±0.027
Phenylalanine	1.31	1.32	1.23	1.20	±0.016
Ethanolamine	2.34	2.26	2.77	2.47	±0.075
γ-Aminobutyric acid	5.28	5.20	4.78	4.48	±0.11
Ammonium	34.9	32.3	35.3	36.4	±1.3
Ornithine	0.80	0.95	0.62	0.62	±0.093
Lysine	1.73	1.81	1.14	1.14	±0.019
Histidine	0.87	1.06	trace	trace	±0.030
Arginine	0.65	0.69	0.49	0.51	±0.011

* Mean of two chromatographic analyses (2.0 g of resin).

so asparagine must have been unaffected. Where 2 *N* ammonium hydroxide was the eluant (Table V) there was no increase in the level of glutamic acid.

A comparison between results obtained on 80% (v/v) ethanolic and on aqueous extracts was included in Table VI. As expected, larger amounts of most amino acids (especially the basics) were found in the water extracts but the chromatographic separations were equally satisfactory, indicating that the ion-exchange procedure could remove the higher levels of impurities extracted by water.

DISCUSSION

The ion-exchange procedure gave solutions of plant amino acids suitable for analysis. Either 0.2 *N* sodium hydroxide or 2 *N* ammonium hydroxide were suitable eluants, the former is better where using ammonia is inconvenient and the latter where a salt-free sample is required. Sodium hydroxide seemed more liable than ammonium hydroxide to hydrolyse glutamine, although Thompson *et al.*[3] noted that ammonia hydrolysed the amides and therefore recommended recovery immediately after elution. Burroughs[13] also obtained only 80% recovery of glutamine although using 1 *N* ammonium hydroxide as eluant.

TABLE VII

DETERMINATION OF FREE AMINO ACIDS IN EXTRACTS OF NEW CONIFER SHOOTS DIRECTLY OR AFTER ION-EXCHANGE PURIFICATION (ELUTION WITH 0.2 *N* NaOH)

Shoots extracted with 80% (v/v) ethanol.

Amino acid	*Amount (μmoles per g dry matter)*											
	Sitka spruce						*Scots pine*					
	*Experiment 1**		*Experiment 2*		*Experiment 3*		*Experiment 1*		*Experiment 2*		*Experiment 3*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Aspartic acid	5.36	5.65	4.25	4.37	5.22	5.21	3.25	3.36	3.43	3.46	3.07	3.12
Serine	3.21	3.34	1.61	1.80	2.11	2.12	5.49	5.63	4.48	4.77	4.05	4.34
Glutamic acid	3.71	4.15	4.32	4.75	3.96	4.41	4.77	5.72	3.63	6.66	3.98	4.87
Proline	7.12	7.35	1.66	1.69	2.85	2.92	1.65	1.76	1.35	1.67	0.84	0.87
Glycine	0.67	0.65	0.35	trace	0.32	0.44	0.41	0.48	0.33	0.39	0.29	0.44
Alanine	3.82	4.01	2.38	2.33	3.24	3.57	3.86	3.75	3.24	3.34	3.05	3.12
Valine	0.57	0.56	0.32	0.32	0.50	0.54	0.58	0.62	0.55	0.60	0.49	0.52
Cystine	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
Methionine	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
Isoleucine	0.25	0.26	0.20	0.19	0.21	0.24	0.21	0.24	0.16	0.20	0.21	0.21
Leucine	0.27	0.31	0.18	0.19	0.24	0.24	0.27	0.27	0.23	0.23	0.24	0.23
Tyrosine	trace	trace	trace	trace	trace	trace	0.24	0.23	0.19	0.20	0.11	0.22
Phenylalanine	trace	trace	trace	trace	trace	trace	0.50	0.38	0.47	0.29	0.48	0.29
Ethanolamine	3.24	3.01	2.08	1.90	3.09	3.05	3.99	3.91	3.21	3.88	3.01	3.21
γ-Aminobutyric acid	8.21	8.42	4.97	4.72	7.39	7.31	7.45	7.69	8.05	9.26	7.43	7.79
Ammonium	–	–	17.4	17.2	42.6	43.3	30.3	40.5	24.2	23.4	10.9	10.7
Ornithine	0.79	0.86	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace
Lysine	1.35	1.34	0.37	0.42	0.49	0.47	0.66	0.63	0.66	0.66	0.40	0.44
Histidine	0.27	–	trace	trace	trace	trace	0.26	0.26	0.14	trace	0.16	trace
Arginine	18.4	19.4	2.95	2.85	5.65	5.55	11.3	11.1	12.8	12.9	3.28	3.13

* a, Chloroform-soluble substances removed; b, purified by ion-exchange resin (2.0 g).

The recovery of basic amino acids was complete if sufficient eluant, relative to the weight of ion-exchange resin, was used. The purification of the particular plant extracts tested gave no visible improvement in the chromatographic separations compared with a chloroform treatment[8], but would be essential for samples containing larger amounts of mucilaginous material or polyvalent cations which could damage the resin and reduce its separating efficiency.

Thompson *et al.*[3] did not recommend purifying the amino acids in a 70–80% ethanol medium and found some loss of glutamic acid, which they attributed to esterification; Plaisted[14] reported that some monoethyl glutamate was formed when using 80% alcohol solutions. No such losses were noted in this work, although the ethanol was not removed from the extracts, possibly because they were substantially diluted with water before purification and the ion-exchange resin was cycled with aqueous solutions. Some gas bubbles released by mixing water and alcohol were observed in the purification column but they did not affect the process.

The oxidation of cystine was a drawback but might be tolerable unless the sample contained much cystine when a separate analysis for this compound might be required.

Using ion-exchange purification for samples containing large amounts of glutamine cannot be recommended, unless the process is carried out at 0°–6° as described by Thompson *et al.*[3].

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