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GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF COMMON AMINO ACIDS IN PINE NEEDLE EXTRACTS

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SUMMARY

An improved gas–liquid chromatographic method based on the separation of N-trifluoroacetyl *n*-butyl esters of amino acids on a "two-column" setup —Tabsorb and Tabsorb HAC— was developed for the identification and estimation of amino acids in pine needles (*Pinus banksiana* Lamb.). A comparative study was made of various available gas–liquid chromatographic methods for separation and estimation of amino acids from pine needle extracts.

INTRODUCTION

Our research on the impact of air pollutants on amino acid metabolism in pine seedlings dictated the need for an analytical technique to separate and identify various plant amino acids. A gas–liquid chromatographic (GLC) method has a two-fold advantage over the ion-exchange method: (1) it requires a less expensive set-up and (2) it is sensitive enough to detect amino acids in the picomolar range rather than the usual nanomolar range. However, one obvious complication with the GLC method is that the amino acids as such are not volatile enough to be chromatographed, and therefore require conversion to more volatile derivatives before injection into the column. This is usually accomplished by converting them to either trimethylsilyl (TMS) derivatives of the amino acids as described by Gehrke and Leimer[1] or to one of the various N-acyl (in NH_2) and COO-alkyl (in COOH) derivatives such as N-trifluoroacetyl (N-TFA) *n*-butyl esters[2], N-heptafluorobutyryl *n*-propyl esters[3], N-heptafluorobutyryl isopentyl esters[4] and N-heptafluorobutyryl isobutyl esters[5].

The distribution pattern of amino acids in pine needle tissues is different from the ones described by the above-mentioned authors. In pine needle tissues the amounts of aspartic and glutamic acids are much larger than those of other amino acids. This distribution pattern is normal for plant extracts, and except for alanine and glycine, quite similar to the one reported for seed proteins[5]. The quantitation of several pine needle amino acids by various established methods is difficult for the following reasons: (1) the close proximity of methionine and aspartic acid peaks

in the method of Moss *et al.*[3], and (2) the close proximity of lysine, tyrosine and glutamic acid peaks in the method of Zanetta and Vincendon[4]. Similarly, the presence of a large alanine peak, particularly in extracts from SO_2-treated pine seedlings, makes it difficult to separate alanine from glycine in the method of MacKenzie and Tenaschuk[5]. In this paper, we describe our evaluation of different liquid phases (OV-11, Apiezon M, Tabsorb and Tabsorb HAC) for identifying and estimating individual amino acids based on the separation of their N-TFA *n*-butyl esters.

EXPERIMENTAL

Reagents

Amino acid standards, acetonitrile, bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). Trifluoroacetic anhydride (TFAA) and 3 *N* HCl in *n*-butanol were obtained from Regis Chemical Co. (Chicago, Ill., U.S.A.). Methylene chloride was a "Baker analyzed" chemical; the bottle containing it was stored in a molecular sieve. The Tabsorb and the Tabsorb HAC columns were both obtained from Regis Chemical Co.

Plant material

Jack pine (*Pinus banksiana* Lamb.) seedlings were grown in styroblock trays as described previously[6]. The seedlings were grown at 22–24° under 10,500 lx light source and 18-h photoperiod. Needles from 6-month-old seedlings were utilized for experimental purposes.

Preparation of derivatives

Stock solutions of standard amino acids were prepared in 0.05 *N* HCl and kept frozen. Depending on the molar response, either a 0.1- or 0.2-mg sample of amino acid was transferred to a 150 × 13 mm screw-cap test-tube. The solution was evaporated to dryness at 60° in an oil-bath under a stream of dry N_2. Last traces of water were removed azeotropically by adding methylene chloride (2 × 1.5 ml) to the residue and then by evaporating it to dryness under a stream of dry N_2. The residue was converted to trimethylsilyl (TMS) or N-TFA *n*-butyl esters of amino acids. TMS derivatives were prepared according to Gehrke and Leimer[1] using BSTFA with or without TMCS.

In order to prepare N-TFA *n*-butyl esters of amino acids, 1.5 ml of 3 *N* HCl in *n*-butanol was added to the tube containing the amino acid residue. After the PTFE-lined cap was secured, the tube was placed in an ultrasonic bath and sonicated for varying lengths of time starting from 15 sec, as recommended by Roach and Gehrke[7]. Observing that 15-sec sonication was not long enough to produce a molar response comparable to that reported by Cancalon and Klingman[8], we therefore sonicated our samples for 30 min. The rest of the derivatization procedure was similar to that of Roach and Gehrke[7].

Gas–liquid chromatography

Analyses were carried out in a Hewlett-Packard Model 5830A gas chromatograph equipped with dual-flame ionization detectors and multifunction digital processor. A thick-walled glass column (183 × 0.2 cm) was used in all cases.

OV-11 column. A prepacked column of 10% OV-11 Chromosorb W (100–120

mesh) obtained from Chromatographic Specialties (Brockville, Canada) was used for the analysis of TMS derivatives of amino acids. The column was conditioned as suggested by Gehrke and Leimer[1]. The chromatographic conditions were as follows: temperature program from 125 to 225° at the rate of 5°/min with an initial hold at 125° for 3.0 min; the injector and detector temperatures were set at 275° and 300°, respectively. The N_2 carrier flow was adjusted to 17 ml/min.

Apiezon M column. For the analysis of N-trifluoroacetyl (TFA) *n*-butyl esters of amino acids on a single column, 10% Apiezon M on Chromosorb W (80–100 mesh) was used. The column wzs packed under suction with gentle tapping and conditioned as recommended by Gehrke and Takeda[9]. The chromatographic conditions were as follows: temperature program from 90 to 260° at the rate of 6°/min with an initial hold period of 6.0 min at 90°; the injector and detector temperatures were set at 275° and 300°, respectively. The N_2 carrier flow was maintained at 17 ml/min.

Tabsorb and Tabsorb HAC columns. For the complete analysis of N-TFA *n*-butyl esters of all amino acids, a two-column setup was employed. The first column was packed with the Tabsorb under suction with gentle tapping and conditioned at 220° for 2 days with a N_2 flow of 30 ml/min. The second column was packed similarly with the Tabsorb HAC and conditioned, as recommended by the supplier, by heating from 50° to 250° with an increase of 1°/min and then by holding at 250° overnight. The N_2 flow was maintained at 30 ml/min. The chromatographic conditions for the Tabsorb column were as follows: temperature program from 100 to 200° at 4°/min after an initial hold of 100° for 7 min; injector temperature, 250°; detector temperature, 250°. The chromatographic conditions for the Tabsorb HAC column were as follows: temperature program from 110 to 210° at 4°/min after an initial hold at 110° for 7.0 min; injector and detector temperatures were the same as for the Tabsorb column. The N_2 carrier flow in both cases was set at 30 ml/min.

The H_2 and air flows for the flame were set at 60 and 240 ml/min, respectively, for all columns. The recorder chart speed was always 1 cm/min.

Extraction of amino acids from pine needle tissues. About 5 g fresh pine needles were cut into small pieces and dropped into a flask containing boiling 95% ethanol. After the ethanol boiled for 5 min, the flask was allowed to cool down, and the liquid was filtered under suction. The needle tissue was homogenized for 5 min in 50 ml of 60% ethanol. The homogenate was filtered with a büchner funnel and the residue washed with 60% ethanol. The pigments were removed from the combined 60% and 95% ethanol extracts with petroleum ether. After the removal of pigments, the extract was evaporated to dryness with a rotary evaporator. The residue was redissolved in 10 ml water and clarified by centrifugation. The phenolic compounds from the supernatant were removed with insoluble polyvinylpyrrolidone, which was purified according to McFarlane and Vader[10]. The phenol-free extract was filtered through a 0.8-μm Millipore filter to obtain a clear solution. This solution was passed through a Dowex 50W-X8 (H^+, 20–50 mesh) cation-exchange column (30 × 1 cm I.D., 16 cm bed height) at a rate of 20 drops/min. After the cation-exchange column had been washed with water to remove organic acids and neutral compounds, the amino acids were eluted with 2 *N* NH_4OH (50 ml). The eluate was evaporated to dryness and the residue redissolved in 5 ml water. An aliquot equivalent to 2 μmoles total amino acids was derivatized for gas chromatographic analysis.

RESULTS AND DISCUSSION

OV-11 column. Our efforts to obtain reproducible analyses of TMS derivatives of amino acids on OV-11 column were unsuccessful. Three serious problems were encountered: (1) multiple peaks for proline, arginine, methionine, aspartic acid, phenylalanine, lysine and tryptophan (Table I); (2) lack of reproducibility from one day to the next and (3) the complete absence of a histidine peak. Our results were different from those obtained by Gehrke and Leimer[1]; they reported smooth and trouble-free derivatization of all the amino acids, including arginine and histidine, which were found by us to be the most difficult ones to derivatize. It is still not understood why there was so much variability from one day to the next, but the sensitivity of the reagents to moisture could have been an important factor. However, no improvement was noticed after taking precautions to prevent interference from the moisture. It is therefore suggested that this GLC technique needs further improvement before it can be applied for quantitative estimation of amino acids.

Apiezon M column. The single-column analysis of N-TFA *n*-butyl esters on an Apiezon M column was also tested. The N-TFA *n*-butyl esters of amino acids

TABLE I

RETENTION TIMES AND TEMPERATURES OF THE TRIMETHYLSILYL DERIVATIVES OF AMINO ACIDS ON THE OV-11 COLUMN

Amino acid	*Retention time (min)*	*Retention temperature (°C)*
Alanine	2.83	125.0
Valine	4.85	134.0
Proline (peak 1)	5.66	138.0
(peak 2)	7.30	146.5
Leucine	5.97	140.0
Isoleucine	6.61	143.0
Glycine (peak 1)	3.29	125.0
(peak 2)	6.84	144.0
Serine	8.19	151.0
Threonine	8.59	153.0
Aspartic acid (peak 1)	11.21	166.0
(peak 2)	12.44	172.0
Arginine (peak 1)	12.07	170.0
(peak 2)	15.75	189.0
(peak 3)	18.39	202.0
(peak 4)	19.38	207.0
Methionine (peak 1)	12.07	170.0
(peak 2)	13.33	176.5
Hydroxyproline	12.28	171.5
Cysteine	13.72	178.5
Glutamic acid (peak 1)	14.31	181.5
(peak 2)	14.90	184.5
Phenylalanine (peak 1)	15.24	186.0
Lysine (peak 1)	19.79	209.0
(peak 2)	20.09	210.0
Tyrosine	21.94	220.0
Tryptophan (peak 1)	32.11	isothermal
(peak 2)	34.51	at 225.0

were prepared as suggested by Cancalon and Klingman[8]. One of the major difficulties encountered was the inability of cystine to dissolve in *n*-butanol–hydrochloric acid even after being heated to 100° for 15 min. Furthermore, after N-derivatization, a tiny residue was always left in methylene chloride with cystine, arginine, histidine and tryptophan. Longer sonication for 30 min did not improve the solubility of the residues. The results of analysis on Apiezon M column (Table II) are similar to those obtained by Gehrke and Takeda[9], except that we failed to obtain a peak for histidine. Also, as shown in Table II, three distinct clusters of peaks were obtained that could not be resolved further. This was an impediment toward the quantitation of alanine, leucine and lysine because of interference from the neighbouring peaks. It is believed that this method needs further improvement in its application for the single-column analysis of amino acids.

TABLE II

RETENTION TIMES AND TEMPERATURES OF THE N-TRIFLUOROACETYL *n*-BUTYL ESTERS OF AMINO ACIDS ON THE APIEZON M COLUMN

Amino acid	*Retention time (min)*	*Retention temperature (°C)*	
Alanine	15.71	148.0	
Threonine	15.90	149.0	Cluster
Glycine	16.36	152.0	
Serine	16.57	153.0	
Leucine	21.26	181.5	
Isoleucine	21.59	183.5	Cluster
Cysteine	21.91	185.5	
Phenylalanine	29.74	232.5	
Lysine	30.17	235.0	Cluster
Tyrosine	30.57	237.0	
Glutamic acid	31.94	245.6	

Tabsorb and Tabsorb HAC columns. The N-TFA *n*-butyl esters of amino acids were analyzed on the Tabsorb and the Tabsorb HAC columns to ameliorate the above-mentioned difficulties. Although a two-column setup was more time-consuming, the results (Table III and Fig. 1) were rewarding. Most of the derivatization and separation problems associated with OV-11 and Apiezon columns were eliminated. All the amino acids except arginine and histidine were analyzed and detected on the Tabsorb column; a duplication injection into the Tabsorb HAC column was necessary to obtain peaks from arginine and histidine. The possibility of using the Tabsorb HAC column for the analysis of the remainder of amino acids was also explored. As is evident from Table III, valine and threonine appear as one peak, and so do leucine and isoleucine, and phenylalanine and aspartic acid. Tryptophan, however, produced two peaks. It was, therefore, quite obvious that a combination of the Tabsorb and the Tabsorb HAC columns was essential for separating all 20 protein amino acids.

Separation and quantitation of pine needle amino acids. An aliquot of the soluble amino acid fraction of pine needle tissues was converted to N-TFA *n*-butyl esters and analyzed by GLC on the Tabsorb and the Tabsorb HAC columns. The

TABLE III

RETENTION TIMES AND TEMPERATURES OF THE N-TRIFLUOROACETYL *n*-BUTYL ESTERS OF AMINO ACIDS ON THE TABSORB AND THE TABSORB HAC COLUMNS

Amino acid	*Retention time (min)*	*Retention temperature (°C)*	*Amino acid*	*Retention time (min)*	*Retention temperature (°C)*
Tabsorb column					
Alanine	7.23	101.0	Methionine	20.61	154.5
Valine	9.10	108.0	Hydroxyproline	21.09	156.0
Glycine	10.41	113.5	Phenylalanine	22.17	160.5
Isoleucine	11.49	118.0	Aspartic acid	22.92	163.5
Leucine	12.97	124.0	Glutamic acid	26.51	178.0
Proline	13.77	127.0	Tyrosine	28.85	187.5
Threonine	15.20	133.0	Lysine	32.86	isothermal
Serine	17.15	140.5	Tryptophan	34.17	at
Cysteine	19.91	151.5	Cystine	50.87	200.0
Tabsorb HAC column					
Alanine	3.65	isothermal	Phenylalanine	20.88	165.5
Glycine	4.73	at	Aspartic acid	20.88	165.5
Valine	6.02		Tyrosine	24.35	179.5
Threonine	6.02	110.0	Glutamic acid	24.65	180.5
Serine	7.58	112.0	Lysine	27.41	191.5
Leucine	8.86	117.5	Arginine	30.02	202.0
Isoleucine	8.86	117.5	Tryptophan 1	31.28	207.0
Cysteine	12.59	132.0	Tryptophan 2	39.18	239.0
Proline	15.96	146.0	Histidine	32.35	211.5
Hydroxyproline	17.43	152.0	Cystine	43.67	256.5
Methionine	18.52	156.0			

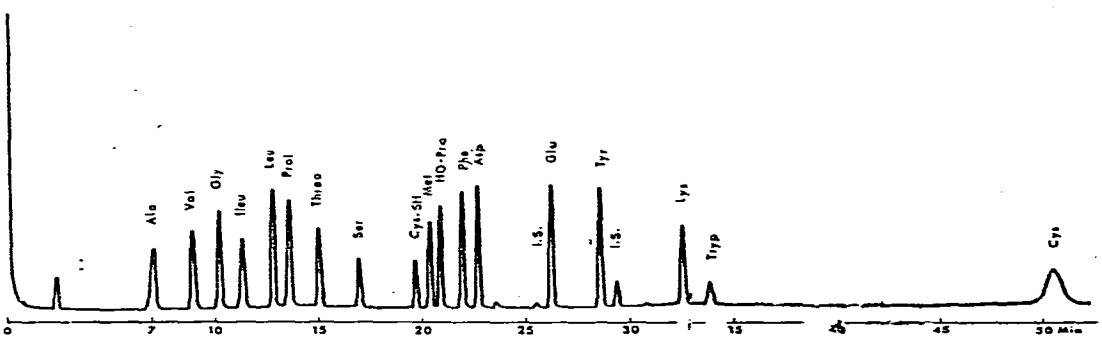

Fig. 1. Gas chromatogram of the N-TFA *n*-butyl esters of the amino acids from the Tabsorb column. I.S. = Internal standard, butyl stearate. Temperature program 100–200° at 4°/min after an initial hold at 100° for 7 min. Internal standard amount injected was 100 ng; other amino acids were 200 ng each, except cystine, histidine, tyrosine, tryptophan and arginine, each of which was 400 ng. GLC conditions are given in the text.

chromatogram from the Tabsorb column (Fig. 2 and Table IV) shows that most of the pine needle amino acids were present in sufficient quantities to be detected by this procedure. Only cystine, hydroxyproline and tryptophan were not present in detectable quantities. Glutamic acid was the most abundant amino acid, followed

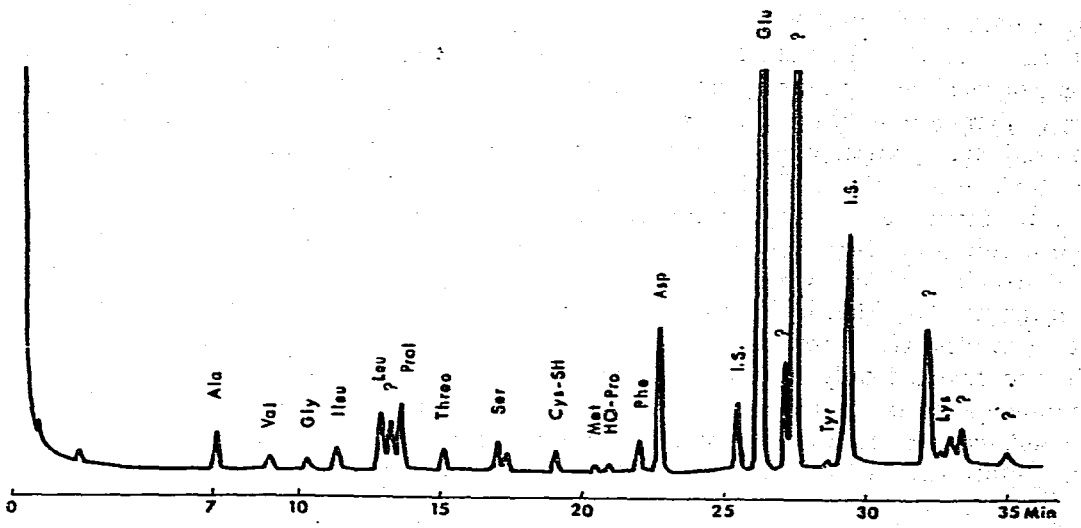

Fig. 2. Gas chromatogram of the N-TFA *n*-butyl esters of free amino acids from pine needle extract from the Tabsorb column. I.S. = Internal standard, butyl stearate. Temperature program: 100–200° at 4°/min after an initial hold at 100° for 7 min. GLC conditions are described in the text.

TABLE IV

RETENTION TIMES AND TEMPERATURES OF PEAKS FROM PINE NEEDLE EXTRACT ON THE TABSORB COLUMN

Identity	*Retention time (min)*	*Retention temperature (°C)*
Alanine	7.39	101.5
Valine	9.25	109.0
Glycine	10.56	114.0
Isoleucine	11.63	118.5
Leucine	13.17	124.5
?	13.53	126.0
Proline	13.87	127.5
Threonine	15.37	133.5
Serine	17.29	141.0
?	17.61	142.5
Glutamine	19.32	149.0
Methionine	20.71	155.0
Phenylalanine	22.26	161.0
Aspartic acid	22.99	164.0
Glutamic acid	26.55	178.0
?	27.48	182.0
?	27.71	183.0
Tyrosine	28.96	188.0
?	32.43	
Lysine	32.92	isothermal
?	33.27	at
?	33.69	200.00
?	35.27	

by aspartic acid and leucine; the remaining amino acids were present in smaller amounts. Due to the nature of derivatization, asparagine and glutamine show up as aspartic acid and glutamic acid, respectively. Parallel experiments conducted with ion-exchange-based high-pressure liquid chromatography and paper chromatography showed that both asparagine and glutamine are present in pine needles. The peaks at 13.53, 17.61, 27.48, 27.71, 32.43, 33.27, 33.69 and 35.27 min retention times could not be attributed to any of the common amino acids (Table IV). However, the extraction procedure may have extracted some amino compounds other than the amino acids, which, when derivatized, would appear as extra peaks on the chromatogram. The identity of the amino acids was established by enriching the injection samples with appropriate standard amino acid derivatives. The Tabsorb HAC column detected only arginine and not histidine or cystine. The amino aicd analyses of pine needle extracts were always reproducible. Although we had wanted to develop a single-column technique, the two-column method proved to be fully satisfactory for quantitative analysis of amino acids from pine needles.

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REFERENCES

1 C. W. Gehrke and K. Leimer, *J. Chromatogr.*, 57 (1971) 219.
2 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 55 (1971) 267.
3 C. W. Moss, M. A. Lambert and F. J. Diaz, *J. Chromatogr.*, 60 (1971) 134.
4 J. P. Zanetta and G. Vincendon, *J. Chromatogr.*, 76 (1973) 91.
5 S. L. MacKenzie and D. Tenaschuk, *J. Chromatogr.*, 97 (1974) 19.
6 S. S. Malhotra, *New Phytol.*, 76 (1976) 239.
7 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 44 (1969) 269.
8 P. Cancalon and J. D. Klingman, *J. Chromatogr. Sci.*, 12 (1974) 349.
9 C. W. Gehrke and H. Takeda, *J. Chromatogr.*, 76 (1973) 63.
10 W. D. McFarlane and M. J. Vader, *J. Inst. Brew.*, 68 (1962) 254.