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Note

Determination of mimosine by ion-exchange chromatography

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The amino acid (*S*)-1,3-[N-(3-hydroxy-4-pyridone)]-2-aminopropanoic acid (mimosine) is found in large quantities (20–100 g kg^{-1} of dry matter)[1] in the tropical shrub *Leucaena leucocephala*. Mimosine, when ingested by animals, either alone or as a constituent in *Leucaena* diets, has been found to be toxic[2–6]. The need for a rapid and specific method for the estimation of mimosine in *Leucaena leucocephala* is therefore apparent.

The method most frequently employed in the quantitative determination of mimosine in *Leucaena* is based on the formation of a violet octahedral tris-iron(III) chelate at low pH[7]. Mimosine is then estimated spectrophotometrically as the iron(III) complex. This method, however, is influenced by factors such as: pH, the presence of other phenolic compounds such as tannins which form chelates with iron(III), and the adsorption of mimosine by charcoal (used in the clarification of the *Leucaena* extract[8,9]).

This report describes an ion-exchange chromatographic (IEC) method for the estimation of mimosine in 6 *M* HCl extracts of *Leucaena* leaf meal. The extracted mimosine was eluted isocratically from the column and detected colorimetrically as its 2,4,6-trinitrobenzene sulphonic acid (TNBS) derivative. The IEC method for the determination of mimosine in 6 *M* HCl extracts has also been applied to 6 *M* HCl hydrolysates and to 0.1 *M* HCl extracts of *Leucaena*. The results are compared with those obtained using an iron(III) chelation procedure[8,9].

EXPERIMENTAL

Extraction of mimosine

Samples (*ca.* 0.8 g) of finely ground *Leucaena* leaf meal were weighed into 100-cm^3 polypropylene centrifuge tubes and 30 cm^3 of 6 *M* HCl were added. The stoppered tubes were shaken on a flatbed shaker for 10 min, then centrifuged (1900 *g*) for 5 min. The resulting supernatant was decanted through a 9-cm filter paper (Whatman No. 41) under reduced pressure. This extraction was repeated, and the residue was transferred to the filter paper with two 10-cm^3 aliquots of 6 *M* HCl. The residue on the filter was washed with distilled water (*ca.* 100 cm^3) and the filtrate made up to 250 cm^3. About 30 cm^3 of the resultant solution were filtered under reduced pressure using 2.1-cm Whatman GF-A papers and about 2 g of Celite filter aid. A

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mixture of a 2-cm³ aliquot of the extract and 2 cm³ of norleucine (0.25 m*M*) was dried down at 40°C under reduced pressure. The residual material in the flask was then dissolved in 2 cm³ of sucrose solution (0.292 *M* in 0.1 *M* HCl) and 0.5 cm³ taken for analysis.

The method of extraction of mimosine with hot 0.1 *M* HCl, for analysis by both iron(III) chelation and IEC, was essentially that described by Megarrity[9]. Hot 0.1 *M* HCl extracts, without treatment with charcoal, were also obtained and analysed by IEC.

Recovery experiments

The recovery of mimosine was determined by adding known quantities of mimosine (Sigma, Poole, Great Britain) to ground dried *Leucaena* leaf meal followed by subsequent acid extraction. Hydrolysis (22 h with 6 *M* HCl at 110°C) of mimosine, mimosine + *Leucaena*, and *Leucaena* alone, also allowed comparative recoveries to be made.

Chromatography

A hybrid semi-automated amino acid analyser, consisting of a 75-cm column packed with Technicon C-2 cation-exchange resin (Technicon, Basingstoke, Great Britain) and maintained at 62°C, was used. Mimosine was eluted isocratically from the column using a pH 3.69 sodium citrate buffer (Table I) 45 min after loading. Regeneration was achieved by pumping 0.2 *M* NaOH solution and pH 3.1 sodium citrate buffer (Table I) for 20 min and 30 min respectively.

TABLE I

COMPOSITION OF BUFFERS USED FOR ION-EXCHANGE CHROMATOGRAPHY

*pH**	*Citric acid (M)*	*Sodium citrate (M)*	*Sodium hydroxide (M)*	*Na_4 EDTA (M)*	*2-Methoxy ethanol (M)*	*2,2-Thio diethanol (M)*	*Phenol (M)*
3.10	—	0.050	0.050	0.001	—	0.0483	0.0106
3.69	0.470	—	0.506	—	0.190	0.0097	0.0053

* Adjusted to the required pH with 6 *M* HCl.

Norleucine was used as an internal standard and was eluted 70 min after loading. The ratio of norleucine peak area to mimosine peak area for isomolar solutions gave a norleucine equivalent (NLE) of 0.9534 (S.D. ±0.0247) for seven analyses.

RESULTS AND DISCUSSION

Our preliminary work indicated that attempts to include the estimation of mimosine with the conventional amino acid analysis of 6 *M* HCl hydrolysates of *Leucaena*, using a standard gradient buffer system, were ineffective since mimosine eluted simultaneously with isoleucine. Simultaneous elution of mimosine and isoleucine may account for the high isoleucine values which have been reported for *Leucanena*[5,10]. Our recently published results[11] tend to confirm this premise.

The sample chromatogram (Fig. 1) for the isocratic elution (pH 3.69) of mimosine, isoleucine, leucine, norleucine and phenylalanine shows that near baseline separation was achieved for these amino acids, within 80 min of loading.

TABLE II

MIMOSINE CONTENT (g kg^{-1} OF DRY MATTER) OF *LEUCAENA* ESTIMATED BY ION-EXCHANGE CHROMATOGRAPHY (IEC) AND BY CHELATION WITH IRON(III)

Leucaena leucocephala	*IEC method*				*Iron(III) method*
	6 M HCl		*0.1 M HCl*		*0.1 M HCl*
	Hydrolysis	*Extraction*	*Extraction without charcoal*	*Extraction with charcoal*	*Extraction with charcoal*
Sample 1 "Peru" cultivar from Malawi (1977 batch)	8.86 (S.D. ± 0.345)	10.26 (S.D. ± 0.079)	10.17 (S.D. ± 0.624)	9.47 (S.D. ± 0.221)	29.25 (S.D. ± 1.038)
Sample 2 "Peru" cultivar from Malawi (1979 batch)	23.86 (S.D. ± 0.405)	24.27 (S.D. ± 1.17)	24.27 (S.D. ± 1.12)	23.05 (S.D. ± 0.203)	33.51 (S.D. ± 0.463)

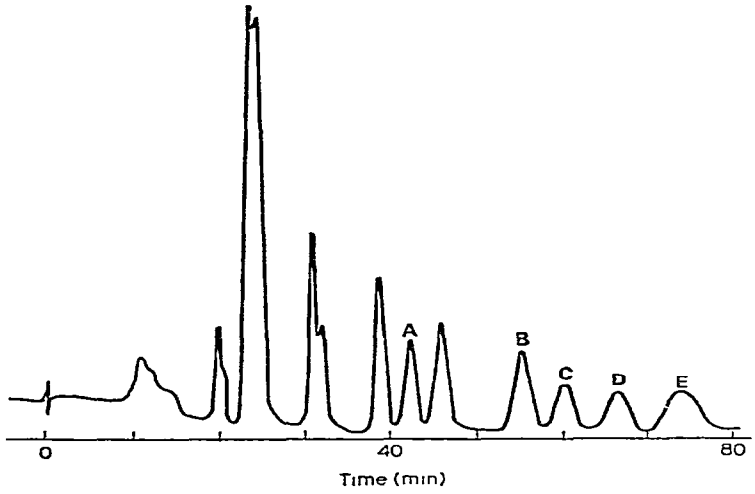

Fig. 1. The isocratic separation of mimosine (A), isoleucine (B), leucine (C), norleucine (D) and phenylalanine (E) by ion-exchange chromatography.

Results of analysis of two sun-dried *Leucaena* samples from Malawi are presented in Table II. The most significant feature in Table II is the much higher mimosine values obtained by iron(III) chelation compared with those obtained by IEC. The higher values are considered to be overestimates due to the chelation of iron(III) by other phenolic compounds such as tannins and 3-hydroxy-4(1H)-pyridone (3,4-dihydroxypyridine; DHP), a major degradation product of mimosine. Since the iron(III)–DHP complex has similar optical properties to the iron(III)–mimosine chelate, any DHP which is not adsorbed by the charcoal will affect the estimation of mimosine[9]. In the case of the two samples obtained from Malawi it is suggested that sample 1 may have a greater proportion of DHP —relative to its mimosine content— than sample 2.

Comparison of the mimosine values obtained using IEC show fairly good agreement between 6 *M* HCl and 0.1 *M* HCl extracts, producing almost identical values for both samples. This close agreement was unexpected because the stability of mimosine in concentrated HCl has been reported[12] to be greater than in 0.1 *M* HCl. Charcoal-treated 0.1 *M* HCl extracts yielded slightly lower values indicating adsorption of mimosine. Adsorption of mimosine by charcoal has been clearly demonstrated in this laboratory when mimosine standards were shaken with charcoal. Comparison of the values for charcoal-treated and untreated mimosine standards (0–1.5 m*M*) showed that variable losses, in excess of 50%, occurred. *Leucaena* hydrolysates show slightly lower values for mimosine than the 6 *M* HCl extracts due to decomposition of mimosine during hydrolysis. The mean recovery of mimosine from hydrolysates of mimosone, mimosine + *Leucaena* and *Leucaena* was 96.18% (S.D. ±1.154). Comparison of the mimosine values obtained from hydrolysates with those from 6 *M* HCl extracts show a similar loss for sample 2, but a higher loss (14%) for sample 1. Recoveries of mimosine from 0.1 *M* HCl extracts (estimated using iron(III)), and from 6 *M* HCl extracts (using IEC), were 91.63% (S.D. ±4.295) and 100.37% (S.D. ±2.056), respectively. The former recovery is similar to that reported elsewhere[9] for the iron(III) chelation method. The estimation of mimosine by IEC is not novel.

Reis *et al.*[13] achieved resolution of mimosine, isoleucine and leucine in ovine plasma in 120 min using a sodium-citrate buffer, while Mzik[14] obtained resolution of these amino acids and tyrosine in 80 min using two lithium citrate buffers. No recoveries of mimosine were reported by Reis *et al.*[13] while Mzik[14] recovered 100.3 ± 1.6% of added mimosine. Shiroma and Akashi[15] described a method for the determination of mimosine in digestive tract fluids; however, they did not provide details of recoveries, analysis times or buffer composition. The estimation of mimosine in *Leucaena* by paper chromatography[12] gave recoveries of 98–102% after a 15 h development period. The rapid (7 min) gas–liquid chromatography of the N-trifluoroacetyl butyl ester of mimosine, in 0.1 *M* HCl extracts of *Leucaena*, has also been achieved[16] although no results or recoveries were reported. The vigorous conditions required for derivatisation may be a disadvantage of this method. Mee and Brooks[16] regarded the absence of both of the alanine esters as an indication that hydrolysis of mimosine in 0.1 *M* HCl[12] had not occurred. In our preliminary investigations we found that, although complete hydrolysis of mimosine had occurred with 0.1 *M* HCl, neither of the two alanine isomers could be detected by IEC.

From the results presented here, 6 *M* HCl extraction of mimosine from *Leucaena* leaf meal followed by IEC using a relatively inexpensive buffer provides a rapid and specific method for the estimation of mimosine in *Leucaena* giving recoveries comparable to those observed with other chromatographic methods. The method reported here is not influenced by the various factors normally associated with the estimation of mimosine using the iron(III) chelation technique.

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