HPLC Determination of Neurotoxin β-N-oxalyl-L-α,β-diaminopropionic acid and Its α-Isomer 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 β-N-oxalyl-α,β-diaminopropionic acid (β-ODAP) and its much less toxic α-isomer (α-ODAP) 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 chromatographic method for the separation of the derivatives in the pmol range is reported.

Keywords: HPLC, *Lathyrus sativus*, 1-fluoro-2,4-dinitrobenzene, α-and β-N-oxalyl-α,β-diamino propionic acid, neurotoxin.

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 area of Africa and Asia. The crop is produced with minimum amount of care and successfully grown in a variety of climate. The edible seeds are very important nutrition since they contain 26-30% protein. However, excessive ingestion of *Lathyrus sativus* seeds can lead to a disease known as lathyrism. The lathyrism is caused by nonessential amino acid β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP)[1-3]. For this reason, its cultivation is prohibited in some places.

Our group is exploring to breed low or zero toxin varieties of *Lathyrus sativus* through cell culture and transgene technique and other methods. So it is very necessary to develop a fast and selective method to determine β-N-oxalyl-α,β-diamino propionic acid. The most common method utilizes the reaction of *o*-phthalaldehyde (OPT) with α,β-diaminopropionic acid (DAP) formed on hydrolysis of both β- and α-isomers of ODAP[4,5]. But it can not be used to analyze α- and β-ODAP, respectively. 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. Euerby *et al*[7]. separated ω-N-oxalyl diamino acids with *o*-phthaldialdehyde chiral thiols, using fluorescence detector, but α-ODAP was not mentioned and the procedures are time-consuming. Khan *et al*[8,9]. used 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]. In order to rapidly analyze α-and β-ODAP of *Lathyrus sativus*, respectively, we utilized precolumn derivatization with FDNB, followed by reversed-phase HPLC with UV detection. The method reported here is sensitive, reproducible and rapid, it is suitable for the determination of β-ODAP and its α-isomer in seed, foliage of *Lathyrus sativus* and animal tissue.

The HPLC system consisted of a Waters Model 600E pump, a Model 2487 dual wavelength absorbance detector set at 360 nm. The gradient was controlled by a Dell computer using Millennium 32 gradient manager software. Solvent A consisted of 0.03 mol/L K_2HPO_4 and 1% dimethyl formamide (DMF) in water (v/v) with varying pH (3.50-6.50) adjusted by phosphoric acid, solvent B was acetonitrile, solvent C was water. All separations were performed on Cartridge 5 μm C_{18} column 3.9×150 mm at ambient temperature 26℃ at a flow rate of 1 mL/min. In order to shorten analytical time and improved resolution of α- and β-ODAP-DNB, different pH values of mobile phase and gradient conditions were attempted. About 20 mg sample was accurately weighed and added to 2 mL of ethanol-water (3:7,v/v), shaken briefly and sonicated for 30 min, then left overnight, centrifuged for 15 min at 15,000g and filtered. A known amount of ODAP or sample extract was dried under vacuum. The residue was dissolved in 100μL of $NaHCO_3$ solution (0.5 mol·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℃. After cooled to room temperature, it was added with 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. To optimize the derivatization conditions, concentrations of FDNB and different derivatization times were studied.

The influence of buffer pH and percentage of acetonitrile is significant on retention time and resolution of α- and β-ODAP-DNB. Two simple gradient conditions were attempted. Standard ODAP derivative was injected into the column, α- and β-ODAP derivatives were eluted before 2,4-dinitrophenol (DNB-OH). The retention time of α- and β-ODAP-DNB in the two systems gradually decreased when pH of solvent A was increased. The retention time of α- and β-ODAP-DNB was decreased when the percentage of acetonitrile was increased at the same pH value of solvent A. According to the optimized result, all the subsequent experiments used A-B (85:15) with pH 5.50 of solvent A. To reduce the total elution time, different flush-times were selected. If it only needs to analyze α- and β-ODAP, the total elution time could be shorten after the product was injected to column and runned for 2 min. Then column was flushed with B-C (60:40) for 5min, followed by a 10 min equilibration with A-B (85:15) before the next run. Major free amino acids were eluted before 11.5 min, so the column was flushed at 11 min for analyzing most amino acids. A typical chromatogram of α- and β-ODAP derivatives is shown in **Figure 1**.

Figure 1. Chromatograph of α - and β -ODAP standard mixture derivatized with FDNB. Peaks: 1= β -ODAP; 2= α -ODAP; 3=DNB-OH.

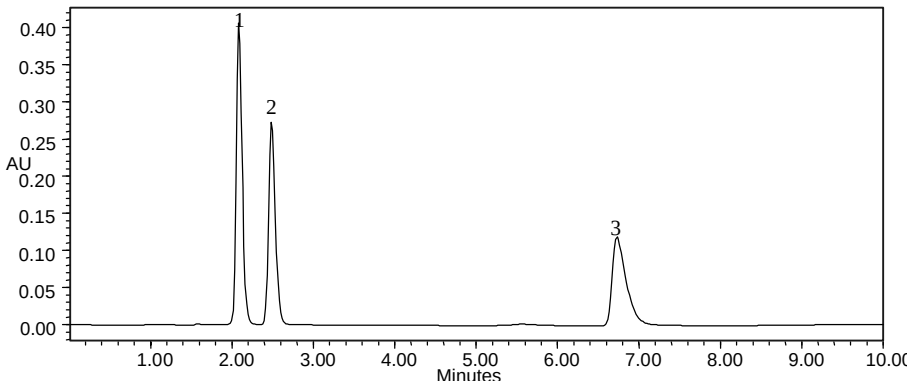
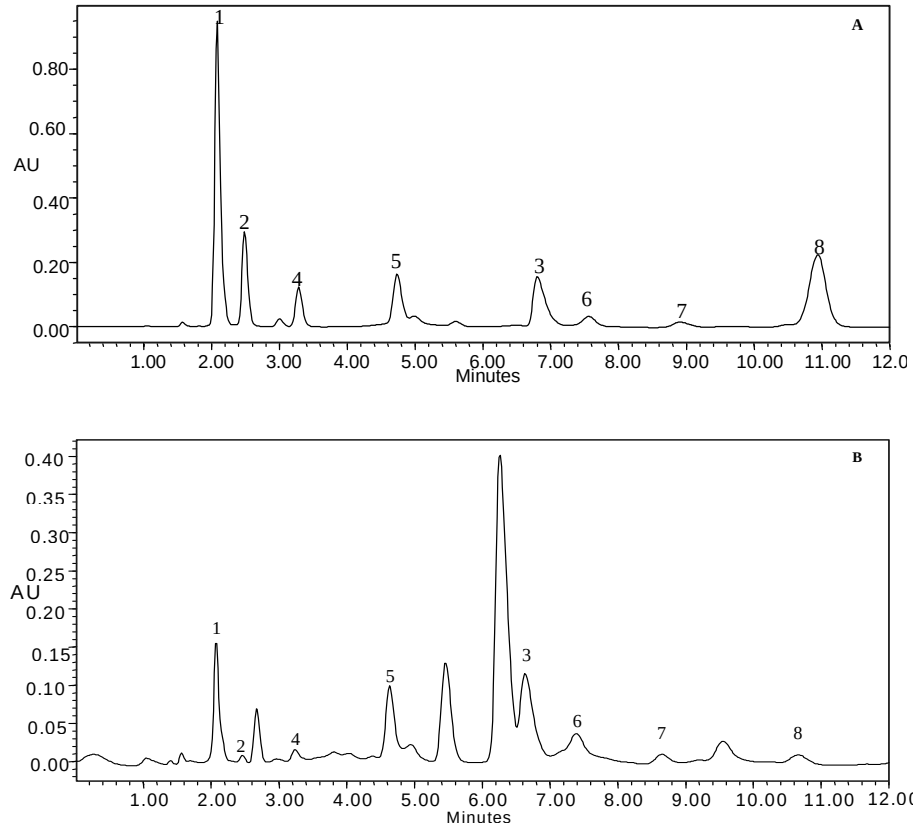


Figure 2. Typical chromatograms of the FDNB-derivatized seed(A) and foliage(B) extract. Peaks: 1= β -ODAP; 2= α -ODAP; 3=DNB-OH; 4=Glu; 5=Ser; 6=Arg; 7=Gly; 8= homoarginine.



The maxium percentage of reaction between ODAP and FDNB was reached for 30 min at 60℃. A very slow but measurable decrease in yield occurs as the reaction was allowed to proceed for longer time, presumably due to the hydrolysis of ODAP-DNB.

The amount of FDNB selected for derivatization was based on preliminary experiments which shown that molar concentration of FDNB should be at least 20 times than that of ODAP. Typical chromatograms of the derivatized seed and foliage extracts are shown in **Figure 2**. Homoarginine, α- and β-ODAP are major free amino acids in the dry seed. In the 30% ethanol extracts of foliage, the components are relatively complex.

Concentrations were determined by estimation of peak area with reference to calibration curve for derivatized α- and β-ODAP. Good limit of detection and reproducibility of analysis were obtained. Peak area for derivatized α- and β-ODAP were essentially unchanged for at least one week at room temperature, only DNB-OH peak area increased because of hydrolysis of FDNB.

The contents of α- and β-ODAP determined by HPLC method were compared with those by OPT method[5]. 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 β-ODAP to α-ODAP is accelerated by heating, the reported equilibrium concentrations ratio of α- and β-ODAP is between 35-40% to 65-60%[8], thus prolonged cooking of the *Lathyrus* seed can obviously decrease the content of β-ODAP.

The procedure described here can be considered as a useful alternative to the methods previously reported, with regard to its reliability and rapidity. It is suitable for the routine determination of *Lathyrus sativus* and related *Lathyrus* species available in formulations.

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