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Determination of neurotoxin 3-*N*-oxalyl-2,3-diaminopropionic acid and non-protein amino acids in *Lathyrus sativus* by precolumn derivatization with 1-fluoro-2,4-dinitrobenzene

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Abstract

A rapid and simple method is presented for determining neuro-excitatory nonprotein amino acid 3-*N*-oxalyl-2,3-diaminopropionic acid (β-ODAP) and non-protein amino acids in *Lathyrus sativus*. Seed and foliage extracts of *Lathyrus sativus* were treated with 1-fluoro-2,4-dinitrobenzene (FDNB) and a reversed-phase high-performance liquid chromatography method (RP HPLC) for the separation of the derivatives in the pmol range is reported. The RP HPLC method and a colorimetric method were compared for measuring ODAP.

Keywords: Derivatization, LC; *Lathyrus sativus*; 3-*N*-Oxalyl-2,3-diaminopropionic acid; Non-protein amino acids; 1-Fluoro-2,4-dinitrobenzene

1. Introduction

Lathyrus sativus (shan li dou in China, khesari in India and Bangladesh, pois carré in France, guaya in Ethiopia) is a popular drought tolerant crop and important grain legume in drought areas of Africa and Asia. The crop is produced with a minimum amount of care and can be successfully grown in a variety of climates. The edible seeds are very important nutritionally since they contain 26–30% protein. However, excessive ingestion of *Lathyrus sativus* seed can lead to an upper motor neuron degenerative disease known as lathyrism. Lathyrism is caused by a neuro-excitatory nonprotein amino acid 3-*N*-oxalyl-L-2,3-diaminopropionic acid (β-ODAP) [1–3]. For this reason, its cultivation is prohibited in some places.

Our group is seeking to breed low or zero toxic varieties of *Lathyrus sativus* through cell culture and transgene techniques and other methods. Therefore it is imperative to develop a fast and selective method to determine the neurotoxin in the large number of samples that are processed in our research.

The most common method utilizes the reaction of *o*-phthalaldehyde (OPA) with 2,3-diaminopropionic acid (DAP) formed on hydrolysis of both α- and β-isomers of ODAP, and the derivative is detected at 420 nm [4,5], since free DAP is not a natural constituent of *Lathyrus sativus* seeds. Because α- and β-ODAP are hydrolyzed to DAP, the method can not be used to analyse α- and β-ODAP, respectively. It also can not determine the extract of foliage

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accurately, owing to pigment interfering with the analysis. Recently, a few HPLC methods for ODAP have been developed. 9-Fluorenyl methylchloroformate (FMOC) [6] derivatization can be used for the detection and quantitative estimation of pmol amounts of ODAP, but it is nonselective between the toxic β-ODAP and its nontoxic isomer, α-ODAP. Buerby et al. [7] separated β-ODAP with *o*-phthaldialdehyde chiral thiols by reversed-phase chromatography, using fluorescence detection, but α-ODAP was not mentioned and the procedures are time-consuming. Khan et al. [8,9] used precolumn derivatization with phenyl isothiocyanate (PITC). In the literature [8], the separation of α- and β-ODAP was achieved; the peak of α-ODAP was not pointed out in the literature [9] and the procedures were also time-consuming.

Precolumn derivatization with 1-fluoro-2,4-dinitrobenzene (FDNB) prior to HPLC has become an important analytical technique for the assay of the amino acids [10–12]. However, a drawback is its toxicity; it must be handled with protective gloves [13]. In order to rapidly analyse β-ODAP and non-protein amino acids of *Lathyrus sativus*, we utilized precolumn derivatization with FDNB, followed by reversed-phase HPLC with UV detection. The method reported here is sensitive, reproducible and rapid, which is suitable for the determination of β-ODAP and non-protein amino acids in the seed and foliage of *Lathyrus sativus*.

2. Materials and methods

2.1. Reagents

α- and β-ODAP were purified from *Lathyrus sativus* extracts [14], L-homoarginine was obtained from Sigma (St. Louis, MO, USA) and amino acid standards were purchased from BDH Chemicals, Poole, England. Ultra-pure water was obtained by means of a Milli-Q System purchased from Millipore (Milford, MA, USA). Acetonitrile (HPLC grade) was locally produced.

2.2. Chromatographic systems

The HPLC system consisted of a Waters Model 600E pump, a Cartridge C_{18} (5 μm) column 3.9× 150 mm, a column heater and a Model 2487 dual wavelength absorbance detector set at 360 nm (all from Waters). Millennium 32 software, from Waters, was used to control system operation and collect and analyse data.

Mobile phase A consisted of 0.03 $mol \cdot l^{-1}$ K_2HPO_4 and 1% dimethyl formamide (DMF) in water (v/v) and was adjusted to pH 5.60 with glacial acetic acid. Mobile phase B was acetonitrile. The mobile phase solutions were filtered through a 0.45 μm membrane filter and degassed before use. For binary gradient elution, the program is shown in Table 1. The column was thermostated at 26°C and operated at a flow-rate of 1.0 ml/min.

2.3. Preparation of sample

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 (with ice added and the water temperature kept at about 8°C), then agitated with a magnetic stirrer for 2 h, centrifuged for 15 min at 15 000 *g* and filtered. The extract was stored at 4°C.

2.4. Precolumn derivatization procedure

The derivatization reagents were freshly prepared every day by dissolving 100 mg of FDNB in 10 ml of acetonitrile. A known amount of ODAP or sample extract was dried under vacuum. The residue was dissolved in 100 μl of $NaHCO_3$ solution (0.5 mol·

Table 1
Chromatographic gradient conditions for the analysis of β-ODAP and non-protein amino acids

Time (min)	A	B
0	85	15
4	85	15
7	73	27
10	70	30
13	65	35
15	65	35
18	55	45
18.1	85	15

l^{-1}), then, 100 μl of the derivatizing reagent was added and the solution was mixed well. The derivatization was complete in 30 min at 60°C. After cooling to room temperature, it was added to 0.8 ml of KH_2PO_4 solution (0.01 mol.l^{-1}), vortexed for several seconds and 20 μl of the product was taken for HPLC analysis.

3. Results and discussion

β-ODAP and standard non-protein amino acids were derivatized using the procedure described for precolumn derivatization. The separation of 20 compounds is shown in Fig. 1. Each component was identified by spiking standard amino acid. α- and β-ODAP–DNB were eluted at 2.5 min and 2.3 min, respectively, and were not interfered with by any of the compounds used.

The maximum percentage of reaction between ODAP and FDNB was reached for 30 min at 60°C (Fig. 2). A very slow but measurable decrease in yield occurred as the reaction was allowed to proceed for a longer time, presumably due to the hydrolysis of ODAP–DNB (Fig. 3).

The amount of FDNB selected for derivatization was based on preliminary experiments which showed that molar concentration of FDNB should be at least 20 times that of ODAP. Hence, 0.1 ml FDNB solution (0.05 mol·l^{-1}) was used and found adequate for the reaction to be complete. Typical chromatograms of the derivatized seed and foliage extracts are shown in Fig. 4. Homoarginine, α- and β-ODAP are major free amino acids in the dry seeds [18]. In the foliage, the components are relatively complex in the 30% enthanol extracts.

Analysis of dilutions of α- and β-ODAP from 60 to 250 pmol showed a linear response in this range with $r>0.999$. Good limit of detection and reproducibility of analysis were shown in Table 2.

Peak areas for derivatized α- and β-ODAP were essentially unchanged for at least one week at room temperature, only the DNB–OH peak area increased because of hydrolysis of FDNB. Thus the α- and β-ODAP derivatives have more than sufficient stability to allow for several days chromatographic analysis. The accuracy of the method is also sup-

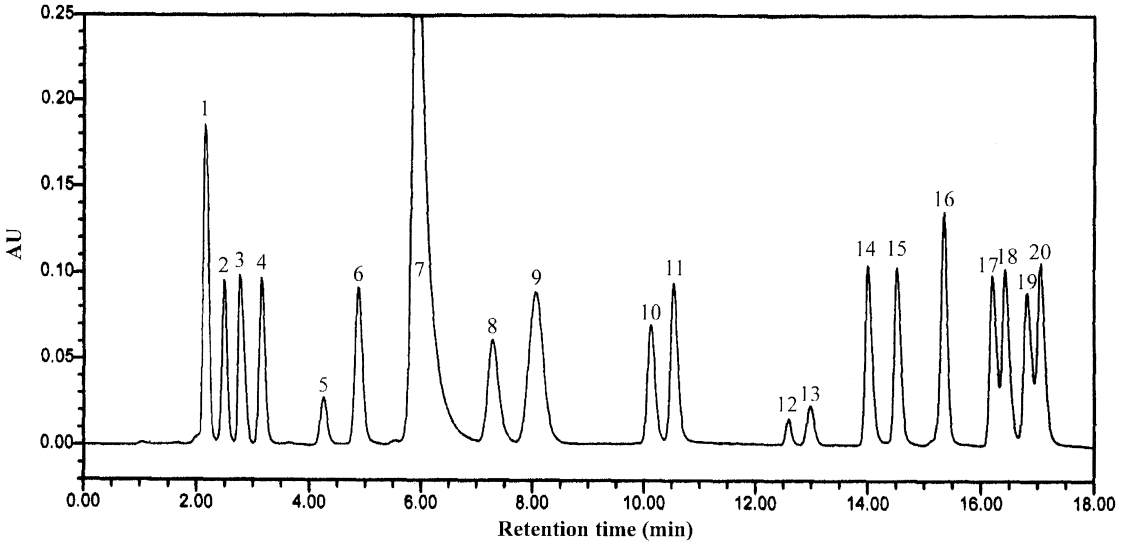


Fig. 1. Chromatogram of an amino acid standard mixture derivatized with FDNB. Preparation of β-ODAP and amino acid standard and chromatography are described in the text and Table 1. The column was thermostated at 26°C. Detection by UV at 360 nm. Peaks: 1=β-ODAP; 2=α-ODAP; 3=Asp; 4=Ser; 5=Glu; 6=Arg; 7=DNB–OH; 8=His; 9=homoarginine; 10=Thr; 11=Ala; 12=Pro; 13=Cys; 14=Tyr; 15=Val; 16=Met; 17=Lys; 18=Ile; 19=Leu; 20=Phe.

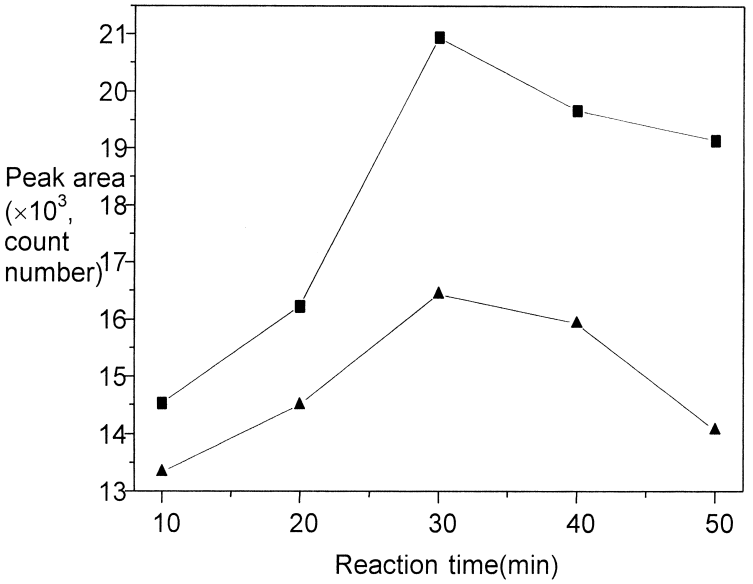
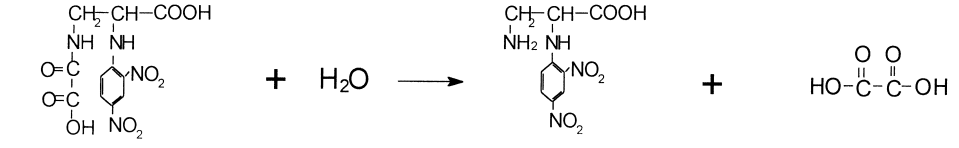


Fig. 2. Peak area vs. reaction time at 60°C for the FDNB derivatization of α- and β-ODAP. (■) β-ODAP, (▲) α-ODAP.

ported by the recovery of α- and β-ODAP from spiked seed extracts. When accurate amounts α- and β-ODAP were added to seeds of know toxin level, the added compounds were analysed as an increase of the α- and β-ODAP peak area. About 99% recovery was possible after extraction and derivatization.

The contents of α- and β-ODAP determined by the HPLC method were compared with those by the OPA method [5] as shown in Table 3. The results of the two methods were consistent on the whole. The content of β-ODAP is about 3–14 times as much as that of α-ODAP in the *Lathyrus sativus* seed and foliage samples. The interconversion of α- and β-ODAP is accelerated by heating [19], the reported equilibrium concentrations of α- and β-ODAP are between 30 and 35% and 70–65%, respectively [8,15]. Thus prolonged cooking of the *Lathyrus* seed can obviously decrease the content of neurotoxin, β-ODAP.

Homoarginine is another non-protein amino acid in *Lathyrus sativus*. There are several different opinions about it. Breitner et al. [16] presented a theory that existence of homoarginine in gene activitor–repressor histones may be a direct cause of most cancers. It is also a modulator of nitric oxide (NO) which mediates glutamate neurotoxicity [17]. However, homoarginine has been considered as a



β-ODAP-DNB

Fig. 3. The presumed reaction leads to a decomposition of β-DNB–ODAP.

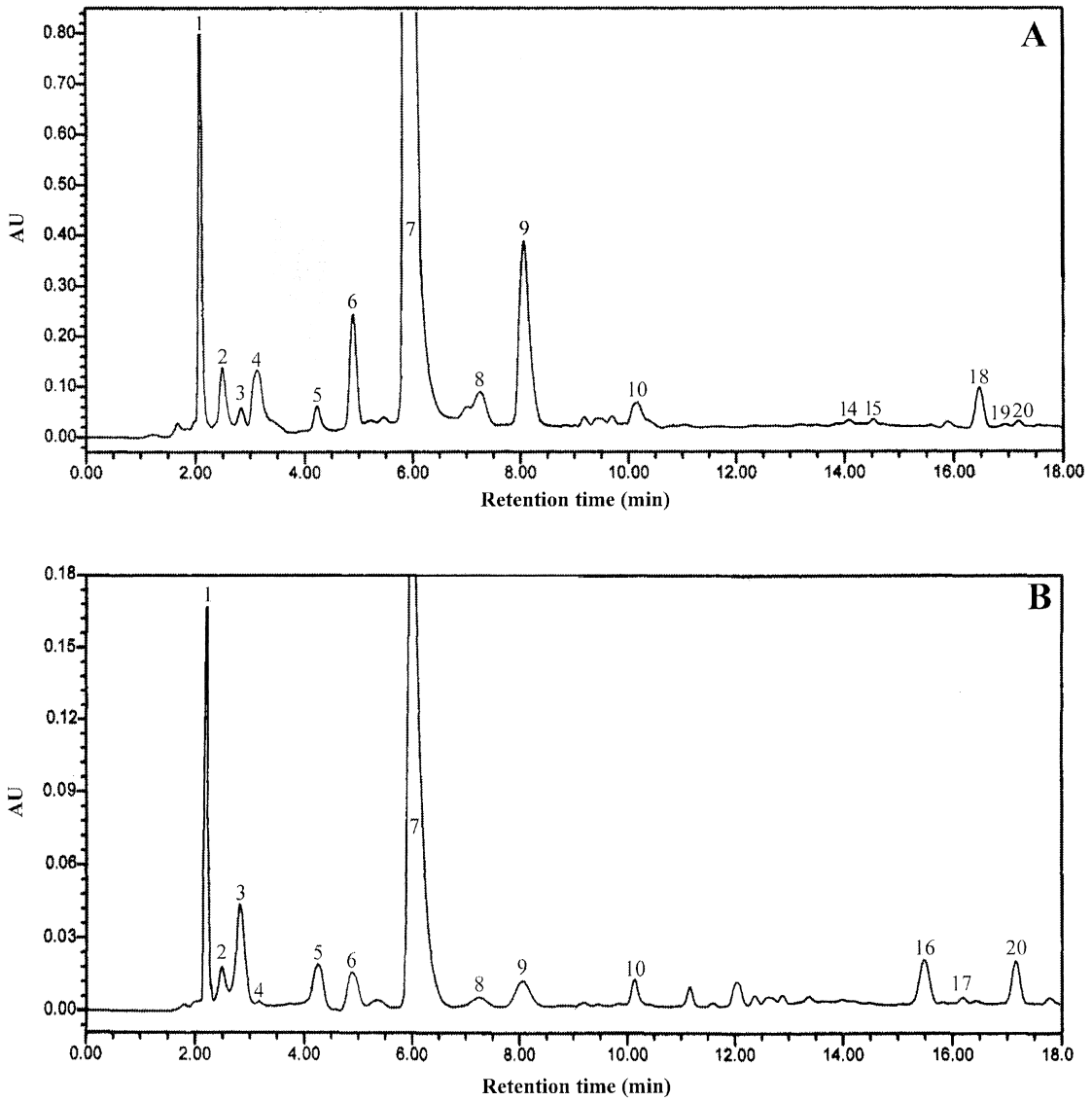


Fig. 4. Typical chromatograms of the FDNB-derivatized seed (A) and foliage (B) extracts. Sample preparation and chromatography are described in the text and Table 1. The column was thermostated at 26°C. Detection by UV at 360 nm. Peak numbers as in Fig. 1.

positive factor because it can be converted into the essential amino acid, lysine, by the mammalian liver. Thus homoarginine is also another important amino acid in *Lathyrus sativus*. From Fig. 4 of *Lathyrus salivus* samples, it can be found that the peak of homoarginine appears at 8.1 min. So the method also may be used to analyse homoarginine rapidly.

4. Conclusion

FDNB reagent is a useful alternative derivatizing reagent for the determination of β-ODAP and non-protein amino acids of *Lathyrus sativus*. The relatively short time for analysis and good sensitivity should make the method preferable over some of the

Table 2
Linear relationship, correlation coefficient, limit of detection, retention time (RT) and reproducibility for α- and β-ODAP in optimum conditions

Analytes	Linear relationship $Y = A + B \times X$[a]		Correlation coefficient ($n = 1$)	Limit of detection (pmol)
	A	B		
α-ODAP	−0.212	1.04×10^5	0.99916	10
β-ODAP	−0.289	1.05×10^5	0.99908	10
Analytes	RT(min)	RSD% for retention time[b]	RSD% for peak area[b]	
α-ODAP	2.55	0.93	3.09	
β-ODAP	2.16	0.82	2.47	

[a] X is peak area; Y is the concentration of analytes ($mol \cdot l^{-1}$).
[b] RSD is relative standard deviation.

Table 3
α- and β-ODAP content of the *Lathyrus sativus* seed and foliage samples

Method		Seed (w/w%)					Foliage (w/w%)				
		S1	S2	S3	S4	S5	F1	F2	F3	F4	F5
HPLC[a]	α-ODAP	0.073	0.021	0.011	0.005	0.015	0.022	0.005	0.013	0.007	0.006
	β-ODAP	0.752	0.334	0.147	0.073	0.137	0.349	0.097	0.253	0.104	0.119
OPA[b]	ODAP	0.80	0.34	0.16	0.08	0.14	0.35	0.10	0.25	0.11	0.12

[a] 20 μl injected, $n = 5$.
[b] OPA is the *o*-phthalaldehyde method (colorimetric method) ($n = 3$).

earlier methods used in studies of neurotoxin evaluation in *Lathyrus sativus*.

Acknowledgements

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