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Identification and quantification of natural isoxazolinone compounds by capillary zone electrophoresis ☆

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Abstract

A capillary zone electrophoresis (CZE) method that is specific, simple, rapid and also cheap was developed to analyse some natural UV-absorbing isoxazolinone compounds with toxic potential present in legume seedlings. The six most common natural isoxazolinone compounds were separated within 10 min with 25 m*M* potassium phosphate (pH 7.5) containing 8% 1-propanol as running buffer. A 60 cm coated fused-silica capillary (52.6 cm effective length×75 μm I.D.), with an electric field of 375 V/cm at 30°C was used. The limit of detection ranged from 0.01 m*M* (3.0 μg/ml) to 0.03 m*M* (7.7 μg/ml). Linearity between peak areas and concentrations ranging from 0.05 m*M* to 1.75 m*M* were determined for each isoxazolinone. The correlation coefficient was 0.9954 or greater. Both relative migration time and peak area were reproducible. The RSD of relative migration time is between 0.44 and 1.94% and RSD of peak area is between 1.26 and 6.86%. The concentrations of isoxazolinones in *Lathyrus odoratus* and *L. sativus* seedlings obtained by CZE were in agreement with the previous results from HPLC.

Keywords: *Lathyrus* spp.; Plant materials; Isoxazolinones; Amino acids

1. Introduction

Legume seeds and seedlings are rich in secondary metabolites especially nonprotein amino acids [1,2]. Recent reports showed the importance of these compounds for human health [3]. A simple and efficient method to analyse and quantify these secondary compounds is essential and capillary zone electrophoresis (CZE) was recently used for this purpose [4]. Isoxazolinones are *N,O*-heterocyclic natural compounds with high sensitivity to UV irradiation and to alkaline conditions [5]. As far as known, the occurrence is limited to the subfamily *Vicieae* of the *Leguminosae* plant family [6], to some leaf beetles of the family *Chrysomelidae* [7] and to the fungus *Streptomyces platensis* [8]. Especially in the seedlings of some *Lathyrus* species the concentration can be very high: up to 10% of the dry mass [9]. Recently, attention was focused on these

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compounds as the biochemical precursors of a group of toxic metabolites present in some *Lathyrus* species [10] and responsible for the human diseases neurolathyrism [11] and osteolathyrism [12]. Some of these natural compounds have physiological activity on isolated neurones [13] and on cloned neuronal receptors [14,15] or are of ecological importance [16,17].

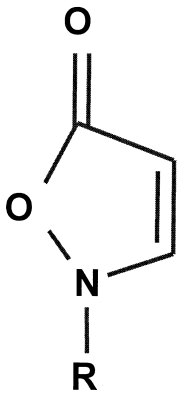
From the genus *Lathyrus*, 10 isoxazolinone derivatives have been isolated [10]. Some of these isoxazolinones are specific for certain plant species. β-(Isoxazolin-5-on-2-yl)propionitrile (BIP) and 2-(3-amino-3-carboxypropyl)isoxazolin-5-one (ACI) are only found in two *Lathyrus* species (*L. sativus* and *L. odoratus*) out of 47 species analysed [6,10]. The occurrence of 2-carboxymethylisoxazolin-5-one (CMI) and γ-glu-AEI (2-γ-glutamylaminoethylisoxazolin-5-one) was originally limited to *L. odoratus*. But CMI was recently also detected in five species of the genus *Lens* [18]. The structures of the isoxazolinone compounds studied in this report are shown in Fig. 1.

These compounds can be identified and quantified in plant extracts by high-performance liquid chromatography (HPLC) with precolumn PITC (phenyl isothiocyanate) derivatisation [19]. However, the procedure for PITC derivatisation is time-consuming and analysis time for each run plus regeneration of the column is 65 min. In addition, the numerous amino acid peaks in the chromatogram sometimes overlapped with the minor peaks and made the accurate determination of the minor peaks very difficult. It is essential to have a more efficient and selective method for the quick screening of these isoxazolinone compounds in different legume seedlings. This information will be useful for chemotaxonomic studies as well as for breeding programmes, and for nutritional, ecological and pharmacological research.

2. Experimental

2.1. Samples of legume seedlings

Seeds of grass pea (*Lathyrus sativus* L.) were received from the Indira Gandhi Agricultural University in Raipur, India. Seeds of sweet pea



R = CH_2-COOH: 2-carboxymethyl-isoxazolin-5-one (CMI)

R = CH_2-CH_2-CN: β-(isoxazolin-5-on-2-yl)-propionitrile (BIP)

R = CH_2-$CH(NH_2)$-COOH: β-(isoxazolin-5-on-2-yl)-L-alanine (BIA)

R = CH_2-CH(COOH)-NH-CO-$(CH_2)_2$-$CH(NH_2)$- COOH: γ-glutamyl-BIA (γ-glu-BIA)

R = $(CH_2)_2$-$CH(NH_2)$-COOH: 2-(3-amino-3-carboxypropyl)-isoxazolin-5-one (ACI)

R = $(CH_2)_2$-NH-CO-$(CH_2)_2$-$CH(NH_2)$-COOH: 2-(γ-glutamyl-aminoethyl)-isoxazolin-5-one (γ-glu-AEI)

Fig. 1. Structures of natural isoxazolinone derivatives.

(*Lathyrus odoratus* L. cv Spencer) were purchased from Supergrain Professional (Mechelen, Belgium). The etiolated seedlings were harvested after germination for 3 days in vermiculite in the dark at room temperature, weighed and stored at −20°C. Samples were extracted with 70% aqueous ethanol overnight. The extracts were centrifuged at 27 000 *g* for 20 min. The pellets were washed twice with 70% aqueous ethanol. The supernatants were pooled together, concentrated under vacuum and adjusted to 1 ml aqueous solution.

2.2. Standards

All the isoxazolinone compounds were purified from *Lathyrus* or lentil (*Lens culinaris*) seedlings in our laboratory as previously reported. BIA, ACI, BIP and γ-glu-BIA are from *Lathyrus sativus* [5,20]; γ-glu-AEI and CMI from *Lathyrus odoratus* [20,21]. They were injected individually or combined at concentrations from 0.005 to 2 m*M*.

2.3. Instrumentation

2.3.1. Capillary zone electrophoresis

A Quanta 4000E system (Waters, USA) equipped with a Mercury lamp and filter at 254 nm was used. The capillary (AccuSep CE/CIA, P/N 250-05, Waters) of coated fused-silica was 60 cm (effective length 52.6 cm)×75 μm. The detection was done at 254 nm. The results were analysed and calculated using Millennium software (version 3.1) from Waters.

The analyses were performed at a constant voltage of 22.5 kV at 30°C in an electrolyte of 25 m*M* KH_2PO_4 (Sigma) buffer containing 8% 1-propanol (Merck), pH 7.5 adjusted by 3 *M* KOH. The electrolyte was prepared daily and Millipore filtered (0.22 μm). The electrolyte was replaced by fresh solution after every three injections. Millipore-filtered samples (0.22 μm) were injected by a hydrostatic method with sample time of 10 s. Before each injection the capillary is regenerated by purging 2 min with 0.1 *M* KOH followed by 3 min with deionised water and finally 2 min with the electrolyte.

Sodium diethylbarbiturate (UCB, Belgium) was chosen as internal standard (I.S.) because of its suitable charge-to-mass ratio and its UV absorption property. This is not a natural compound of legume plants, it is stable and gives a symmetrical peak under the conditions used. Its migration time does not interfere or overlap with the migration time of any of the studied compounds. The optimal concentration for CZE analysis is 4 m*M* in the samples.

2.3.2. HPLC

A Waters 625 LC System with Waters 991 photodiode array detector was used. For reversed-phase HPLC an Alltima C_{18} column (250×4.6 mm I.D., 5 μm particle size) from Alltech (USA) was used. A guard column cartridge (Alltima C_{18}, 5 μm) was directly connected to the column. The column temperature was 43°C, the buffer gradient system and the procedure for derivatisation with PITC (99%, Aldrich) were as reported before [19]. A 50-μl volume of seedling extract was derivatised, dissolved in 1 ml buffer A and Millipore filtered. Buffer A (0.1 *M* ammonium acetate, pH 6.5) and buffer B (0.1 *M* ammonium acetate–acetonitrile–methanol, 44:46:10, v/v, pH 6.5) were prepared and Millipore filtered. The gradient system included buffer A (100–0% after 50 min) and buffer B (0–100% after 50 min). Another 15 min is necessary for the regeneration of the column before the next injection. Millennium software (Waters, version 1.10) was used to analyse the results.

3. Results and discussion

To reach the optimal separation of these six compounds, we optimised the concentration of phosphate buffer, pH value as well as temperature (results not given). The lower pH (3.0) of the phosphate buffer increased to almost double the migration time and the separation was less efficient. The increase of run voltage and the increase of ionic strength of the electrolyte resulted in the increase of run current. To compromise the run current to be close to but not exceed 100 μA to avoid outgassing of the electrolyte, we selected a run voltage of 22.5 kV and a potassium phosphate buffer concentration of 25 m*M*. The higher temperature shortens the migration time, at 40°C BIA and γ-glu-BIA were not separated. The addition of β-cyclodextrin in the buffer did not improve the separation but the peaks were sharper.

The addition of 8% 1-propanol in the buffer improved both the separation and the peak shape. The higher boiling point of 1-propanol (97.4°C) compared to that of methanol (65.5°C) made the electrolyte more stable with the former at 30°C and the results were more reproducible.

Fig. 2a and b present chromatograms of the standard isoxazolinone compounds analysed by CZE (a) and by HPLC (b). The results showed that the CZE method is much shorter than the HPLC method. With CZE we obtained a good separation of six compounds within 10 min. These peaks were not overlapped with peaks of UV-absorbing protein amino acids such as Trp, Phe and Tyr. The migration time of the later compounds is very short and they appeared immediately after the reagent and BIP peak. Since those protein amino acids are generally very low in protein-free extracts of soluble compounds in the seedling stage, they will not influence the analysis of isoxazolinone compounds. Some other UV-absorbing secondary metabolites present in some legume seedlings such as trigonelline (*N*-methylnicotinic acid), isowillardiine (β-uracil-3-yl-alanine) and lathyrine [β-(2-aminopyrimidine-4-yl)-alanine] were also checked. The peak of trigonelline overlapped with the peak of BIP, lathyrine was between γ-glu-AEI and ACI while isowillardiine was between ACI and I.S. Normally there were two small front reagent peaks, BIP co-migrated with the second reagent peak when BIP was in low concentration but co-migrated with both reagent peaks when it was in higher concentration. For correct BIP area calculation, the area of the reagent peak (single or double) was subtracted from the area of BIP peak. The area of reagent peak was constant and could be determined by injection of the buffer alone. This explains the rather high peak area RSD obtained for BIP.

Each isoxazolinone standard was injected 10 times at different concentrations between 0.005 and 2 m*M*. A linear correlation between 0.05 and 1.75 m*M* was observed between the concentration and the area of the peaks. The slope, intercept, the corresponding coefficient of correlation (r^2) and the detection limit of each compound are shown in Table 1.

The migration time of all the compounds slightly decreased after each run. Therefore the inclusion of I.S. in the samples was essential and the use of the value of relative migration time (the ratio between the migration time of a given compound and the migration time of the I.S.) is more correct. The relative migration time of each isoxazolinone compound was calculated and the value is reproducible for each compound (Table 2). The RSD of the relative migration time in CZE (0.44–1.94%) is lower than the RSD of the retention time in HPLC (0.57–3.6%). The RSD of the peak area in CZE is also lower than the RSD of the peak area in HPLC except for BIP and CMI. The fact that CMI in HPLC comes out very early (3.8 min) while in CZE it comes out last (8.3 min) might explain this difference. This result suggests that the CZE method is more selective and accurate for determining the UV-absorbing isoxazolinone compounds. Within 10 min, the six compounds studied can be separated, while for HPLC a minimum of 50 min is required for each amino acid sample. If other amino acids are disregarded, an elution time of 30 min and analysis time of 45 min including 15 min regeneration would still be needed for the HPLC analysis of the known natural isoxazolinone compounds.

The electropherograms of 3-day-old seedling extracts of grass pea (*Lathyrus sativus*) and of sweet pea (*Lathyrus odoratus*) analysed by CZE are shown in Figs. 3 and 4, respectively. This method permits one within 10 min to confirm the previous results obtained by HPLC that *L. sativus* seedlings contain BIP, ACI, BIA and γ-glu-BIA with higher concentrations of BIA and that *L. odoratus* seedlings contain BIP, γ-glu-AEI, ACI, BIA and CMI with higher concentrations of ACI [10]. The concentrations of these isoxazolinones were comparable to the results obtained by HPLC method shown in Table 3. In the seedling extracts we find higher values by HPLC for BIP, γ-glu-AEI and γ-glu-BIA while we find higher values by CZE for BIA in *L. sativus* and for CMI and ACI in *L. odoratus*. We cannot exclude the presence of minor unknown compounds which overlap with the isoxazolinone compounds in these seedlings that are unusually rich sources for nonprotein amino acids and isoxazolinones.

4. Conclusion

The advantages of CZE are mainly in the speed of

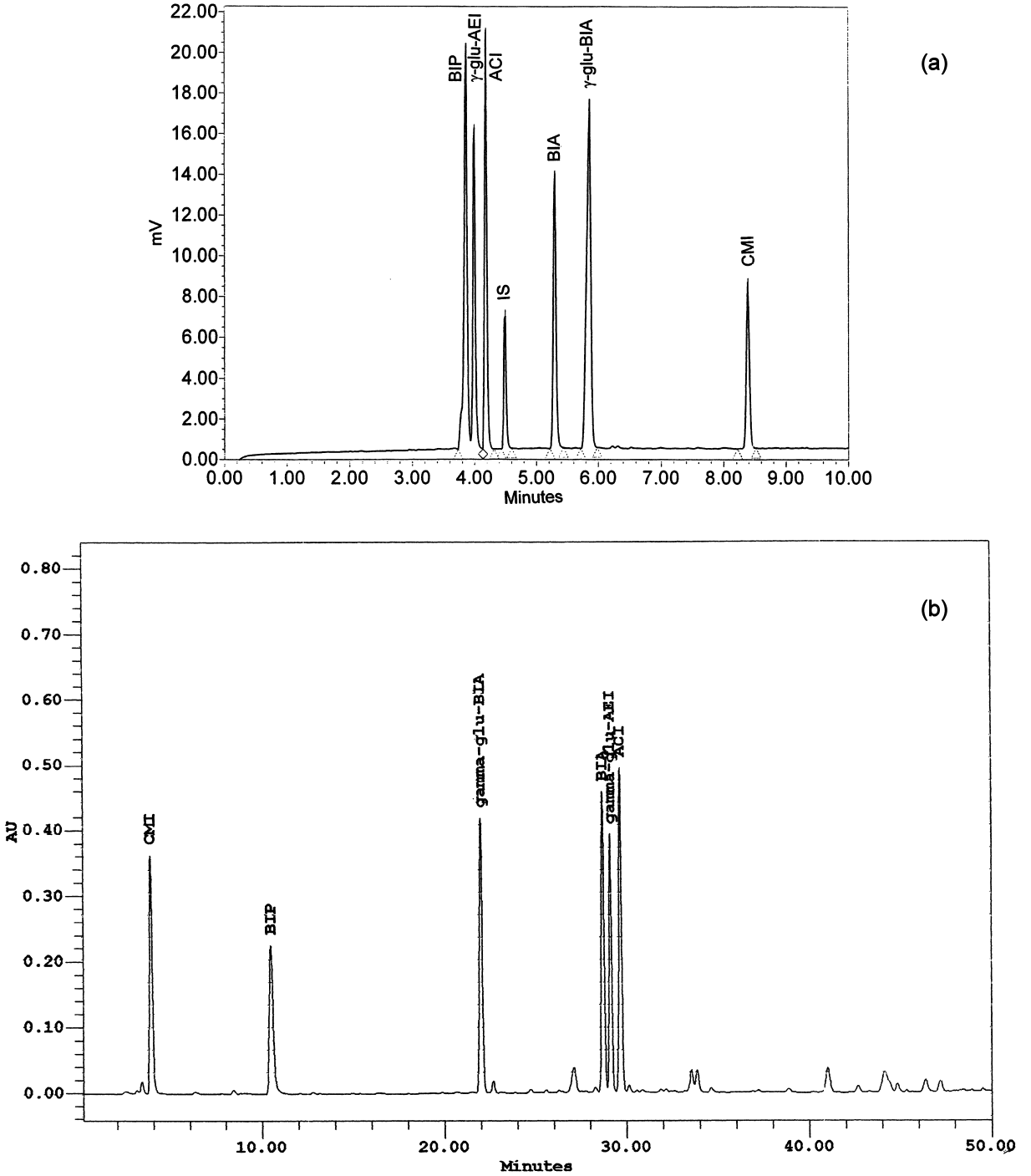


Fig. 2. Chromatogram of standard isoxazolinone compounds analysed by CZE (a) and by HPLC (b). BIP: β-(Isoxazolin-5-on-2-yl)-propionitrile; γ-glu-AEI: 2-(γ-glutamylaminoethyl)isoxazolin-5-one; ACI: 2-(3-amino-3-carboxypropyl)isoxazolin-5-one; I.S. (internal standard): Na-diethylbarbiturate; BIA: β-(isoxazolin-5-on-2-yl)L-alanine; γ-glu-BIA: γ-glutamyl-BIA; CMI: 2-carboxymethylisoxazolin-5-one.

Table 1
Linearity and correlation coefficients between the peak areas and the concentration of isoxazolinones by the CZE method

Isoxazolinone compound	Slope ($\cdot 10^3$)	Intercept ($\cdot 10^3$)	Correlation coefficient	Detection limit (μg/ml)
BIP	53.4	−1.75	0.9975	4.14
γ-Glu-AEI	34.5	−0.43	0.9967	7.70
ACI	40.1	−0.26	0.9966	4.60
BIA	42.5	+1.16	0.9954	4.30
γ-Glu-BIA	42.8	+1.84	0.9960	3.00
CMI	57.6	−1.12	0.9990	4.30

Table 2
Comparison of the reproducibility of the CZE method and the HPLC method

Compound	CZE			HPLC	
	Relative migration time ($n=10$)	RSD (%) for RMT ($n=10$)	RSD (%) for peak area ($n=5$)	RSD (%) for t_R ($n=6$)	RSD (%) for peak area ($n=6$)
BIP	0.862±0.010	1.16	4.97	3.60	2.10
γ-Glu-AEI	0.897±0.004	0.44	3.60	1.10	5.70
ACI	0.933±0.007	0.75	1.26	1.20	3.00
BIA	1.192±0.010	0.84	1.55	1.30	2.90
γ-Glu-BIA	1.337±0.026	1.94	2.98	1.70	3.40
CMI	1.901±0.028	1.47	6.86	0.57	0.83

RMT: Relative migration time; t_R: retention time.

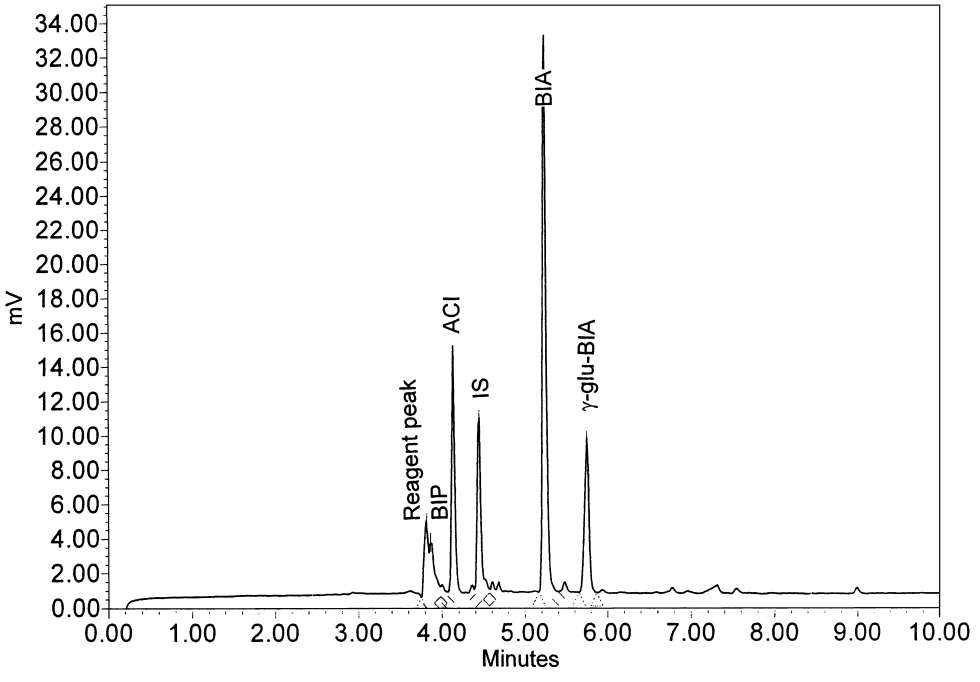


Fig. 3. Electropherogram of isoxazolinone compounds in 3-day-old seedlings of *Lathyrus sativus* analysed by CZE. Reagent peak; BIP: β-(isoxazolin-5-on-2-yl)propionitrile; ACI: 2-(3-amino-3-carboxypropyl)isoxazolin-5-one; I.S. (internal standard): Na-diethylbarbiturate; BIA: β-(isoxazolin-5-on-2-yl)L-alanine; γ-glu-BIA: γ-glutamyl-BIA.

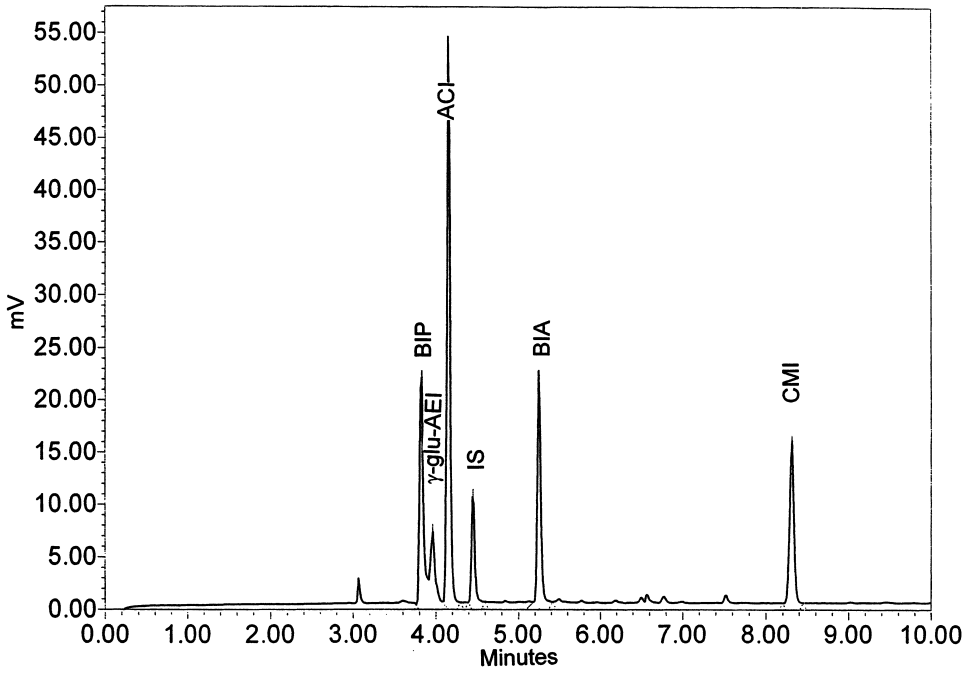


Fig. 4. Electropherogram of isoxazolinone compounds in 3-day-old seedlings of *Lathyrus odoratus* analysed by CZE. BIP: β-(Isoxazolin-5-on-2-yl)propionitrile; γ-glu-AEI: 2-(γ-glutamylaminoethyl)isoxazolin-5-one; ACI: 2-(3-amino-3-carboxypropyl)isoxazolin-5-one; I.S. (internal standard): Na-diethylbarbiturate; BIA: β-(isoxazolin-5-on-2-yl)L-alanine; CMI: 2-carboxymethylisoxazolin-5-one.

analysis and the low cost and ecological acceptability of water based buffers as compared to HPLC solvents. For the analysis of the compounds studied, an additional advantage of the CZE method is that the crude plant extract after Millipore filtration can be used directly in the system for injection. No time-consuming precolumn derivatisation procedure is required. After injection the same sample can be used for other purposes, which is not the case with HPLC.

The relative migration time is very reproducible for each compound and can be used to screen the legume seedlings and as an additional identification of UV-absorbing compounds in plant extracts. With this method we confirmed the presence of CMI in the seedling of *Lens* species for the first time [18].

Table 3
Concentrations of isoxazolinones in 3-day-old seedlings of *Lathyrus odoratus* and *L. sativus* analysed by CZE and by HPLC methods

Compound	Concentration (mg/g)			
	Lathyrus odoratus		*Lathyrus sativus*	
	CZE	HPLC	CZE	HPLC
BIP	0.72	0.75	0.23	0.33
γ-Glu-AEI	0.82	0.96	ND	ND
ACI	2.59	2.24	0.52	0.59
BIA	1.34	1.36	1.47	0.84
γ-Glu-BIA	ND	ND	0.67	1.08
CMI	0.50	0.31	ND	ND

ND: Not detected.

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