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HPLC Determination of the Major Non-protein Amino Acids and Common Biogenic Amines in *Lathyrus sativus* Using a Novel Extraction Method

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Abstract: An assay is presented for simultaneously determining 5 biogenic amines and the major non-protein amino acids: the toxin β-N-oxalyl-L-α,β-diaminopropanoic acid (β-ODAP), its isomer α-ODAP and homoarginine in *Lathyrus sativus* extracts using the HPLC system after derivatization with *para*-nitrobenzyloxycarbonyl chloride (PNZ-Cl). However, it is more worthy of noting that this paper also describes a new extraction method using 0.2 mol/L $HClO_4$. The new method has some advantages: shorter extraction-time, simultaneous extraction of free amino acids and polyamines, better inhibiting the isomerization of β-ODAP to α-ODAP, and so on.

Keywords: ODAP, homoginine, HPLC, biogenic amines, new extraction method.

Lathyrus sativus (*L. sativus*) which is known as Shan li dou in China, is cultivated as a drought tolerant food crop requiring minimal care in semi-arid areas of Africa, Asia and Europe. But, 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) in seeds, if consumed in large quantities and long-time, which can cause irreversible paralysis known as lathyrism or neurolathyrism[1-2]. In addition, it is also reported that β-ODAP in solution undergoes a conversion to non-toxic α-ODAP *via* an unstable intermediate[3-4]. Because the β-isomer can be isomerized to the non toxic α-isomer, the explored various processing and cooking methods were as a means of thermal detoxification of the legume. Therefore, it is essential to quantify isomers β-, α-ODAP in order to lead to *L. sativus* consumption at a relatively safe level for human food and animal feed. Other key non-protein amino acid in the seeds is L-homoarginine, in *Lathyrus sativus*, which has been reported that homoarginine can neutralize the toxic action of β-ODAP in 3 day-old chicks[5]. Considering the potential role of non-protein amino acids in the biosynthesis of *Lathyrus* toxins as well as in lathyrism, it is essential to examine the major free amino acids presented in the ripe seeds and the seedlings of *L. sativus.*

In higher plants, biogenic amines (BAs) have been implicated in molecular signaling events in plant pathogen interactions, which involve in plant response to

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microbial symbionts, and are important in plant nutrition[6]. As we known, major shifts in nitrogen and BAs metabolism can occur when plants are starved for nutrients, or exposed to osmotic shock or atmospheric pollutants. Further, as reported that during the development of *L. sativus* seedling, substantial amounts of BAs including agmatine, putrescine, cadaverine, spermidine and spermine, were accumulated progressively[7]. To clarify the biological function of biogenic amines and the relationship between metabolism of BAs and accumulation of toxin ODAP, it is essential to develop a simple and sensitive assay for quantifying these compounds in different developing stage of *L. sativus*.

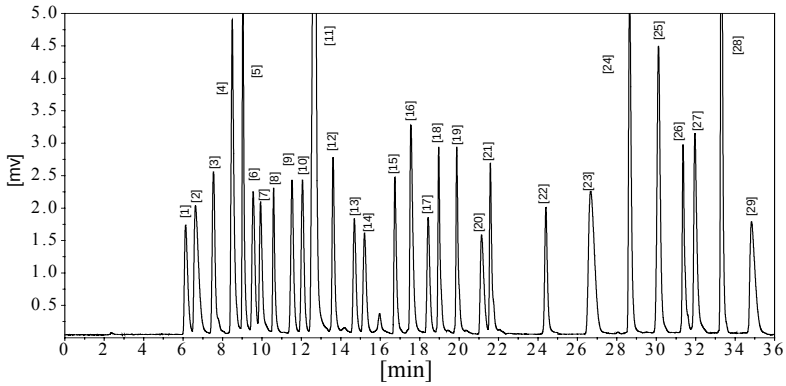
Recently, HPLC methods[8-12] have widely been used to quantify β-ODAP and its isomer. These methods were specific, convenient and sensitive, but they also suffered some drawbacks. *Para*-nitrobenzyloxycarbonyl chloride (PNZ-Cl) was used as selectively cleavable amino protecting group in peptide chemistry. Brückner and Lüpke[13] used it as reagent for analysis of amino acids based on reversed-phase C_8 column. However, to our best knowledge, no method was proposed to simultaneously determine β-, α-ODAP, homoarginine and biogenic amines ODAP by pre-column derivatization with PNZ-Cl.

In the present work, we found that PNZ-Cl was a suitable reagent for quantitative determination of biogenic amines and the major non-protein amino acids presented in *L. sativus*: the neurotoxin β-ODAP, its α-isomer and homoarginine by reversed-phase C_{18} column. The derivative procedure was completed within 6 min at ambient temperature under ultrasound irradiation. Compared with the extraction method before, the new method gave more satisfactory results. Furthermore, due to the isomerization between β-ODAP and α-ODAP, the content of α-ODAP increased. The natural abundance of α-ODAP and β-ODAP can not be accurately quantified. Based on the fact that ODAP and BAs easily dissolve in acid solution, we explored a new extraction method. The results exhibited that the proposed assay can more accurately quantify β-ODAP and α-ODAP.

For HPLC a Model 10ATvp series was used in experiments (Shimadzu, Japan). Non-protein amino acids and biogenic amines derivatives were resolved on a 250×4.6 mm Luna-C_{18} column (5 μm) from Phenomenex, USA, equipped a C_{18} guard column. The two solvent reservoirs containing (A) 0.1 mol/L sodium acetate buffer (pH: 4.6) and (B) acetonitrile (MeCN) were used throughout. The elution program was as follows: 20 to 28 % B (0-3 min), 28 to 50 % B (3-25 min), 50 to 100 % B (25-35 min), 100 % B (35-40 min), 100 to 20 % B (40-48 min). The column was then equilibrated for 12 min with 20 % B. The eluted PNZ-Cl derivatives were detected by their UV absorption at 260 nm.

Conventional procedure for extraction of β-, α-ODAP of *L. sativus* samples is under ethanol-water solution in the ratio 3:7 to 7:3 (V / V) for about 24 h. Conventional extractions for a period of 24 h in ethanolic solution, will inevitably lead to the contents of α-ODAP were increased in the extraction period. We found that perchloric acid solution can perfectly extract β-, α-ODAP and homoarginine as well as biogenic amines of *Lathyrus sativus* samples, preventing the transformation of β-ODAP to α-ODAP. A typical chromatogram of β-, α-ODAP, homoarginine, common amino

Figure 1 RP-HPLC chromatogram of a standard mixture of major non-protein amino acids and 5 biogenic amines detected by UV at 260 nm.



Peaks: [1] = Asp; [2] = Ser; [3] = Arg; [4] = Homoarginine; [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] = Agmatine; [25] = Spermine; [26] = Putrescine; [27] = Cadaverine; [28] = PNZ-Cl; [29] = Spermidine.

Figure 2 Typical chromatogram of PNZ-derivatives from *L. sativus* seed. Peaks as in **Figure 1**.

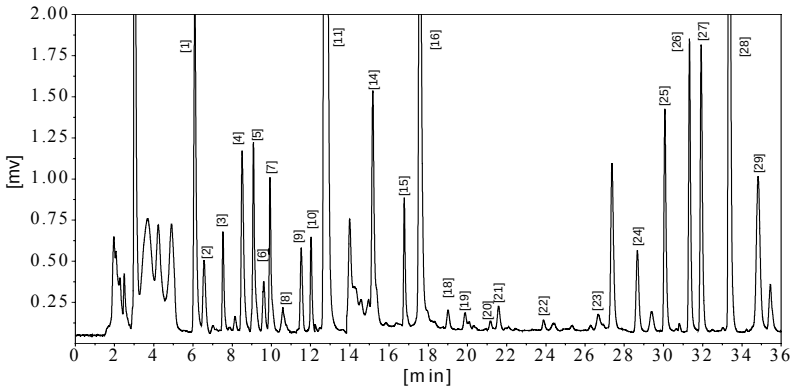


Table 1 Contents of β-, α-ODAP, homoarginine in *Lathyrus sativus via* two extraction methods.

Extraction	Compound	Seeds (mg / 1 g dry weight)				Seedling (mg / 1 g fresh weight)				
		I	II	III	IV	I	II	III	IV	V
ethanol	β-ODAP	5.32	4.98	4.76	5.67	2.39	3.85	2.97	1.27	2.62
	α-ODAP	0.794	0.599	0.562	0.703	0.289	0.497	0.306	0.173	0.306
	Homoarginine	6.57	5.61	5.19	6.88	1.89	1.92	1.76	1.52	1.46
$HClO_4$	β-ODAP	5.95	5.39	5.04	6.14	2.58	4.07	3.21	1.35	2.86
	α-ODAP	0.280	0.302	0.265	0.333	0.139	0.267	0.181	0.085	0.153
	Homoarginine	6.72	5.65	5.17	7.05	1.99	1.85	1.73	1.50	1.58

acids and five biogenic amines is shown in **Figure 1**. It is very evident that all the protein amino acids do not interfere with the determination of non-protein amino acids and biogenic amines.

Our results also showed that the total content of ODAP is in good agreement with both methods (**Table 1**). **Figure 2** demonstrated a typical chromatogram of the derivatized extract of *L. sativus* seedling using the proposed method.

In conclusion, the proposed method effectively prevented the conversion between the two isomers, and is very suitable for quantification the natural abundance of β-, α-ODAP. On the one hand, under the strong acid condition, β-, α-ODAP react rapidly with $HClO_4$ to form the corresponding perchlorate and prevent the isomerization of β-ODAP to α-ODAP. The developing method is suitable for processing a large amount of samples, because the extracts were stable at storing for two weeks at 4°C, even at room temperature. However, as the traditional method, the ethanol-water extract solution must be derivatized immediately, otherwise higher content of α-ODAP is inevitable. Finally, the new method can simultaneously extract β-, α-ODAP, homoarginine and biogenic amines within 1 h, while the conventional method needs 22-24 h. Therefore, the new PNZ-Cl assay was considered a simple, efficient, rapid method that can be applied directly to the simultaneous determination of β-, α-ODAP, homoarginine and biogenic amines of extracts from *L. sativus* seedlings and seeds.

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