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Analysis of β-*N*-oxalyl-L-α,β-diaminopropionic acid and homoarginine in *Lathyrus sativus* by capillary zone electrophoresis

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Abstract

A simple capillary zone electrophoresis (CZE) method has been developed for the simultaneous quantitative determination of β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) and homoarginine in *Lathyrus sativus* (LS; grass pea). A new $Na_2B_4O_7$–Na_2SO_4 run buffer was used and the pH was 9.20, contents of β-ODAP and homoarginine in crude extracts of LS plant material were determined with this method, the RSDs of peak areas of β-ODAP and homoarginine were 2.62% and 3.61%, respectively. It was found that the equilibrium concentration ratio of α- and β-ODAP decreased from 34.5/65.5 to 28.6/71.4 when the pH of the solution increased from pH 3.0 to pH 11.0.

Keywords: *Lathyrus sativus*; Plant material; Oxalyldiaminopropionic acid; Homoarginine; Arginine; Amino acids; Neurotoxin

1. Introduction

With the uncontrolled increase of the population, the short supply of water resources and the more frequent occurrence of disasters, the problem of foodstuffs has become critical in some regions. *Lathyrus sativus* (LS; grass pea) is a popular drought- and flood-tolerant crop and foodstuff in drought-prone areas of northwest China, India, Pakistan, Ethiopia etc. The seeds of LS may be the only food available during periods of famine. However, the excessive consumption of seeds of LS can cause a neurological disorder (lathyrism), which is believed to be caused by the non-protein amino acid β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) [1]. The disease sometimes reaches epidemic proportions following drought- and flood-triggered famine in those areas. Homoarginine is another non-protein amino acid in LS [2]; it has been considered as a positive factor because it can be converted into lysine by the mammalian liver. However, it is also a precursor of nitric oxide (NO) which mediates glutamate neurotoxicity [3]. And Breitner [4] presents a theory that presence of homoarginine in gene activator–repressor histones may be the direct cause of most cancers. So the concentration level of these two compounds in LS is very important. In dry seeds of LS, β-ODAP and homoarginine are major free amino acids, which together make up over 90% of the ninhydrin-reacting compounds in the 70% ethanol extracts [5]. The level of both compounds in the dry seeds varies widely depending on genetic factors and environmental conditions.

Intensive research for developing low- or zero-

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toxin varieties of LS seeds is going on in several institutes focusing on agricultural reserves. The most obvious option to achieve this goal entails plant breeding and post-harvest processing. This involves processing of a large number of samples, requiring a fast and selective method for monitoring the toxic compounds. The most widely used method for determining the neurotoxin utilizes the reaction of *o*-phthaladehyde (OPA) with 2,3-diaminopropionic acid (DAP) formed on hydrolysis of ODAP and measurement of the absorbance at 420 nm [6,7], since free DAP is not a natural constituent of LS seeds. But this method is nonselective between β-ODAP and its innoxious isomer α-*N*-oxalyl-L-α,β-diaminopropionic acid (α-ODAP). A few HPLC methods for ODAP have been developed in recent years, including dansyl-Cl [8] and 9-fluorenylmethyl chloroformate [9] derivatization methods which do not differentiate between the two isomers. Separation of α- and β-ODAP was achieved by HPLC [10], based on precolumn phenyl isothiocyanate derivatization, this method also can determine the concentration of homoarginine. All these methods are based on inconvenient and time-consuming off-line pretreatments. Selective and speedy determination of β-ODAP could be achieved by capillary zone electrophoresis (CZE) [11] or a LC–biosensor method [12], but both these methods could not determine homoarginine at the same time. This paper presents a new CZE method for the determination of homoarginine, α- and β-ODAP simultaneously. The method is sensitive, fast, and simple.

2. Experimental

2.1. Instruments

CZE has been carried out using a Waters Quanta 4000 system (Waters, Milford, MA, USA) and UV detecting at 185 nm. The temperature is 22±1°C. Separation was performed in uncoated fused-silica capillaries manufactured by Waters Accasep. Capillaries of 55 cm (47.4 cm effective length)×75 μm I.D., were used. Samples were introduced from the anodic end of the capillary by hydrostatic injection where the sample vial was raised by 10.0 cm for 5 s. Data acquisition was carried out with a Maxima820 chromatography workstation. A 1 min wash cycle with 0.5 *M* NaOH solution followed by 1 min distilled water and 1 min separation buffer was necessary to condition the capillary.

2.2. Reagent

LS and its plant material were obtained from the State Key Laboratory of Arid Agroecology, Lanzhou University, Lanzhou, China. L-Homoarginine, L-arginine, L-glycine, L-alanine, L-tyrosine and L-phenylalanine were obtained from Sigma (St. Louis, MO, USA). β-ODAP was extracted from LS as in the method of Ref. [13]; in most cases this method resulted in a mixture of α-ODAP and β-ODAP (Fig. 1) rather than in pure β-ODAP because the isomerization of β-ODAP in aqueous solution. At higher temperatures, the reaction speed of the transformation of β-ODAP into α-ODAP considerably accelerated, the reaction speed at 95°C was 84 times faster than at 75°C [14]. Thus to keep the temperature of the ODAP solution low in the process of the preparation of β-ODAP, and decrease the heating time in the recrystallization step would help to bring about a pure β-ODAP.

The structures of homoarginine, arginine, α-ODAP and β-ODAP were shown in Fig. 2.

2.3. Sample extraction

0.020 g powder of LS seeds or its plant material was soaked with 2 ml ethanol–water (30:70, v/v) solution and shaken for 24 h. After ultracentrifugation, the upper clear solution was injected directly into the CZE system.

2.4. Preparation of electrolyte

Buffers were mixed from 0.10 *M* $Na_2B_4O_7$ and 0.10 *M* Na_2SO_4 solutions and diluted with distilled water; the required pH of buffer was adjusted by H_3PO_4. Unless otherwise specified, all chemicals were of analytical-reagent grade.

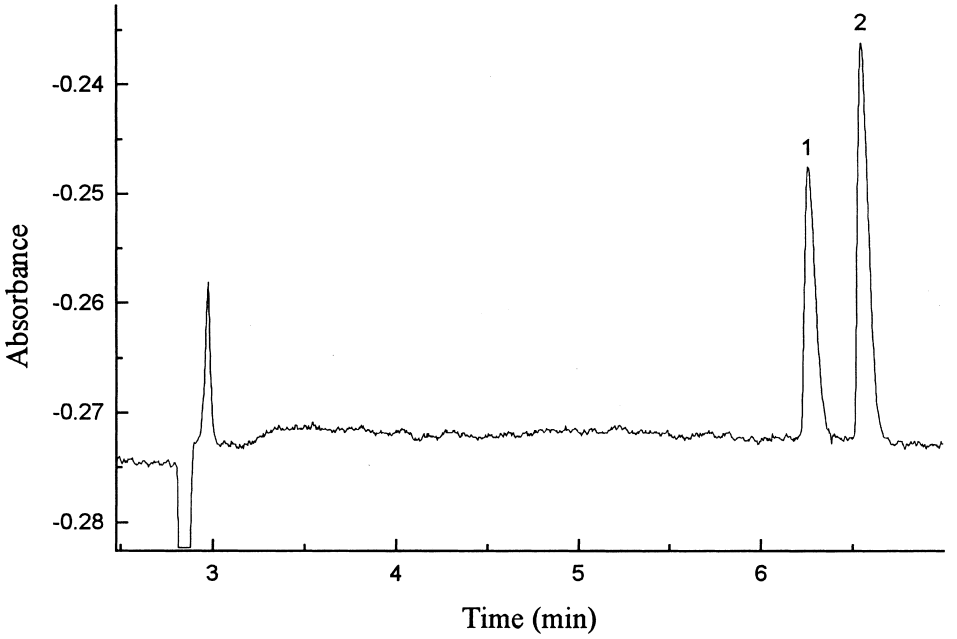
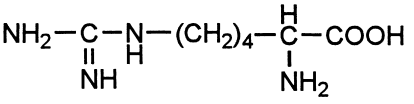
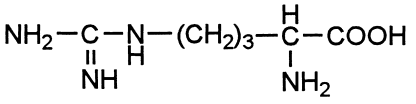


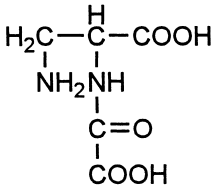
Fig. 1. Electropherogram of aqueous solution of ODAP prepared by the method of Ref. [13]. Concentration of borate 0.0010 *M*, Na_2SO_4 0.005 *M*, pH 9.2, applied voltage: 21 kV, UV detection at 185 nm. Peaks: 1=α-ODAP; 2=β-ODAP.



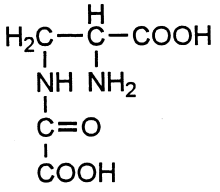
Homoarginine



Arginine



α-ODAP



β-ODAP

Fig. 2. Structures of homoarginine, arginine, α-ODAP and β-ODAP.

3. Results and discussion

3.1. Calculations

The separation efficiency was calculated by:

$$N = 5.54 \left(\frac{t_R}{w_{1/2}} \right)^2 \quad (1)$$

where N is the theoretical plate number, t_R is the migration time of analyte, $w_{1/2}$ is the peak width at half height.

3.2. Effect of different buffers and pH

Solutions such as barbiturate, acetate, Tris–H_3PO_4, phosphate etc. were investigated as buffers. All of these buffers have strong UV absorption at 185 nm except borate. So the borate was selected as buffer. With increasing of the borate concentration, the background of UV absorption enhanced, and hence the sensitivity decreased, so the concentration of the borate buffer in this paper was kept at 10 mmol/L.

In the pH range examined, there were positive charges on the arginine and homoarginine molecules, and negative charges on α- and β-ODAP. A marked positive and negative mobility could be observed. Experimental results showed that α- and β-ODAP are separated well and show satisfactory peak shapes at pH 7.8; however, the peaks of arginine and homoarginine overlapped at this pH. When the pH increased from pH 7.8 to pH 10.0, the peaks of homoarginine, arginine, α- and β-ODAP tail. At pH 8.50, α- and β-ODAP cannot be separated, and the peaks of arginine and homoarginine begin to be separated into two peaks, but not completely. At pH 9.20, α- and β-ODAP separated completely although their peaks are tailing; arginine and homoarginine are relatively separated better. When the pH increased, the difference in mobilities of arginine and homoarginine decreased, and their separation was declined. So pH 9.20 was selected as the optimal buffer pH.

3.3. Effect of ion strength of buffer

To obtain a better separation of homoarginine and arginine, we tried to increase the ion strength of the buffer, because it was found that when the concentration of buffer increased, peak tailing of homoarginine and arginine decreased, and they separated better. To increase the ion strength and not change the pH of the buffer, the only way is to add another salt into the buffer. Solutions such as KCl, NaCl, $NaNO_3$, KNO_3, Na_2SO_4, etc. have been tried to add into buffer, the former four salts have a strong UV absorption, only Na_2SO_4 has a very low UV absorption, and Na_2SO_4 was added into buffer in concentration from 5 mmol/L to 25 mmol/L. The relation between the numbers of theoretical plates for homoarginine and β-ODAP and the concentration of Na_2SO_4 is shown in Fig. 3. It was shown that when the ion strength increased, the numbers of theoretical plates of homoarginine increased, but for β-ODAP the numbers of theoretical plates decreased. The choice of 10 mmol/L borate and 5 mmol/L Na_2SO_4 as the buffer was a compromise of the experimental results.

3.4. Applied voltage

A higher applied voltage resulted in a lower migration time, and also the peak broadened due to the excess Joule heat and diffusion. 21 kV was selected as the optimized applied voltage in the method presented.

3.5. Peak identification

Peaks were identified by the addition of standard amino acids to the sample solution: by the increase of peak area the amino acid peak could easily be identified. The peak of α-ODAP was identified by adding a mixture of α- and β-ODAP to the sample solution, except the peak of β-ODAP which have been identified, another area increased peak is α-ODAP. Or it could be identified by the peak area increase of α-ODAP after the sample solution was heated for several hours. Because α- and β-ODAP have an equilibrium concentration ratio of about 35:65, but in LS sample, the concentration of α-ODAP never exceeded 5% of the total concentration of α- and β-ODAP, however, undergoes a slow transformation from β-ODAP to α-ODAP in water at room temperature. A higher temperature will accelerate this transformation process.

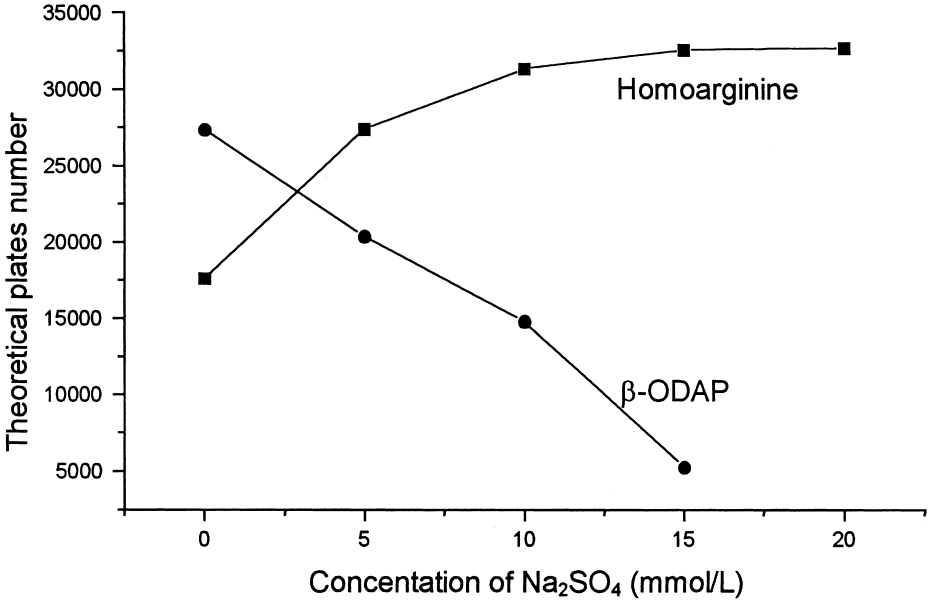


Fig. 3. Influence of concentration of Na_2SO_4 (mmol/L) in buffer on the theoretical plate number. Concentration of borate 10 mmol/L, pH 9.2, applied voltage: 21 kV.

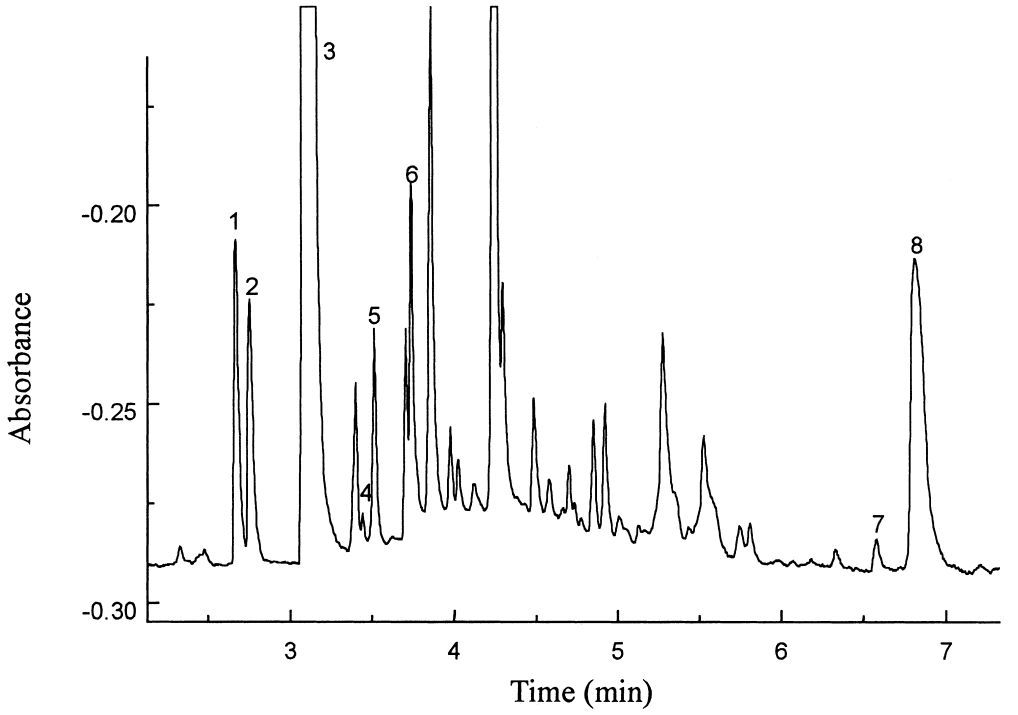


Fig. 4. Electropherogram of 30% (v/v) aqueous ethanol extract of dried seedling of LS. Concentration of borate 0.0010 *M*, Na_2SO_4 0.005 *M*, pH 9.2, applied voltage: 21 kV, UV detection at 185 nm. Peaks: 1=homoarginine; 2=arginine; 3=ethanol solvant; 4=alanine; 5=glycine; 6=phenylalanine and tyrosine; 7=α-ODAP; 8=β-ODAP.

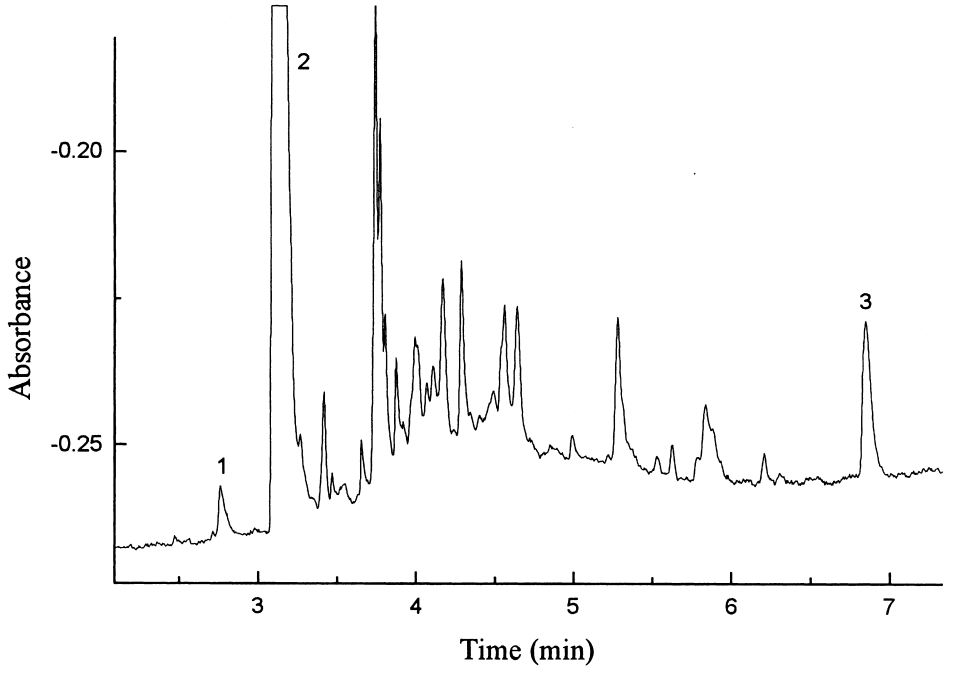


Fig. 5. Electropherogram of 30% (v/v) aqueous ethanol extract of leaf powder of LS. Concentration of borate 0.0010 *M*, Na_2SO_4 0.005 *M*, pH 9.2, Applied voltage: 21 kV, UV detection at 185 nm. Peaks: 1=arginine; 2=ethanol solvant; 3=β-ODAP.

Typical electropherograms were shown in Figs. 4 and 5. From the electropherogram of the LS sample, it can be seen that the peak of homoarginine appears first, and the peak at the end is β-ODAP. At the optimal buffer pH 9.2, other amino acids such as asparagine and serine etc. were added to the LS sample, the migration time of asparagine was longer with these amino acids present, but much shorter (2 min) than that of β-ODAP. The contents of these amino acids were so low that the UV detector could not detect them. In addition, the possibility of co-eluting peaks with β-ODAP and homoarginine was excluded by a recovery test. So there are no amino acids or other unknown substances in samples interfering with the separation of homoarginine and β-ODAP at optimal separation conditions.

3.6. Linearity and reproducibility

A mixture of α- and β-ODAP was used to get the curve of α-ODAP because of a lack of pure α-ODAP; the concentration ratio of α- and β-ODAP of the mixture was determined by NMR spectroscopy [15,16].

A linear relationship between the concentration of homoarginine, α- and β-ODAP and the corresponding peak area was found. The result was shown in Table 1. The reproducibility of analytes [relative standard deviations (RSDs) of relative migration times and peak areas], limits of detection, and concentration ranges were also shown in Table 1.

4. Applications

4.1. Analysis of LS samples

This method was applied to the separation and determination of homoarginine and β-ODAP in the leaf, seedlings and seeds of LS. The typical results of different samples were listed in Table 2. It was apparent that the concentration of homoarginine in LS leaf was much lower than that in LS seeds, the concentration of β-ODAP in the seedling significantly increased compared with that in the seeds;

Table 1
Linear relationships, correlation coefficients, limits of detection, concentration ranges, migration times and reproducibilities for the three analytes under optimum conditions

Analytes	Linear relationship[a] $S=A+B\times C$		Correlation coefficient	Limit of detection ($\mu g\ ml^{-1}$)	Concentration range ($\mu g\ ml^{-1}$)	Migration time (min)	RSD (%) for migration time ($n=5$)	RSD (%) for peak area ($n=5$)
	A (mV s)	B (mV s/$\mu g\ ml^{-1}$)						
Homoarginine	$0.26\cdot10^2$	$1.50\cdot10^2$	0.9985	1.5	4–200	2.57	0.89	3.61
α-ODAP	$2.26\cdot10^2$	$2.91\cdot10^2$	0.998	2.5	5–90	6.28	0.71	3.37
β-ODAP	$1.99\cdot10^2$	$3.25\cdot10^2$	0.9998	2.5	5–600	6.70	0.92	2.62

[a] S is peak area (mV·s); C is the concentration of analytes ($\mu g\ ml^{-1}$).

Table 2
Assay results of LS sample

Sample[a]	Homoarginine (%, w/w)	β-ODAP (by CZE method) (%, w/w)	β-ODAP (by OPA method) (%, w/w)
1	0.01	0.47	0.46
2	0.02	0.57	0.54
3	1.06	0.47	0.45
4	0.43	0.58	0.56
5	0.32	0.16	0.18
6	1.02	0.53	0.54
7	0.90	1.95	–
8	1.00	2.27	2.51
9	0.11	0.25	–
10	0.12	0.27	–

[a] Samples 1 and 2 were powder of LS leaf of different periods of growth. Samples 3–6 were powder of LS seeds. Samples 7 and 8 were dried seedling of seed of sample 6 that has been grown for three days. Samples 9 and 10 were fresh seedling of samples 7 and 8.

however, the concentration of homoarginine was nearly unchanged from seeds to seedling. Also, the contents of β-ODAP determined by CZE was compared with that using the OPA method [6] as shown in Table 2. The results by the two methods were consistent.

4.2. Thermal isomerization of β-ODAP

In the aqueous solution of β-ODAP, there exists a slow transformation from β-ODAP to its nontoxic isomer α-ODAP, so thermal isomerization is one of the detoxification methods used. Using NMR spectroscopy, Abegaz [16] found that the equilibrium concentration ratio of β-ODAP and α-ODAP is 3/2 at 55°C; the transformation reaction from β-ODAP to α-ODAP in solution of β-ODAP (pH 2.3) was quicker than in solution of sodium salt of β-ODAP (pH 6.6). The CZE method in this paper was employed to detect the concentration ratio of β-ODAP and α-ODAP. The thermal isomerization of β-ODAP has been studied in buffers of various acidities at 55°C, the results were shown in Table 3

It was shown in Table 3 that not only the transformation reaction is slower at higher pH, but also the equilibrium concentration ratio of α- and β-ODAP was different at different acidity. The equilibrium concentration ratio of α- and β-ODAP decreased when the pH increased. In the cooking process, the pH of food made by LS seeds is around 7, so it is important to use the equilibrium ratio at pH 7.0 to calculate the efficiency of thermal isomerization detoxification.

Table 3
Experimental results of thermal isomerization of β-ODAP

Heating time (h) at 54.2°C	Concentration ratio of α-ODAP (%, w/w)		
	pH 3.0	pH 7.0	pH 11.0
0	0.5	0.5	0.5
7	12.8	11.4	10.7
80	34.6	31.1	24.6
136	34.5	31.0	28.6

5. Conclusions

The CZE method could conveniently determine the concentration of homoarginine, α- and β-ODAP in crude extracts of LS. The simple and fast method brought less pollution to the environment than the traditional methods [6]. The method could also be employed to determine thermodynamic constants of the thermal isomerization of β-ODAP because the concentration ratio of α- and β-ODAP in aqueous solution could be measured expediently by this method.

Acknowledgements

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