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Determination of Neurotoxin β-ODAP and Non-protein Amino Acids in *Lathyrus Sativus* by High-Performance Liquid Chromatography with Precolumn Derivatization with 6-Amino quinolyl-N-hydroxysuccinimidyl Carbamate (AQC)

Fei WANG[1,2], Xiong CHEN[2], Qian CHEN[1], Xing Chen QIN[2], Zhi Xiao LI[1]*

[1]National Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000
[2]State Key Laboratory of Arid Agroecology, Lanzhou University, Lanzhou 730000

Abstract: A new method was developed for the quantitative determination of the neurotoxic non-protein amino acid, 3-N-oxalyl-L-2,3-diaminopropionic acid (β-ODAP), its nontoxic α-isomer and other non-protein amino acids in the plant samples of *Lathyrus sativus* after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) by reversed-phase high-performance liquid chromatography (HPLC). 2-Amino butyric acid (ABA) was used as an internal standard. The RP HPLC detection limit for both isomers is 1.8 ng with good response linearity. The results are compared with a colorimetric method.

Keywords: RP HPLC, *Lathyrus sativus*, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate.

Lathyrus sativus (*L. sativus*) is leguminous crop cultivated in India, Bangladesh and Ethiopia, where it is a major protein source for people in the lowest income groups[1,2]. 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 non-protein amino acid, 3-N-L-oxalyl-2,3-diaminopropionic acid (β-ODAP, sometimes, referred to as BOAA)[3,4]. The α-isomer of ODAP, 2-N-oxalyl-2,3-diaminopropionic acid, has been shown to be nontoxic to chicks and mice[5].

L. sativus (shan li dou in China) shows good adaptation to the low rainfall conditions of northwestern China. Our group was exploring to breed low or zero toxin varieties of *L. sativus* as grain crops for human consumption and as protein-rich feed for animals. So it is necessary to develop a method to determine the toxin or other amino acids suitable for different purposes. The colourimetric method that used phthalaldehyde (OPT) is the most widely method for determining the neurotoxin in food and seed samples[6]. 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[7] in recent years. 9-Fluorenyl methylchloroformate (FMOC) derivatization, though suitable for detection of ODAP in the picomolar range in plant and animal tissues, can not differentiate between β-ODAP and α-ODAP[8]. Separation of α- and β-ODAP was achieved by Khan *et al*[9] using

reversed-phase HPLC, precolumn derivatization with phenyl isothiocyanate. However, excess coupling reagent has to be evaporated off before analysis.

Recently, Cohen and Michaud[10] developed a precolumn derivatization method in which a new reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), was used for amino acid derivatization. In order to determine the α- and β-ODAP in *L. Sativus* by AQC derivatization, we have developed a HPLC method that can simultaneously determine α-, β-ODAP and other non-protein amino acids. The method provides a simple accurate alternative to existing methods for plant screening purposes.

Amino acid standard (containing 2.5 μmol of seventeen amino acids) was obtained from Pierce (Rockford, IL USA). L-homoarginine was obtained from Sigma (St. Louis, MO, USA). Standard α- and β-ODAP was purified from *L. sativus*[11-13] and its purity was identified by capillary zone electrophoresis (CZE)[14]. 2-Amino butyric acid (ABA) 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, USA). Eluent A (concentrated sodium acetate buffer) was also obtained from Millipore. Water was supplied by a Milli-Q system from Millipore (Milford, MA, USA).

A known amount of sample was accurately weighed and added to 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 15,000 g) and subsequently filtered. A 10 μl of the sample extract or standard amino acid (with the internal standard added) was delivered to a 6×50 mm tube and buffered at pH 8.8 with 70 μl of 0.2mol/L sodium borate solution. The derivatives were formed with 20 μl of AQC in acetonitrile and heated for 10 min at 55 ºC.

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 254nm. Millennium 32 software, all from Waters, was used to control system operation and collect and analyze data. Mobile phase A was prepared by mixing 100ml of Eluent A (concentrated acetate buffer, 0.14 mol/L sodium acetate and 0.02 mol/L triethylamine at pH 5.02) with 1000 ml water. Mobile phase B was acetonitrile and mobile C was water. A ternary gradient system was used to analyze α-, β-ODAP and other non-protein amino acids (**Table 1**). The AccQ·Tag column was thermostated at 37 ℃and operated at a flow-rate of 1.0ml/min.

Table 1. Chromatographic gradient conditions for AQC-ODAP and non-protein amino acids analysis

Time(min)		0	0.5	18	19	29.5	33	36	50
	A(%)	100	99	95	91	83	0	0	100
Mobile	B(%)	0	1	5	9	17	60	60	0
Phase	C(%)	0	0	0	0	0	40	40	0

Figure 1A shows the chromatogram of α-, β-ODAP and other amino acids standard mixture derivatized with AQC. The α- and β-ODAP were eluted at 17.16min and 13.83min, respectively, and were not interfered with any of the compounds used. ABA was considered as internal standard, which was added to samples with different amino acid concentrations, showed that ABA gave very reproducible results. Therefore,

ABA could be used as the internal standard for quantitation. **Figure 1**B shows a typical chromatogram of the α- and β-ODAP and other non-protein amino acids in the dry seeds of *L. sativus* after AQC derivatization.

The HPLC detection limit for both isomers is 1.8 ng (signal:noise ratio=2:1) which, when taking the prepurification procedure into account, would give an apparent detection limit of 0.15μg/g in the *L. sativus* samples. 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 OPT method. Both results are in good agreement (**Table 3**). The accuracy of the method was also supported by measuring a known amount of α- and β-ODAP added to the seed samples. About 98% recovery was possible after extraction and derivatization.

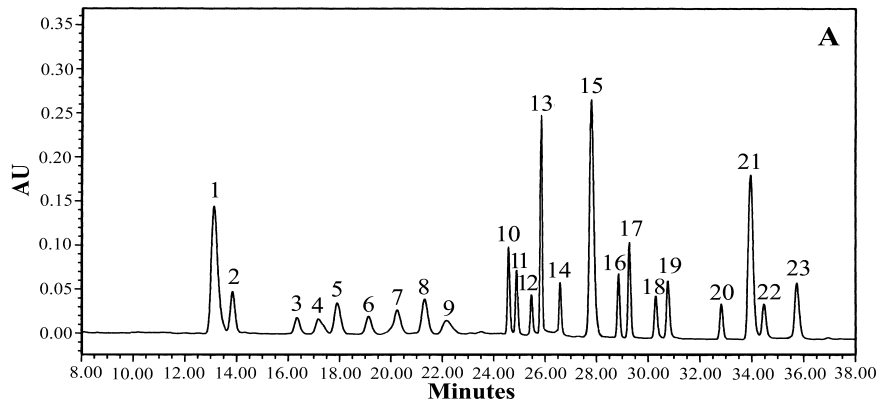
Table 2. Linear reponse (concn.=A+B×area) and Correlation Coefficient, Comparison of reproducibilities of α- and β-ODAP(n=7).

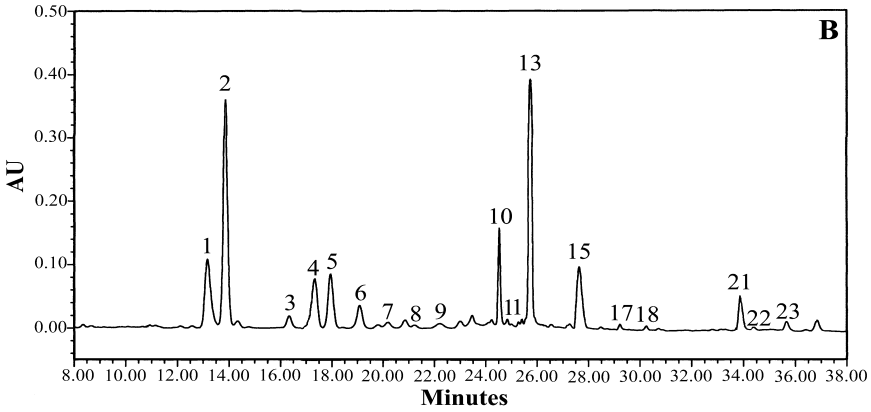
Compound	A	B×10^{-16}	r	R.S.D.	
				External calibration method	Internal calibration method
α-ODAP	3.86	6.85	0.9991	2.07	0.73
β-ODAP	5.79	6.86	0.9992	1.89	0.54

Table 3. α-and β-ODAP content in the *L. sativus* seed samples

Method	Compound (w/w, %)	Seed		
		1	2	3
HPLC	α-ODAP	0.07	0.08	0.07
	β-ODAP	0.31	0.39	0.48
OPT	ODAP	0.37	0.45	0.53

Figure 1. Chromatograph of (A) standard amino acid 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.

The α- and β-ODAP-AQC derivatives were easy to decompose when they were irradiated by UV light (254 nm). So the amino acid-AQC derivatives should be stored at 0ºC and refrained from light. The results showed the peak area was almost unchanged until five days after derivatization. When the derivatives were stored at -20ºC, it decomposed much more slowly and the peak area decreased insignificantly after one month.

In conclusion, AQC reagent is a useful alternative derivatizing reagent for determination of α-, β-ODAP and other non-protein amino acids of *L. sativus*. The method presented here gives an advantage which is important for research involved in pluse processing and preparation and toxin removal aspects of *L. sativus* and related *Lathyrus* species available.

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