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Analysis of Neurotoxin 3-*N*-Oxalyl-L-2,3-diaminopropionic Acid and Its α-Isomer in *Lathyrus sativus* by High-Performance Liquid Chromatography with 6-Aminoquinolyl-*N*-hydroxysuccinimidyl Carbamate (AQC) Derivatization

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A method was developed for the quantitative determination of the neurotoxic nonprotein amino acid, 3-*N*-oxalyl-L-2,3-diaminopropionic acid (β-ODAP), and its nontoxic α-isomer, 2-*N*-oxalyl-L-2,3-diaminopropionic acid (α-ODAP), in the plant samples of *Lathyrus sativus* after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) by reversed-phase high-performance liquid chromatography (HPLC). Hippuric acid was used as an internal standard. A linear reponse was recorded in the concentration rang 0.32−32 nmol with $r > 0.999$. The RP HPLC detection limit for both isomers is 1.8 ng. According to different experimental needs, a ternary gradient system can be used to determine toxin and other nonprotein amino acids. The RP HPLC method and a colorimetric method were compared for measuring ODAP.

Keywords: *RP HPLC; neurotoxin; Lathyrus sativus; 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate*

INTRODUCTION

Lathyrus sativus (*L. sativus*) is a leguminous crop cultivated in India, Bangladesh, and Ethiopia, where it is a major protein source for people in the lowest income groups (Spencer et al., 1986; Tekle-Haimanot et al., 1993). However, excessive ingestion of this pulse can lead to irreversible paralysis of the legs−a disease known as neurolathyrism or lathyrism. The causative agent was reported to be the nonprotein amino acid, 3-*N*-L-oxalyl-2,3-diaminopropionic acid (β-ODAP, sometimes referred to as BOAA) (Rao et al., 1967; Ross et al., 1985). The α-isomer of ODAP, 2-*N*-oxalyl-2,3-diaminopropionic acid, has been shown to be nontoxic to chicks and mice (Wu et al., 1976).

L. sativus (shan li dou in China) shows good adaptation to the low rainfall conditions of northwestern China. It grows also in poor soil and is resistant to salt and flood. Our group is exploring the breeding of low or zero toxin varieties of *L. sativus* as grain crops for human consumption and as protein-rich feed for animals. Thus, it is necessary to develop a method to determine the toxin or other amino acids suitable for different analyses. The most widely used method for determining the neurotoxin in foods and seed samples is a colorimetric method using *o*-phthalaldehyde (OPA) (Rao et al., 1978). However, the nontoxic α-ODAP is determined along with β-ODAP. High-performance liquid chromatographic methods have been developed for screening plant samples and analyzing animal tissues containing ODAP (Shah et al., 1992) in recent years. 9-Fluorenyl methylchloroformate (FMOC) derivatization is suitable for detection of ODAP in the picomolar range in plant and animal tissues. The derivatized solution must be extracted with hexanes−ethyl acetate to remove excess of reagent because it interferes with the separation of the amino acid derivaties and is detrimental to column performance (Geda et al., 1993). Separation of α- and β-ODAP was achieved by Khan et al. (1993) by reversed-phase HPLC using precolumn derivatization with phenyl isothiocyanate, but excess coupling reagent has to be evaporated before chromatographic analysis.

Recently, Cohen and Michaud (1993) developed a precolumn derivatization method in which a new reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), was used for amino acid derivatization. AQC can react quantitatively with all primary and secondary amino acids in a few seconds with little matrix interference and form single, very stable derivatives. To determine the α- and β-ODAP in *L. sativus* by AQC derivatization, we have developed an HPLC method that can simultaneously determine α- and β-ODAP. The method provides a simple, fast, and accurate alternative to existing methods for plant screening purposes. According to different experimental needs, it is also important to develop some new methods for the simultaneous determination of toxins and other nonprotein amino acids in the plant samples of *L. sativus* by changing eluting conditions.

EXPERMENTAL PROCEDURES

Reagents. Amino acid standard (containing 2.5 μmol of 17 protein amino acids) was obtained from Pierce (Rockford, IL). L-Homoarginine, hippuric acid, and *o*-phthalaldehyde were obtained from Sigma (St. Louis, MO). 2,3-Diaminopropionic acid was purchased from Aldrich. Standard α- and β-ODAP were purified from *L. sativus* (Rao et al., 1964; Harrison et al., 1977; Li et al., 1992) and their purity was confirmed by

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Table 1. HPLC Gradient Conditions

time (min)	mobile phase composition (%)		
	A	B	C
0	100	0	0
0.5	99	1	0
18	95	5	0
19	91	9	0
29.5	83	17	0
33	0	60	40
36	0	60	40
50	100	0	0

capillary zone electrophoresis (CZE) (Zhao et al., 1999). 2-Aminobutyric acid was purchased from BDH Chemicals Ltd., Poole, England.

AQC (Waters AccQ Fluor reagent) and borate buffer were obtained as a kit from Millipore (Milford, MA). Eluent A concentrate (sodium acetate buffer) was also from Millipore. Water was supplied by a Milli-Q system from Millipore (Milford, MA).

Sample Preparation. About 20 mg of sample was accurately weighed and added to 2 mL of ethanol−water (3:7,v/v), shaken briefly, and sonicated for 30 min, then agitated with a magnetic stirrer for 2 h. The solution was separated after centrifugation (15 min at 15000*g*) and subsequently filtered through a 0.22-μm membrane.

Precolumn Derivatization Procedure. A 10-μL aliquot of the sample extract or amino acid standard solution (with the internal standard added) was delivered to a 6- × 50-mm tube and buffered at pH 8.8 with 70 μL of 0.2 M sodium borate solution. The derivatives were formed with 20 μL of AQC in acetonitrile and heated for 10 min at 55 °C.

Chromatographic Instruments. The HPLC system consisted of a Waters model 600E pump, an AccQ-Tag C_{18} (4 μm) column (15 × 0.39 cm), a column heater, and a model 2487 dual wavelength absorbance detector set at 254 nm (all from Waters). Millennium 32 software, from Waters, was used to control system operation and collect and analyze data.

Chromatographic Conditions. Eluent A (concentrated sodium acetate buffer) was obtained from Millipore. Mobile phase A was prepared by mixing 100 mL of eluent A (concentrate) with 1000 mL of water. Mobile phase B was acetonitrile and mobile C was water. For analysis of samples for only α- and β-ODAP, a mobile phase of A:B (96:4) for 5.5 min and changing to B:C (60:40) for 3 min was used. To analyze α- and β-ODAP along other nonprotein amino acids, a ternary gradient system was used (Table 1). The AccQ-Tag column was thermostated at 37 °C and operated at a flow rate of 1.0 mL/min.

Colorimetric Method. Extracts (0.1 mL) was taken in test tube. Then 0.2 mL of 3 M KOH was added, and the tube was kept in a boiling water bath for 30 min. After the tube was cooled to room temperature, the sample was brought to 1 mL with water and 2 mL of the OPA reagent (0.1 g of OPA, 0.2 mL of mercaptoethanol, 1 mL of absolute ethanol, 99 mL of 0.05 M $K_2B_4O_7$, pH 9.9) was added. The absorbance of the yellow solution was measured after 30 min at 420 nm against a reagent blank made with an equivalent amount of alkali. A standard curve based on dilutions of a DAP solution was used for the quantification.

RESULTS AND DISCUSSION

The AQC derivatives of standard α- and β-ODAP and extracts of *L. sativus* were analyzed and resolved into two peaks with retention times of about 6.07 and 3.85 min for α- and β-ODAP, respectively (Figure 1). Each component was identified by spiking the samples with the pure isomer.

Analysis of dilutions of α- and β-ODAP from 0.32 to 32 nmol showed a linear reponse in this range with $r > 0.999$ (Table 2). The HPLC detection limit for both isomers is 1.8 ng (signal:noise ratio = 2:1) which, when

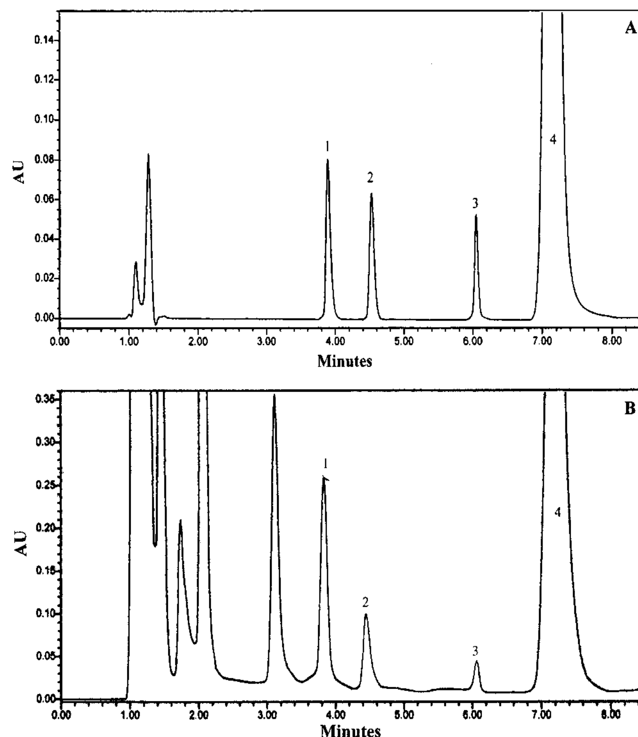

Figure 1. Separation of α- and β-ODAP after derivatization with AQC. Peaks: 1, β-ODAP; 2, hippuric acid; 3, α-ODAP; 4, AMQ; (a) chromatogram of standard α- and β-ODAP derivatives; (b) chromatogram of α- and β-ODAP in dry seeds of *L. sativus* after AQC derivatization.

Table 2. Linear Response[a] and Correlation Coefficient: Comparison of Reproducibilities of α- and β-ODAP ($n = 7$)

compd	$A \times 10^{15}$	$B \times 10^{15}$	r	RSD external calibration	RSD internal calibration
α-ODAP	5.64	1.46	0.9991	2.07	0.73
β-ODAP	8.44	1.46	0.9992	1.89	0.54

[a] Area = A + B × concentrations.

taking the prepurification procedure into account, would give an apparent detection limit of 0.15 μg/g in the *L. sativus* samples. Another validation test for the accuracy of the method was done by measuring a known amount of α- and β-ODAP added to the samples. The average recovery was about 98%. Peak area repeatability, obtained from nine injections of standard on the same day, ranged from 4.5 to 8.7% relative standard deviation (RSD). The reproducibility, from five injections on several days, was slightly higher (RSD 5.4−10.1%). Hippuric acid was studied as internal standard. The results showed that hippuric acid was stable and yielded reproducible results, so it was adopted as internal standard for quantification. The retention time of hippuric acid is about 4.51 min. External calibration was also tested for the analysis of the same sample, and the two sets of data were almost identical. The reproducibility of the results with the internal calibration method was better than with external calibration (Table 2). The same samples were also analyzed using the OPA method. Both results are in good agreement (Table 3).

The α- and β-ODAP-AQC derivatives decomposed easily when they were irradiated by UV light (254 nm) at room temperature 30 °C (Figure 2), so the amino acid-AQC derivatives should be stored at 0 °C and protected

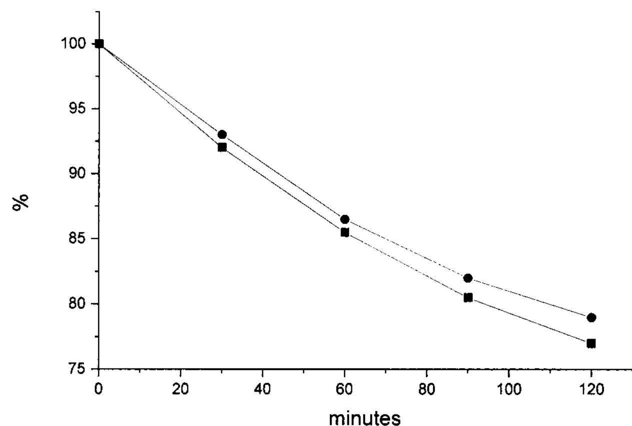

Figure 2. Decomposition of α- and β-ODAP derivatives irradiated by UV light: ●, β-ODAP; ■, α-ODAP.

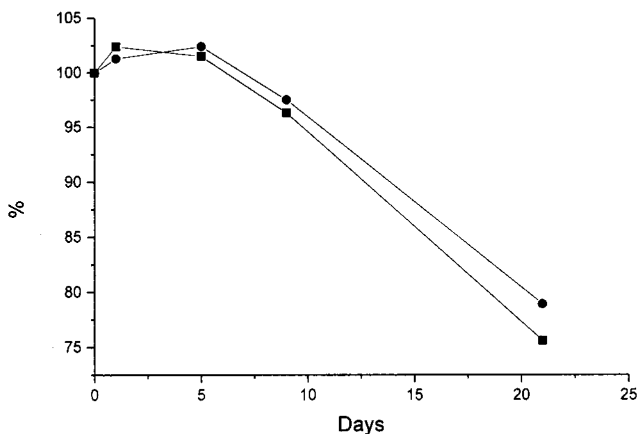

Figure 3. Plot of decrease in derivative stability versus days after derivatization. The derivatives were stored at 0 °C and protected from light: ●, β-ODAP; ■, α-ODAP.

Table 3. α- and β-ODAP Content in *L. sativus* Seed, Leaf, and Root Samples

method	compd (w/w %)	seed 1	seed 2	seed 3	leaf 1	leaf 2	leaf 3	root 1	root 2	root 3
HPLC	α-ODAP	0.07	0.08	0.07	0.08	0.04	0.02	0.12	0.085	0.072
	β-ODAP	0.31	0.39	0.48	0.14	0.17	0.12	0.019	0.022	0.019
OPA	ODAP	0.37	0.45	0.53	0.21	0.19	0.15	0.14	0.10	0.083

from light. If this were done, the peak area was almost unchanged until 5 days after derivatization (Figure 3). When the derivatives were stored at −20 °C, they decomposed much more slowly and the decrease of their peak areas was not important after 1 month (data not shown).

Sometimes it is necessary to simultaneously determine α- and β-ODAP and other amino acids, so a ternary gradient was used (Table 1). Figure 4A shows the chromatogram of an amino acid standard mixture derivatized with AQC. The α- and β-ODAP were eluted at 17.16 and 13.83 min, respectively, and did not interfere with any of the compounds used. 2-Aminobutyric acid (ABA) was used as an internal standard which, when added to samples at different amino acid concentrations, showed that ABA gave very reproducible results. Therefore, ABA could be used as the internal standard for quantification. Figure 4B shows a typical chromatogram of the α- and β-ODAP and other nonprotein amino acids in the dry seeds of *L. sativus* after AQC derivatization.

A lot of leaf, root, seed, and seedling samples were analyzed, and it was found that many amino acids vary

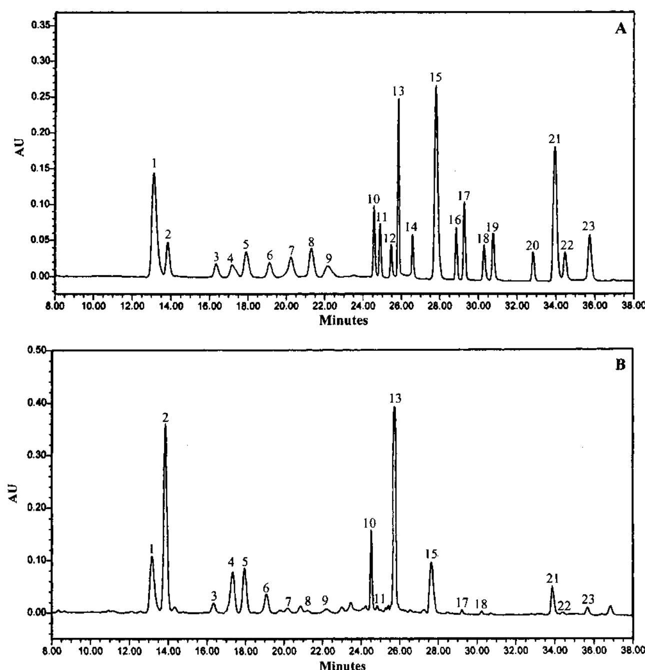

Figure 4. Chromatograph of (A) amino acid standards and (B) seed extract of *L. sativus*. Peaks: 1, AMQ; 2, β-ODAP; 3, Asp; 4, α-ODAP; 5, Ser; 6, Glu; 7, Gly; 8, His; 9, NH_3; 10, Arg; 11, Thr; 12, Ala; 13, homoarginine; 14, Pro; 15, ABA; 16, Cys; 17, Tyr; 18, Val; 19, Met; 20, Lys; 21, Ile; 22, Leu; 23, Phe.

greatly. Usually, the content of β-ODAP is about 3−15 times that of α-ODAP in the leaf, seed, and seedling samples; however, in root sample, α-ODAP is about 2.5−11 times that of β-ODAP. The reasons for this will be the subject of future research.

CONCLUSION

AQC reagent is a useful alternative derivatizing reagent for determination of α- and β-ODAP or other nonprotein amino acids of *L. sativus*. The relatively short time for analysis and good sensitivity for α- and β-ODAP isomers make this method preferable to earlier methods in the studies of the neurotoxin evaluation in *L. sativus*.

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