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ANALYSIS OF CONIFER LEAF FREE AMINO ACIDS BY GAS–LIQUID CHROMATOGRAPHY*

S. L. MacKENZIE* and K. R. HOLME
Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan S7N OW9 (Canada)

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SUMMARY

Free amino acids from the leaves of ten North American conifer species have been analysed by packed-column gas–liquid chromatography as their N(O,S)-heptafluorobutyryl isobutyl esters. The species examined were Balsam fir [*Abies balsamea* (L.) Mill], douglas fir [*Pseudotsuga menzeisii* (Mirb.) Franco], western hemlock [*Tsuga heterophylla* (Raf.) Sarg.], Rocky mountain juniper (*Juniperus scopulorum* Sarg.), western juniper (*Juniperus occidentalis*, Hook), lodgepole pine (*Pinus contorta* Dougl. var. latifolia Engelm.), ponderosa pine (*Pinus ponderosa* Laws.), black spruce [*Picea mariana* (Mill.) BSP], colorado spruce (*Picea pungens* Engelm.), and white spruce [*Picea glauca* (Moench) (Voss)]. All the proteic amino acids and some biologically important non-proteic amino acids were readily assayed. Gas chromatography–mass spectrometry was used to confirm the identity of the proteic amino acids and the homogeneity of the corresponding peaks. Ten unidentified components were partially characterised by their mass spectra.

INTRODUCTION

Several methods have been developed for analysing amino acids as volatile derivatives using gas–liquid chromatography (GLC)[1–7]. However, because of differences in the elution patterns and resolution of specific amino acids, the individual methods have not been equally useful for all kinds of samples. Furthermore, the methods have been applied to a rather limited range of sample matrices, although the resolution of the GLC technique has frequently enabled the assaying of some physiologically important non-proteic amino acids along with the standard proteic amino acids. We analysed free amino acids from the leaves of ten North American conifer species to determine the applicability of the procedures developed in this laboratory for the assaying of conifer needle free amino acids by GLC. The results presented here demonstrate the procedure is entirely suitable for that purpose.

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MATERIALS AND METHODS

Samples of conifer leaves were kindly supplied by Dr. E. von Rudloff. The species examined were Balsam fir [*Abies balsamea* (L.) Mill], Douglas fir [*Pseudotsuga menzeisii* (Mirb.) Franco], western hemlock [*Tsuga heterophylia* (Raf.) Sarg.], Rocky mountain juniper (*Juniperus scopulorum* Sarg.), western juniper (*Juniperus occidentalis,* Hook), Lodgepole pine (*Pinus contorta* Dougl. var. latifolia Engelm.), Ponderosa pine (*Pinus ponderosa* Laws.), Black spruce [*Picea mariana* (Mill.) BSP], Colorado spruce (*Picea pungens* Engelm.), and White spruce [*Picea glauca* (Moench) (Voss)]. Samples were collected in the dormant season as described by von Rudloff[8].

Amino acid extraction

The leaves were removed from the bracts after dipping the branches into liquid nitrogen. All residue of twigs and bark was manually removed. The needles (5 g) were washed thoroughly with distilled water in a coarse sintered glass funnel to remove surface contamination. Free amino acids were then extracted by the following procedures.

Procedure 1. This procedure is based on that described by Sarkar and Malhotra[9]. Washed, air dried leaves (5 g) were cut into small pieces (2–5 mm) and extracted for 5 min in 75 ml boiling 95% ethanol. After cooling, the suspension was filtered through Whatman No. 1 paper using a Buchner funnel and the filtrate retained. The residue was homogenised in a Waring Blendor for 5 min in 50 ml 60% ethanol and filtered as above. The combined filtrates were extracted using 100 ml of leight petroleum (Skelly F) and the petroleum layer discarded. The aqueous layer was evaporated to dryness using a rotary evaporator, dissolved in 10 ml 0.1 *N* hydrochloric acid and "cleaned up" using ion exchange as described below.

Procedure 2. This procedure is based on that described by Nijholt[10]. Washed, air dried leaves (2 g) were freeze dried, ground in a coffee grinder (Micromill) and again freeze dried. The dried pulp (500 mg) was extracted using 8 ml of methanol–chloroform–water (120:50:30, v/v/v) in a 15-ml centrifuge tube. The contents of the tube were agitated three times, each of 1 min duration, using a Vortex Genie (Scientific Industries, New York) with the debris being allowed to settle in between. After centrifuging for 15 min at 3000 *g*, the supernatant was decanted and the extraction procedure repeated three more times. The combined supernatants were extracted with chloroform in a separating funnel. When phase separation occurred readily in the funnel, the lower phase was drained and the upper phase retained; otherwise, centrifugation was required and the upper phase was removed using a Pasteur pipette. The aqueous phase was evaporated to dryness, dissolved in 10 ml of 0.1 *N* hydrochloric acid and "cleaned up" using ion exchange as described below.

Sample clean-up

A column (2.3 × 2.5 cm) of Dowex 50X8, 100–120 mesh was regenerated with 1 *N* hydrochloric acid then rinsed with distilled water to pH 5–6. The conifer leaf extract was applied to the column and washed with 17 ml distilled water followed by 10 ml of acetone–water (1:1) and 10 ml of water, all eluates being discarded. The amino acids were eluted using 2 *N* ammonia, a total of 30 ml being collected. The basic eluate was evaporated to dryness and dissolved in 2 ml 0.1 *N* hydrochloric acid in preparation for analysis.

Amino acid analysis

The N(O,S)-heptafluorobutyryl (HFB) isobutyl esters of the amino acids were prepared and assayed as previously described[6,7].

Mass spectrometry

Amino acids were identified by relative retention times and gas chromatography–mass spectrometry (GC–MS) using a Finnigan Model 3300 or Model 4000 mass spectrometer operated in either the electron impact (EI) or chemical ionization (CI) mode.

RESULTS AND DISCUSSION

The main purpose of this paper is to establish the suitability of GLC for the analysis of free amino acids in conifer leaf extracts. It is nevertheless pertinent to note specific observations relating to sample extraction and preparation. For example, using procedure 1, there was significant precipitation on combination of the 95% and 60% ethanol extracts. This precipitate disappeared on extraction but a green or brown colour remained. When evaporated, all samples formed a syrupy liquid frequently containing some solid material. During ion-exchange purification, the acetone–water wash often generated a white suspension which readily washed through the column.

Some typical chromatograms illustrating the free amino acid profiles obtained for each genus using procedure 1 are shown in Figs. 1–5. Balsam fir is omitted because the profile was very similar to that of Douglas fir. Although the proportions of the amino acids varied considerably, the resolution was more than adequate for the precise quantitation of all the proteic amino acids. Each of these was verified by

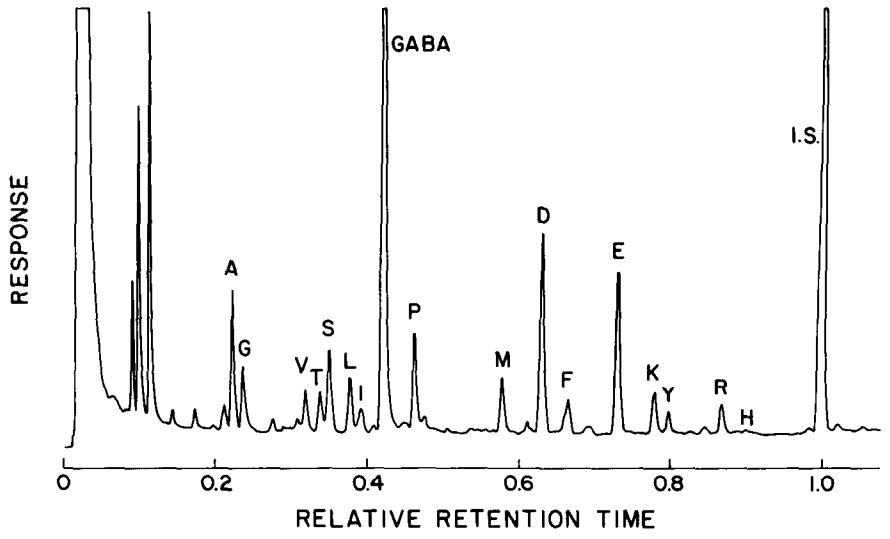

Fig. 1. Chromatogram illustrating separation of free amino acids isolated from the leaves of Douglas fir. See text for chromatographic conditions. The proteic amino acids are designated by the standard single letter code; GABA = γ-aminobutyric acid; I.S. = internal standard.

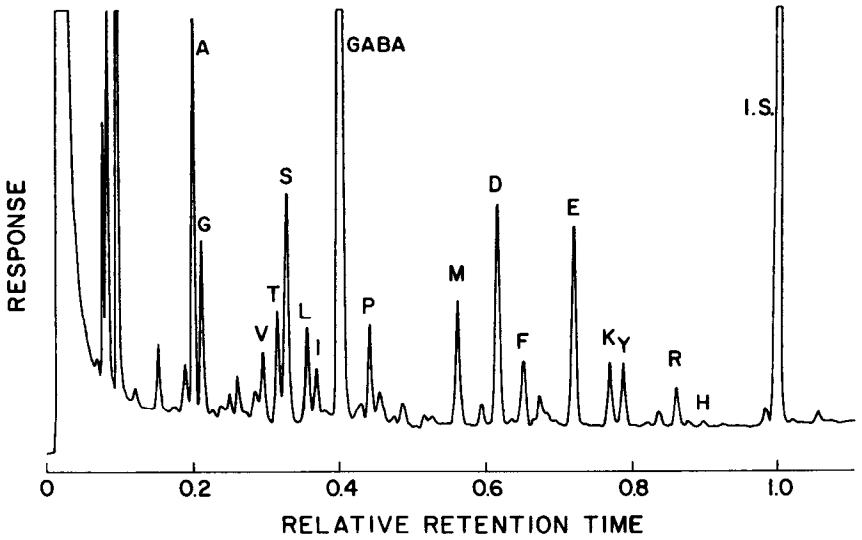

Fig. 2. Chromatogram illustrating separation of free amino acids isolated from the leaves of Western hemlock. See text for chromatographic conditions and Fig. 1 for abbreviations.

GC–MS to be free of contamination beyond that which might be expected from adjacent, clearly visible peaks. Widely different proportions of alanine and glycine were sufficiently well resolved to allow precise quantitation in contrast to the observations of Sarkar and Malhotra[9]. These authors probably used either too short a column or too high a starting temperature. However, since neither a chromatogram

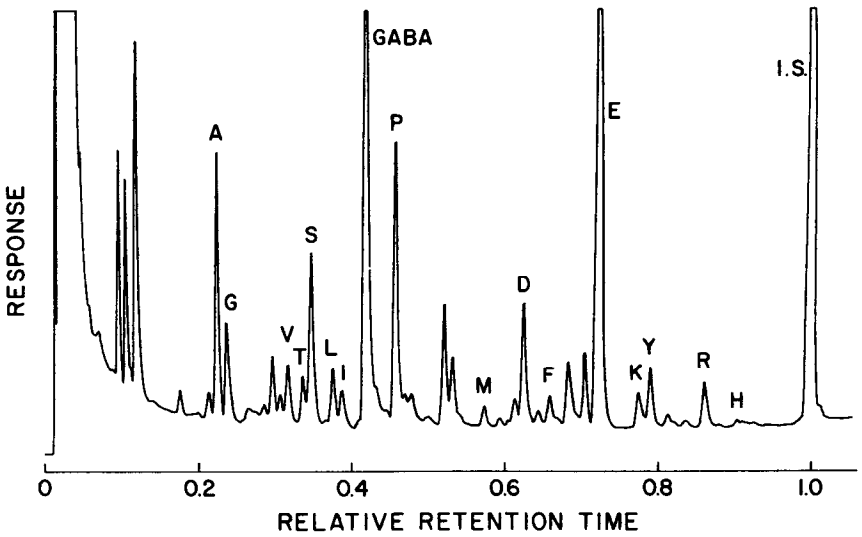

Fig. 3. Chromatogram illustrating separation of free amino acids isolated from the leaves of Western juniper. See text for chromatographic conditions and Fig. 1 for abbreviations.

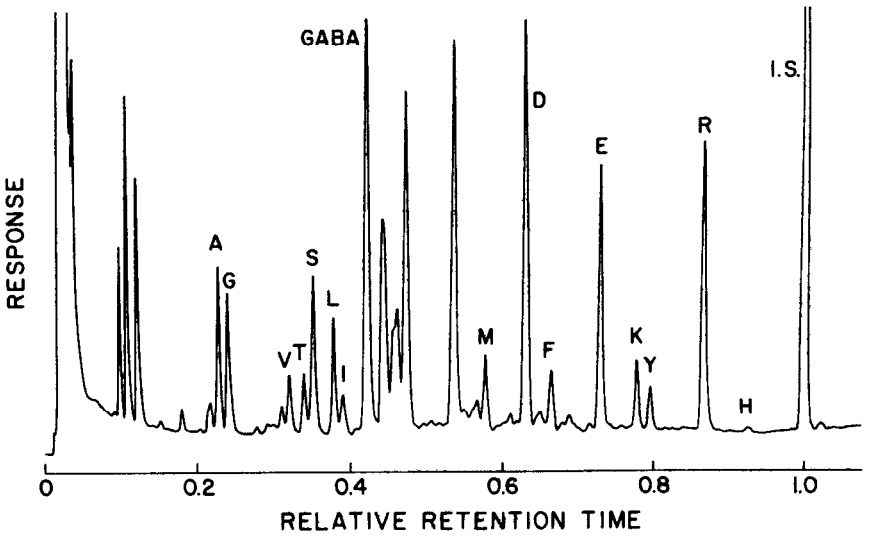

Fig. 4. Chromatogram illustrating separation of free amino acids isolated from the leaves of Ponderosa pine. See text for chromatographic conditions and Fig. 1 for abbreviations.

illustrating the problem nor information on the specific conditions used were presented, it is not possible to provide a definitive explanation. It is possible that vastly disproportionate amounts of alanine and glycine might result in the swamping of the lesser component. Given such a sample, and given that the amounts of alanine and glycine were important, appropriate manipulation of experimental conditions (column length, temperature program rate, carrier gas flow and starting temperature)

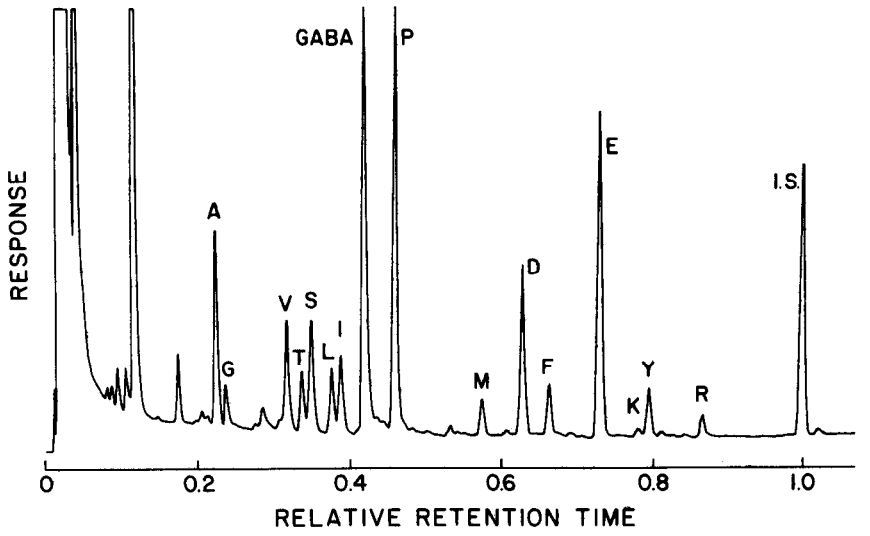

Fig. 5. Chromatogram illustrating separation of free amino acids isolated from the leaves of Black spruce. See text for chromatographic conditions and Fig. 1 for abbreviations.

TABLE I

CONIFER LEAF FREE AMINO ACIDS (nmoles/g)

Abbreviations: the proteic amino acids are represented by the standard three letter code. The other abbreviations represent compounds as follows: Eth = ethanolamine; $CySO_3H$ = cysteic acid; Gaba = γ-amino-butyric acid; Pip = pipecolic acid. – denotes compound not detected.

Compound	*RRT**	*Fir*		*Hemlock Western*	*Juniper*		*Pine*		*Spruce*		
		Balsam	*Douglas*		*Rocky Mountain*	*Western*	*Lodgepole*	*Ponderosa*	*Black*	*Colorado*	*White*
Eth**	0.108	200	290	280	220	160	340	109	190	77	150
$CySO_3H$**	0.212	40	11	26	5	27	20	14	29	50	14
Ala	0.227	800	520	750	210	740	620	280	1900	1030	840
Gly	0.240	150	140	320	100	64	400	114	550	440	130
β-Ala	0.301	–	–	210	40	30	–	–	40	40	–
Val	0.320	200	190	210	150	210	310	140	880	480	280
Thr	0.340	130	80	160	70	76	180	76	450	210	170
Ser	0.352	420	250	510	160	170	480	310	1020	630	430
Leu	0.378	130	130	140	130	170	490	200	430	300	200
Ile	0.390	110	88	83	68	100	120	64	520	240	190
Gaba	0.425	1980	1580	2160	620	1440	1029	400	4900	2800	2100
Pro	0.460	580	350	460	680	840	250	240	4200	35000	1240
Pip	0.502	–	53	150	–	–	–	–	–	510	–
Met	0.576	76	71	156	160	160	84	120	110	170	74
Asp	0.627	410	360	250	110	160	360	340	1060	750	400
Phe	0.661	90	110	130	80	84	190	80	260	240	140
Orn	0.693	55	50	130	24	330	25	–	70	–	40
Glu	0.727	540	300	860	220	270	480	270	2120	4000	720
Lys	0.775	110	80	70	50	50	170	70	150	1160	95
Tyr	0.791	90	50	90	80	120	150	50	270	570	95
Arg	0.861	80	60	80	105	50	50	220	140	6500	80
His		–	34	27	64	46	62	–	–	480	50
Cys	1.145	25	–	–	10	12	15	–	32	–	–

* Retention time relative to methylstearate.

** Response factor = 1.

would overcome most conceivable practical resolution problems. The alternative of using a two-column procedure[9] suffers the severe disadvantage of either requiring two instruments or using each column in an uncompensated mode in a single instrument. Although the latter possibility certainly does not preclude precise analysis, it places a premium on clean, well conditioned columns being used. Furthermore, a two-column procedure imposes an additional requirement to combine the data from two analyses into a single report.

The amounts of the free amino acids extracted from conifer leaves by procedure 2 are shown in Table I. In addition to the proteic amino acids, information is also presented for a few non-proteic amino acids identified by mass spectrometry and by coelution with authentic standards. Procedure 1 consistently extracted about 25% of the amounts extracted in procedure 2, so no data derived from the former procedure are presented. The reason for this difference is not clear but it is possible that the grinding step included in procedure 2 renders the amino acids more accessible to the extraction solvent. Alternatively, the solvent itself may be a more efficient extractant.

With the exception of the spruces, the total amino acids recovered ranged from about 3000 to 7000 nmoles/g of leaves. Black spruce and Colorado spruce yielded respectively about 19,000 and 56,000 nmoles/g of leaves. The basic amino acids constituted less than 7% of the free amino acids except for Colorado spruce (15%), Ponderosa pine (9%) and western juniper (9%). The former two species contained proportionally much more arginine than the other samples and western juniper contained about ten times as much ornithine as the other species.

With two exceptions (Rocky Mountain juniper and Colorado spruce), γ-aminobutyric acid was the predominant free amino acid. For most of the species, alanine, proline, aspartic acid or glutamic acid was the next most abundant free amino acid. Colorado spruce differed from both the other species and the other spruces in that proline constituted more than 60% and arginine and proline together comprised more than 74% of the total free amino acids.

There are few reports of the analysis of conifer leaf free amino acids by GLC with which to compare the quantitative values obtained herein. Sarkar and Malhotra[9] presented chromatograms but no quantitative information. Furthermore, arginine and histidine were not eluted in the chromatographic column system used by these authors. The results for Douglas fir are quantitatively different than those presented by Nijholt[10], but this author did not specify the time of the year at which the samples were collected.

In addition to the compounds indicated in Table I, a number of other compounds were detected but not identified. The relative proportions of the most abundant of these compounds are shown in Table II. Information on the chromatographic and mass spectrometric characteristics of these unknown compounds is presented for completeness and to facilitate further studies of the free amino acids of conifer leaves. Furthermore, it is interesting to note that the extracts contained compounds other than amino acids.

All the conifer leaf extracts contained four compounds having the following characteristic: (1) mol.wt. 100 [relative retention time (RRT) of 0.099 where methyl stearate = 1] corresponding to the composition $C_6H_{12}O$ and representing a completely saturated cyclic structure such as cyclohexanol or a monounsaturated straight-

TABLE II

UNIDENTIFIED COMPOUNDS IN CONIFER LEAVES*

Compound	*RRT***	Mol.wt.****	*Fir*		*Hemlock Western*	*Juniper*		*Pine*		*Spruce*		
			Balsam	*Douglas*		*Rocky Mountain*	*Western*	*Lodgepole*	*Ponderosa*	*Black*	*Colorado*	*White*
1	0.099	100	63	44	93	35	14	72	42	57	31	53
2	0.124	87	1120	630	2460	900	2230	2370	630	3250	1340	2160
3	0.178	99	80	110	150	110	120	140	42	210	145	120
4	0.287	60	43	57	50	88	61	86	31	124	122	81
5	0.443	153	–	–	150	–	–	–	133	–	1410	–
6	0.533	129	74	26	86	37	–	–	300	62	340	40
7	0.615	158	–	–	25	8	28	–	60	–	–	–
8	0.644	174	–	–	–	–	–	10	54	–	52	–
9	0.820	156	–	9	–	21	–	26	–	13	–	13
10	0.838	156	13	6	–	–	–	–	–	–	–	4

* Quantities are relative and based on response factors of 1 relative to methyl stearate.
** Retention time relative to methyl stearate.
*** Molecular weight of original compound.

chain alcohol; (2) mol.wt. 87 (RRT = 0.124) corresponding to the composition $C_5H_{13}N$ and possibly representing a pentylamine; (3) mol.wt. 99 (RRT = 0.178) corresponding to the composition $C_6H_{13}N$ corresponding to a mono-unsaturated amine; (4) mol.wt. 60 (RRT = 0.287) corresponding to the composition $C_2N_2H_8$: since this compound can be di-N-acylated and is, to satisfy the rules of valence, completely saturated, the composition can best be rationalized as corresponding to diaminoethane.

A compound of mol.wt. 153 (RRT = 0.443) was detected in western hemlock, Ponderosa pine and, in much greater amount, in Colorado spruce. The mass spectrum showed no evidence of an ester and the composition, $C_{10}H_{19}N$, would suggest a tetra-unsaturated amine.

Several species contained a compound (RRT = 0.533) having a mol.wt. of 129 and having a single amino and carboxy group. This molecular weight corresponds to pipecolic acid but the retention time would suggest that the compound is a structural isomer such as 4-methyl-proline or 4-keto proline. A straight-chain analogue such as 2-amino-3-methylene pentanoic acid would probably have a retention time significantly different from that observed. This compound was significantly more abundant in Ponderosa pine and Colorado spruce than in the other samples.

A compound at RRT = 0.615 had a mol.wt. of 158 corresponding to an unsaturated C_9 hydroxy acid.

A compound at RRT = 0.644 had a mol.wt. of 174. The mass of the derivative, *m/z* 818, suggested that the compound was a tri-hydroxy C_7 monocarboxylic acid. No evidence for the presence of nitrogen was obtained so that the mass of the original compound would be satisfied by complete saturation.

Two compounds, eluting at RRT 0.820 and 0.838, each had a mol.wt. of 156. Spectral evidence indicated diacylation but not esterification. The similarity of the spectra and proximity of elution would suggest that these were structural isomers. The spectra provided insufficient information to distinguish between N- and O-acylation.

The extract of Colorado spruce leaves contained small amounts of two compounds eluting at RRT = 0.700 and 0.718, just before glutamic acid. These compounds each contained chlorine, a single carboxylic acid group and two acylatable groups and had a mol.wt. of 192. The spectral evidence suggested N-acylation leading to a probable composition of $C_7H_{13}N_2O_2Cl$ which, in turn, requires unsaturation to satisfy the valence rules. Trace amount of the former were also detected in the extracts from Western hemlock and Black spruce.

Other compounds detected in the samples included hydroxypipecolic acid, γ-guanidinobutyric acid, α-aminophenylacetic acid and 2,4-diaminobutyric acid but these were present only in trace amounts.

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