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Short communication

Use of Sep-Pak C_{18} cartridges to clean up free amino acids from coniferous needles☆

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Abstract

Reversed-phase Sep-Pak C_{18} cartridges were investigated to evaluate their ability to purify free amino acids extracted from jack pine (*Pinus banksiana* Lamb.) and white spruce [*Picea glauca* (Moench) Voss] needle tissue samples for HPLC analysis. Twenty-one amino acids from a standard only and amino acids from conifer needles with added standard were eluted through Sep-Pak C_{18} cartridges. An average recovery of 98% was found for all standard amino acids. Using norleucine as an internal standard, recovery for all amino acids except alanine and methionine averaged 104% for jack pine and 97% for white spruce tissue. Alanine co-chromatographed with an unknown peak and recovery appeared to exceed 130%. Methionine, with less than 33% recovery, was probably degraded during the extraction and purification procedures. Aside from alanine and methionine, Sep-Pak C_{18} cartridges appear to be a faster and more effective method for purifying conifer foliage extracts prior to amino acid analysis than the traditional ion-exchange column purification method.

1. Introduction

Extracts of biological samples for amino acid analysis are commonly purified with a strong cation ion-exchange resin [1]. In our laboratory, Amberlite IR-120 is used in an automated ion-exchange column system to clean extracts of samples collected from coniferous tree tissues [2,3].

However, even automated ion-exchange chromatography sample purification is time consuming. Sep-Pak C_{18} cartridges (Sep-Pak) appeared to be a potentially less time-consuming alternative purification method [4,5]. Hart and White [6] used Sep-Paks to purify amino acid samples hydrolyzed from protein using trifluoroacetic acid (TFA) by Waters' method (Waters, Mississauga, Canada) and found different amino acid yields because of retention differences in the Sep-Pak. These retention differences were corrected by Cohen et al. [7] when they modified the procedure to use hydrochloric acid instead of TFA to elute amino acids from the Sep-Pak.

The purpose of this study was to observe the efficacy of the Waters' modified (using HCl) Sep-Pak method [7] for purification of conifer needle

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tissue amino acid extracts for free amino acid analysis by the Waters' Pico-Tag method.

2. Experimental

2.1. Sample preparation

Randomly collected 2 + 0 jack pine and 3 + 0 white spruce seedlings from Midhurst Nursery, Ontario, Canada were rinsed with cold tap water, rinsed with reagent-grade water and drained. Current-year needles were separated from stems, frozen in liquid nitrogen and lyophilized for 72 h. Dry samples were ground in a Wiley mill with a 20-mesh sieve and stored at −20°C until extraction.

2.2. Extraction of amino acids

A 200-mg amount of dried sample was placed in a 15-ml centrifuge tube, 10 ml of distilled water were added, and vortexed. Tube was sealed and shaken at 50°C for 30 min in a horizontal position to extract. Tube was centrifuged at 1850 *g* for 10 min. The supernatant was decanted into a 100-ml evaporating flask. The extraction was repeated twice and the sample was washed with 10 ml of water prior to being decanted for a total of 40 ml of solution after centrifugation. The combined solution was evaporated to dryness in a rotary evaporator at 50°C. To five of ten dried samples, 400 μl of 2.5 μ*M*/ml standard amino acids were added and the samples were redried. The ten samples in the flasks were redissolved with 2 ml of 1.0 m*M* norleucine (internal standard) in water. The aliquots of samples were then centrifuged in 1.5-ml micro tubes at 8160 *g* for 10 min.

2.3. Sep-Pak C_{18} cartridge purification of samples

The Sep-Pak C_{18} Plus cartridge was preconditioned with 10 ml of methanol and 10 ml of water. The Sep-Pak cartridge was loaded with 0.5 ml of 1 *M* HCl and 0.5 ml of sample with internal standard. Amino acids were eluted with 1.5 ml of 1 *M* HCl, and then 2.5 ml of 30% acetonitrile in 1 *M* HCl [7]. Because tryptophan is not stable in acid, the amino acids were eluted into a vial containing 0.5 ml of 1.0 *M* sodium hydrogencarbonate solution.

2.4. Derivatization and separation of amino acids

A 50-μl volume of sample was derivatized using phenylisothiocyanate [7]. A 20-μl volume of the derivatized samples was then injected into the Waters Maxima 820 HPLC system with a 30 × 0.39 cm stainless-steel Pico-Tag column. Calibration was carried out using a Pierce (IL, USA) amino acid standard H with asparagine,

Table 1
Recovery of 250 pmol/injection of each standard amino acid by the Sep-Pak cartridge clean-up method

Amino acid	Recovery (%) ($n = 4$)	R.S.D. (%) ($n = 4$)
Asp	94.2	4.2
Glu	96.0	2.9
Ser	101.1	3.4
Asn	95.8	2.1
Gly	95.6	2.2
Gln	95.8	2.6
His	99.5	8.5
GABA	108.3	4.1
Thr	99.3	1.5
Ala	102.8	3.1
Arg	96.8	2.5
Pro	99.3	2.5
Tyr	95.7	2.8
Val	96.4	3.0
Met	95.4	3.5
Ile	95.9	3.0
Leu	96.7	2.6
Norl	100.5	2.7
Phe	99.2	2.7
Trp	97.0	2.7
Lys	97.3	2.6
Overall average	98.0	3.1

Three-letter abbreviations are standard abbreviations for amino acids. GABA = γ-Aminobutyric acid; Norl = norleucine.

glutamine, γ-aminobutyric acid, tryptophan and norleucine added. The amino acids were separated with solvent 1 [70 m*M* sodium acetate, pH 6.55, 2.5% (v/v) acetonitrile] and solvent 2 (acetonitrile–methanol–water, 45:15:40) [8] using a standard procedure for free amino acid analyses [7]. Blank tests were performed with underivatized samples and with derivatized blanks.

All amino acid concentrations were calculated based on 20 μl of injected samples and the amino acid concentrations in Table 2 were corrected to 100% using norleucine as an internal standard.

3. Results and discussion

Standard amino acid recoveries using Sep-Pak cartridges are shown in Table 1. The average yield of the 21 standard amino acids was 98% with a relative standard deviation (R.S.D.) of 3.1%. The recovery of norleucine, which was used as an internal standard, was 100.5% which confirmed it as a good internal standard.

The recoveries of most amino acids in jack pine needles except alanine and methionine were close to the expected amount with an average of 104% with a R.S.D. of 2.9% (Table 2). The average yield in white spruce was 97% (R.S.D.

Table 2
Sep-Pak purified amino acid concentrations extracted from jack pine and white spruce seedling needles with and without the addition of 250 pmol of standard (std.)/injection

Amino acid	Jack pine		White spruce	
	Concentration without std. (pmol ± S.E.)	Recovery with std. (%)[a]	Concentration without std. (pmol ± S.E.)	Recovery with std. (%)[a]
Asp	24 ± 2	108.5 (3.0)	34 ± 1	77.0 (6.7)
Glu	88 ± 1	104.8 (3.0)	91 ± 2	105.1 (5.0)
Ser	27 ± 1	111.2 (2.6)	24 ± 2	101.1 (4.3)
Asn	5 ± 0	99.8 (1.7)	2 ± 0	99.8 (3.3)
Gly	5 ± 1	93.1 (3.3)	8 ± 1	94.0 (4.0)
Gln	7 ± 1	99.5 (1.9)	21 ± 2	97.2 (5.5)
His	5 ± 1	98.5 (4.4)	8 ± 2	95.4 (5.4)
GABA	61 ± 1	100.9 (3.2)	28 ± 2	97.7 (4.6)
Thr	11 ± 2	103.8 (4.6)	2 ± 0	94.4 (4.6)
Ala	12 ± 2	152.0 (3.2)	7 ± 3	131.8 (8.9)
Arg	129 ± 2	105.9 (3.2)	394 ± 6	99.0 (4.6)
Pro	63 ± 2	115.6 (4.0)	40 ± 2	105.6 (6.2)
Tyr	8 ± 0	109.9 (2.4)	30 ± 3	94.4 (5.6)
Val	8 ± 0	112.4 (3.2)	7 ± 1	95.3 (4.6)
Met	4 ± 2	21.9 (51.4)	5 ± 1	32.8 (55.8)
Ile	4 ± 0	111.4 (2.2)	5 ± 1	102.9 (3.2)
Leu	8 ± 0	108.2 (0.9)	10 ± 1	105.0 (1.0)
Phe	11 ± 0	105.1 (1.1)	12 ± 1	97.8 (2.6)
Trp	38 ± 1	96.1 (1.7)	78 ± 1	97.2 (3.0)
Lys	12 ± 0	83.1 (5.3)	17 ± 1	85.6 (4.0)
Overall average[b]		104 (2.9)		97 (4.3)

S.E. = Standard error.
[a] Relative standard deviations (%) in parentheses; $n = 5$.
[b] Alanine and methionine were excluded from the calculation of the mean.

4.3%) of the expected concentration except for alanine and methionine. White spruce showed a higher R.S.D. than did jack pine. The methionine recovery from jack pine needles was 21.9% and from white spruce needles it was 32.7% while the recovery of the methionine standard was 95.4% (Table 1). R.S.D. in the both species also showed over 50% (Table 2). Although it was not confirmed experimentally, something from the plant extract probably degraded methionine in the acidic eluting solution, a phenomenon that was also observed in extracts purified by the cation ion-exchange resin [9].

Although the Sep-Pak procedure was effective at cleaning up most contaminants, there were some quantification problems usually caused by co-eluting unknown peaks especially in samples from outdoor grown trees. For example, alanine concentration was higher than its actual concentration because of an unknown co-eluting peak found in underivatized samples. In addition, samples from outdoor grown trees showed a higher unknown co-eluting peak near alanine than did greenhouse samples. Some field samples also had unknown co-eluting peaks which, at low concentrations of amino acid (less than 10 pmol per injection), interfered with threonine resolution and quantitation and aspartic acid quantitation. The unknown co-eluting peak interference to resolution could be largely overcome by rerunning samples with a known amount of standard amino acid added to increase the amino acid peak size. After amino acid identity was clearly established the computer was used to reprocess peak data. Lysine, the last peak in the run, showed a reduced yield of 83.1% in jack pine and 85.6% in white spruce. Actual lysine concentration was calculated using the correction factor based on percent recovery of the internal standard norleucine.

The Sep-Pak method requires about 10 min to elute each sample whereas the ion-exchange column method requires 3–4 h when column regeneration time is included. We estimate that in our laboratory for each set of 16 samples, the time savings of the cartridge method over the automated ion-exchange column method (with 10 columns running at one time) is one entire working day. The Sep-Pak method also yields excellent recovery rates for most amino acids and we currently use this method routinely in our laboratory for amino acid analysis of conifer foliage and root tissue and find it yields consistent results. In our evaluation, the Sep-Pak method is superior for cleaning up conifer foliage extracts for amino acid analysis.

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