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Note

Determination of L-canavanine and L-canaline in plant tissues by high-performance liquid chromatography

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The amino acid L-canavanine, an analogue of L-arginine, is accumulated in the seeds of several legumes[1]. In some species the L-canavanine content is over 12% of the seed dry weight, representing more than 95% of the free amino acid nitrogen[2]. This amino acid can be translocated from the cotyledons to the growing seedling tissues[3] and metabolized by arginase to L-canaline and urea[4]. Both L- canavanine and L-canaline are potent antimetabolites, toxic to different types of organisms[5], and accordingly roles have been proposed for L-canavanine as a nitrogen-storing metabolite and as a defence against predator insects[5].

In most studies L-canavanine has been determined using the method reported by Fearon and Bell[6]. However, this method lacks specificity and is relatively insensitive. A method with improved sensitivity has been reported[7], based on the formation of a fluorescent ring system between guanidino compounds and phenanthrenequinone. However, interference by L-arginine cannot be discounted. This method was further improved by the separation of the amino acids using ion-exchange chromatography and post-column derivatization[8].

An indirect spectrophotometric method has been reported for the determination of L-canaline[9], but to our knowledge no data obtained by this method have been published. Indeed, very little information is available concerning L-canaline in plants.

In order to understand the importance of L-canavanine and its metabolites in plants, an appropriate method for their determination is required. This paper describes a method for the simultaneous determination of L-canavanine and L-canaline in samples containing other amino acids.

EXPERIMENTAL

Instrumentation

The equipment consisted of a Varian Model 5060 single-pump liquid chromatograph capable of gradient formation, a Varian Fluorichrom detector set with Corning filters 7-60 and 7-54 (excitation) and 4-76 and 3-71 (emission) and a Varian Vista 401 chromatography data system.

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Chemicals

Methanol (HPLC grade), acetone and acetonitrile (analytical-reagent grade) were obtained from Merck (Mexico). Dansylated and underivatized amino acids and dansyl chloride were obtained from Sigma (St. Louis, MQ, U.S.A.). All other chemicals were obtained from J. T. Baker (Mexico). Water was distilled twice before use.

Columns

Two reversed-phase (C_{18}) columns were tested: a MicroPak MCH-10, particle size 10 μm (30 cm × 4 mm I.D.), from Varian and a Hibar C_{18}, particle size 5 μm (25 cm × 4 mm I.D.), from Merck.

Derivatization

An aliquot of standard amino acid solution or plant extract (usually 20 μl) was mixed with 0.1 *M* sodium hydrogencarbonate (pH 10.5) to give a final volume of 100 μl, then 50 μl of a1.5 mg/ml solution of dansyl chloride in acetone were added and the mixture was shaken vigorously and incubated for 90 min at room temperature. A volume of 30 μl was loaded into the 10-μl loop injector.

Extraction

Canavalia ensiformis (L.) DC. seed parts were finely ground and then shaken for 10 min in water (10 ml/g). The extracts were centrifuged (2000 *g*) for 10 min and the resulting supernatant was extracted with an equal volume of chloroform and centrifuged (2000 *g*) for 10 min. The chloroform extraction was repeated and the aqueous phase was recovered and filtered through a Millipore HAWP filter (0.45 μm). All operations were carried out at 4°C. The extracts were then analysed immediately or stored at −20°C.

Elution gradient

Mobile phase A was methanol in 0.01 *M* Tris–HCl (pH 7.7) (5:95) and mobile phase B was methanol. The gradient used was from 0 to 47% B in 70 min and then from 47 to 100% B in 10 min. Re-equilibration to the initial gradient conditions took 30 min.

RESULTS AND DISCUSSION

There are no previous reports on the dansylation of L-canavanine or L-canaline. The initial protocol used dansyl chloride dissolved in acetonitrile. A dansylated derivative was obtained with L-canavanine but not with L-canaline. However when acetone was used as the solvent, dansylated derivatives of both amino acids were obtained (Fig. 1).

The separation of a mixture of 23 dansyl amino acids, including dansyl-L-canavanine and dansyl-L-canaline, was compared using two different columns, Hibar C_{18} and MicroPak MCH-10. Preliminary tests showed that the resolution was better with the Hibar C_{18} column (data not shown) and all subsequent work was carried out with this column.

The separation of a mixture of dansyl-L-canavanine and dansyl-L-canaline was easily achieved (Fig. 1). However, in a mixture containing the dansyl amino acids

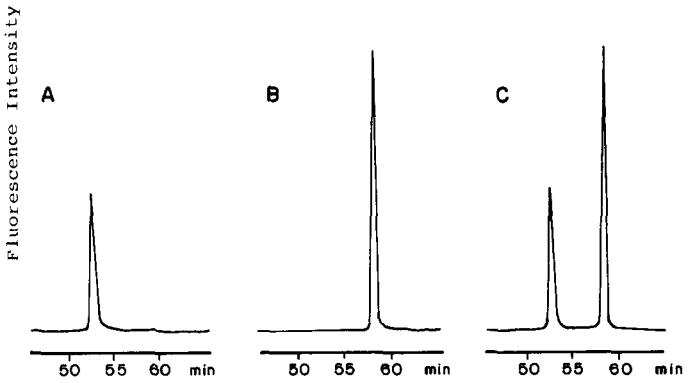

Fig. 1. Reversed-phase HPLC of (A) dansyl-L-canavanine, (B) dansyl-L-canaline and (C) dansyl-L-canavanine with dansyl-L-canaline.

listed in Table I, the best separation was obtained with a gradient from 0% to 47% of solvent B in 70 min and from 47% to 100% in the following 10 min. A typical chromatogram is shown in Fig. 2 with the corresponding retention times listed in Table I. The elution time is long and one sample can be injected every 110 min, but shorter elution times, using steeper gradients, or starting with a higher proportion of

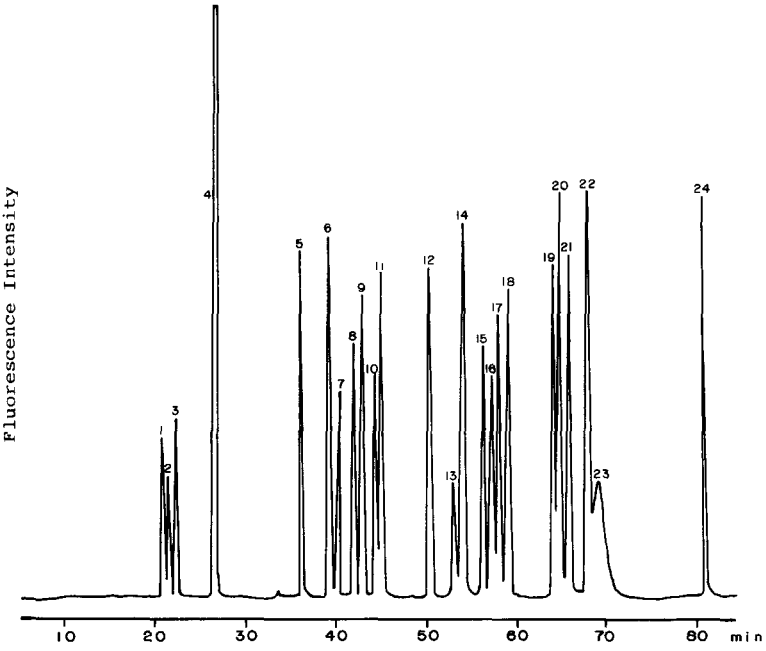

Fig. 2. Gradient separation of dansyl amino acids by reversed-phase HPLC. (1) Dns-Cys; (2) Dns-Asp; (3) Dns-Glu; (4) Dns-OH; (5) Dns-Asn; (6) Dns-Gln; (7) Dns-Ser; (8) Dns-Gly; (9) Dns-Thr; (10) Dns-His; (11) Dns-Ala; (12) Dns-GABA; (13) Dns-canavanine; (14) Dns-Pro; (15) Dns-Val; (16) Dns-Arg; (17) Dns-Met; (18) Dns-canaline; (19) Dns-Ile; (20) Dns-Try; (21) Dns-Leu; (22) Dns-Phe; (23) Dns-Lys; (24) DNS-Tyr.

TABLE I

RETENTION TIMES OF DANSYL AMINO ACID STANDARDS

Amino acid	*Retention time (min)*	*Amino acid*	*Retention time (min)*
Cysteine	20.9	Proline	54.0
Aspartic acid	21.5	Valine	56.5
Glutamic acid	22.5	Arginine	57.3
Asparagine	36.2	Methionine	58.0
Glutamine	39.3	Canaline	58.9
Serine	40.2	Isoleucine	64.0
Glycine	42.4	Tryptophan	64.7
Threonine	43.0	Leucine	65.7
Histidine	44.6	Phenylalanine	67.9
Alanine	45.0	Lysine	69.0
δ-Aminobutyric acid	50.4	Tyrosine	69.0
Canavanine	52.8		

solvent B, always gave a poorer separation of certain derivatives, including dansyl-L-canaline.

The retention times were highly reproducible: 52.8 ± 0.15 min for dansyl-L-canavanine and 58.5 ± 0.18 min for dansyl-L-canaline (data calculated from nine consecutive chromatograms performed within 48 h). Calibration graphs were prepared for L-canavanine and L-canaline. A linear response was obtained wihtin the ranges 0.15–5 and 0.15–3.6 nmol for dansyl-L-canavanine and dansyl-L-canaline, respectively. The detection limits (signal-to-noise ratio = 2) were 50 pmol for dansyl-L-canavanine and 10 pmol for dansyl-L-canaline.

This method was applied to the determination of L-canavanine and L-canaline in seed parts of *C. ensiformis* (Table II). L-Canaline was not detected in any of the parts studied, in contrast to results reported by Rosenthal[10].

Using a paper chromatographic method, Rosenthal detected L-canaline in jack bean cotyledons 24 h after the start of imbibition. The absence of L-canaline in our

TABLE II

CONTENT OF L-CANAVANINE AND L-CANALINE IN SEED PARTS OF *CANAVALIA ENSIFORMIS*

The results shown are averages of analyses of three different samples. Figures in parentheses correspond to mg per pair of cotyledons or mg per embryo.

Part	*Concentration (mg/g fresh weight)*	
	L-canavaline	*L-canaline**
Dry cotyledon	27.0 (48.6)	N.d.
Dry embryo	32.0 (0.45)	N.d.
Hydrated cotyledon**	17.5 (45.5)	N.d.
Hydrated embryo**	7.6 (0.45)	N.d.

* N.d. = not detected.

** After 24 h imbibition.

extracts cannot be explained by an insufficient recovery of L-canaline by the extraction method used, as the recovery of authentic L-canaline added to tissue samples before extraction was 95% (average of two results). On the other hand, we observed that if samples were not adequately stored the L-canavanine peak decreased with a concomitant appearance of an L-canaline peak.

The content of L-canavanine in dry seeds has been previously reported[6,11,12] to range from 25 to 31 mg/g. The analysis reported here gave a content of 27 mg/g. With the dry embryo there have been no previous reports; the L-canavanine content was found to be similar to that of dry cotyledons (Table II). This seems logical, assuming a defence role for L-canavanine, as reduced levels of this amino acid would make the embryo more susceptible than cotyledons to insect attack. The content is nevertheless very high and the total amount present in the embryo after imbibition remains constant for at least 24 h. It will be interesting to study how embryo tissues deal with this highly toxic amino acid. The content of L-canavanine in cotyledons also showed very little change after 24 h of imbibition.

CONCLUSION

A method has been developed that can determine L-canavanine and L-canaline simultaneously in mixtures containing all the common amino acids. Further, it has potential for the determination of each of the common amino acids.

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