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Analysis of Toxin β-N-Oxalyl-α, β-diaminopropionic Acid (β-ODAP), its Isomer α-ODAP and Other Free Amino Acids in *Lathyrus sativus*

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Abstract: A method has been presented for the simultaneous analysis of neurotoxin β-N-oxalyl-α, β-diaminopropionic acid (β-ODAP) and its non- or low-toxic isomer α-ODAP as well as other amino acids in *Lathyrus sativus* extracts after derivatization with *para*-nitrobenzyloxycarbonyl chloride (PNZ-Cl) by reversed-phase (C_{18} column) high performance liquid chromatography (RP-HPLC). Detection limits of isomers were 0.08 μg/mL for both β-, α-ODAP, for homoarginine 0.09 μg/ mL at a signal-to-noise ratio of 3:1.

Keywords: HPLC, β-N-oxalyl-L-α,β-diaminopropanoic acid, α-ODAP, homoarginine, PNZ-Cl.

Lathyrus sativus (*L. sativus*) is an annual leguminous crop for human and animal consumption. It can provide an economic yield under adverse environmental conditions and offer great potential for use in marginal low rainfall areas and may be the only affordable survival food for the poorer sections of the starved, which has made it a popular crop in subsistence farming in many developing countries with abominable weather[1]. However, development of *Lathyrus sativus* into an important food legume has been hindered by the presence of a neurotoxin—β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP), after large quantities or long-time consuming, which can cause irreversible paralysis known as lathyrism or neurolathyrism[1, 2].

The natural abundance of α-ODAP is approximately 5% of the total toxin in seeds of *L. sativus*, and in the animal studies showed α-ODAP to be less or non-toxic[3]. It is also documented that β-ODAP in solution can transform into α-ODAP *via* an unstable intermediate until an equilibrium mixture (3:2 ratio) was formed[4]. Therefore, it is important to quantify isomers β-, α-ODAP for the safety in the consumption of *L. sativus*.

Other key non-protein amino acid is L-homoarginine. In *L. sativus*, it has been reported that homoarginine can neutralise the toxic action of β-ODAP in 3 day-old chicks, this seems that the seed contains an antidote to its own toxin. Considered the potential role of homoarginine in lathyrism, it became essential to examine homoarginine presented in the ripe seeds or during the germination of *L. sativus*[5].

A few high-performance liquid chromatographic (HPLC) methods for ODAP have

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also been developed, including Euerby *et al.*[6] based on *o*-phthalaldehyde, Kisby *et al.*[7] and Geda *et al.*[8] with 9-fluorenylmethylchloroformate (FMOC), Khan *et al.*[9, 10] with phenyl isothiocyanate (PITC), Chen *et al.*[11] with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC), Wang *et al.*[12] with 1-fluoro-2, 4-dinitrobenzene (FDNB). However, the above HPLC methods suffered some disadvantages. For example, FMOC method [8] is nonselective for α-, β-ODAP; although Khan *et al*. separated successfully the two isomers in 1993[9], it is a pity that the peak of α-ODAP did not observe in 1994[10]; Although FDNB method as well as AQC method resolved the isomers in short time, however, the respective derivatives were formed after 30 min at 55°C for AQC, and at 60°C for FDNB. Besides, all these method have a common disadvantage, namely long derivatization time. Therefore, there is still a need for a selective, sensitive, simple, inexpensive and rapid HPLC method for the analysis of neurotoxin ODAP and other free amino acids in seeds and seedlings of *L. sativus*.

Para-nitrobenzyloxycarbonyl chloride (PNZ-Cl) were used to developing as selectively cleavable amino protecting group in peptide chemistry. In analytic chemistry, Brückner and Lüpke used it as reagent for amino acids analysis based on reversed-phase C_8 column[13]. In the present work, we proposed a method to resolve the neurotoxin β-ODAP and its α-isomer as well as other free amino acids with reversed-phase C_{18} column using PNZ-Cl. The derivative procedure is completed within 2 min at ambient temperature. Therefore, a simple, ideal and rapid HPLC method for determination of α-, β-ODAP, homoarginine and other amino acids in *L. sativus* was achieved.

A model LC-10ATvp liquid chromatograph (Shimadzu, Japan) was used throughout. ODAP and other amino acids derivatives were resolved on a 250×4.6 mm Luna-C_{18} column (5 μm) from Phenomenex, USA, which equipped a C_{18} guard column. The two solvent reservoirs containing (A) 0.1 mol/L acetate buffer (pH 4.4) and (B) acetonitrile (MeCN) were used to separate all the amino acids. The gradient program was as follows: 22 % B (0-2 min), 22 to 52 % B (2-6 min), 52 to 60 % B (6-20 min), 60 to 64 % B (20-26 min), 64 to 100 % B (26-30 min), 100 % B (30-35 min), 100 to 22% B (35-40 min). The column was then equilibrated for 12 min with 22 % B. The eluted derivatives were detected by monitoring their UV absorption at 260 nm. The column temperature was kept at 40°C and the flow-rate was fixed at 1.0 mL/ min.

About 100-200 mg of dry sample powder (3-8 days seedlings, the cotyledons were discarded) of *L. sativus* seeds was accurately weighed and soaked into 10 mL of ethanol-water (3:7, v/v), shaken briefly and sonicated for 1 h. After 22-24 h, the extracts were centrifuged at 15000 g for 10 min, and the supernatant was filtered through a 0.45μm membrane. The solution was derivatized immediately in the case of isomerization.

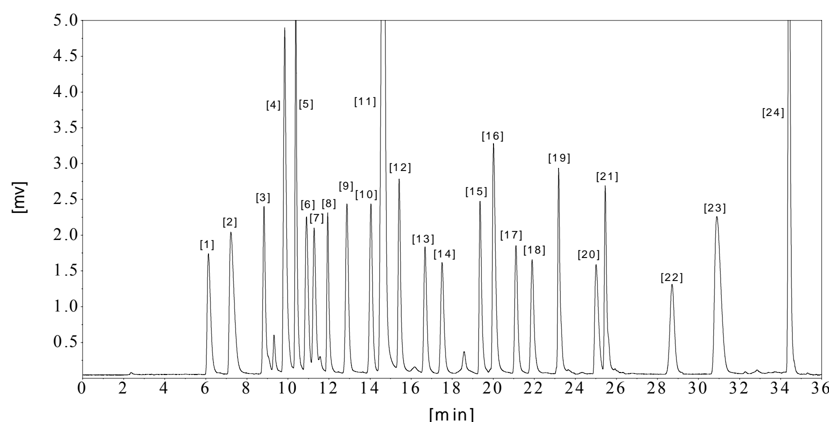
The stability of PNZ-Cl in acetonitrile, the influence of the pH of the mobile phase were studied, and various derivatization times, ultrasound irradiation and reaction temperature were also tested for assessing optimal reaction conditions. The best result was obtained within 2 min at ambient temperature under ultrasound irradiation using acetate buffer at pH 4.4.

Amounts of 150 μL of 0.5 μmol/L $NaHCO_3$, 30 μL ODAP standard mixture and 50

μL amino acids standard mixture, and 300 μL of PNZ-Cl solution (50 mmol/L) were mixed. The derivatization mixture was vortexed briefly and sonicated for 2 min at ambient temperature. In the case of sample, 50-150 μL of extract were used for derivatization, other procedure is in the same way as the standard mixture. The derivatization mixture was filtered and subjected to analysis.

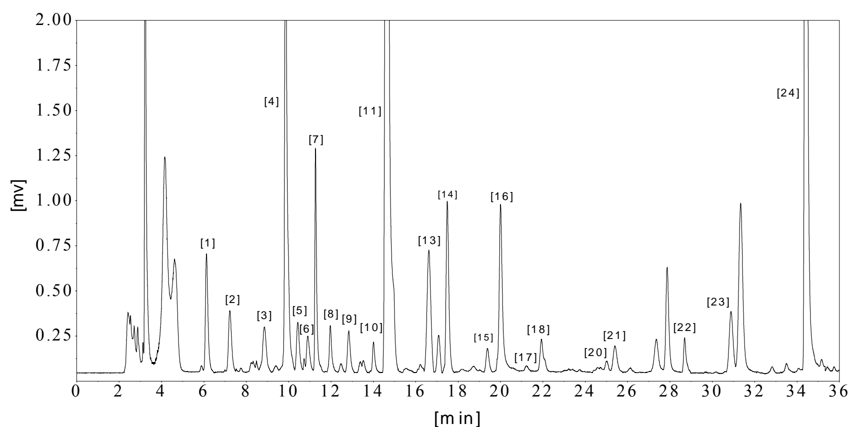
Each component was identified by spiking standards of β-, α-ODAP, homoarginine and amino acids. The derivatives of β-, α-ODAP, homoarginine were eluted at 9.8 min, 20.0 min and 28.7 min, PNZ-Cl at 34.4 min as the final component, respectively. The typical chromatography of standard mixtures is showed in **Figure 1**.

Figure 1 RP-HPLC chromatogram of standard mixture of amino acids derivatized with PNZ-Cl



Peaks: [1] = Asp; [2] = Ser; [3] = Arg; [4] = Homoariginine; [5] = Glu; [6] = Gly; [7] = Thr; [8] = Ala; [9] = Pro; [10] = Minosubstituted Tyr; [11] = PNZ-OH; [12] = Met; [13] = Unknown; [14] = Val; [15] =Phe; [16] = β-ODAP; [17] = Try; [18] = Ile; [19] = His; [20] = Cys; [21] = Lys; [22] = α-ODAP; [23] = Disubstituted Tyr; [24] = PNZ-Cl.

Figure 2 Chromatogram of the PNZ-derivatives from *L. sativus* seed



Peaks as in **Figure 1**

Table 1 The contents of β-, α-ODAP, homoarginine in seeds and seedlings of *Lathyrus sativus*.

Compounds	Seeds (mg / 1 g dry weight)				Seedling (mg / 1 g fresh weight)				
	I	II	III	IV	I	II	III	IV	V
β-ODAP	5.45	4.70	5.27	4.89	1.29	2.31	2.75	2.87	3.79
α-ODAP	0.687	0.551	0.792	0.563	0.177	0.278	0.293	0.291	0.482
Homoarginine	6.74	5.48	5.33	6.40	1.39	1.27	1.59	1.47	1.85

The content of β-, α-ODAP, homoarginine in seeds and seedlings of *L. sativus* are listed in **Table 1**, and typical chromatograms of seed sample is shown in **Figure 2.** The results showed that the proposed method was suitable for the simultaneous determination of β-, α-ODAP, homoarginine from the samples of *L. sativus*.

The new method shows the advantages in following aspects: the derivatization proceeds rapidly and quantitatively at ambient temperature, is suitable for processing a large amount of samples; PNZ-Cl reagent is rather stable to light and air, and can store in the freezer for several months. Further more, its sensitivity is as good as the previously reported methods.

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