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Short communication

Determination of indole-3-acetic acid in plant tissues by capillary electrophoresis

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Abstract

A capillary electrophoresis method with direct fluorimetric detection was developed for the determination of the plant growth regulator indole-3-acetic acid (IAA) in plant tissues of *Zea mays*, *Ricinus communis* and *Kalanchoë daigremontiana*. Extraction of this metabolite from different plant organs, leaves and stem was performed with methanol, followed by a solid-phase extraction procedure. The subsequent capillary electrophoresis step with a phosphate buffer and fluorimetric detection provides sufficient sensitivity to determine IAA in these plant samples. © 1997 Elsevier Science B.V.

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1. Introduction

Over the last few years, capillary electrophoresis (CE) has been shown to be a powerful tool for many complex biological separation problems [1,2], for pharmaceutical analyses [3,4], clinical diagnostics [5–7] or food analyses [8,9]. The high stability of CE against non-ionic matrices as well as the high separation efficiencies (up to 10^6 plates/m) elevates this separation method as an orthogonal system to high-performance liquid chromatography (HPLC) [8,10,11]. In the field of phytochemical analysis, CE is widely used for the determination of plant metabolites, such as flavonoids [12–14], alkaloids [15,16], phenolic acids [12] and others [12,17].

The main challenge in developing CE systems for phytochemical analyses is the occurrence of a complex mixture of various chemically different compounds in plant samples.

The determination of metabolites that are responsible for the regulation of plant growth and differentiation is of particular biological interest. The determination of auxins, such as indole-3-acetic acid (IAA), is important for the investigation of cell division, cell growth and cell differentiation of the vascular bundle with its two elements, phloem and xylem.

The correlation of the concentration of IAA with a physiologically active part within a tissue requires high local resolution for the determination of IAA in plant tissues.

A large variety of methods have been described for the determination of IAA, including chromatographic methods like HPLC [18–22] and gas chromatography (GC) [20,23–26] as well as immunoassays such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) [20,27,28]. In

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most cases, these methods were used in combination with purification steps by thin-layer chromatography (TLC) or HPLC and determination by ELISA [29].

However, high sample purity is required for most of these methods, which demands elaborate and time-consuming sample preparation procedures. Moreover, for GC and immunoassay determinations, derivatisation of IAA is generally required.

CE with amperometric detection [30] or with fluorescence detection seems to be a useful alternative for the analysis of indole derivatives. The high separation efficiency of CE and its low absolute detection limits provide a simplified purification procedure compared to other methods. In this contribution, the determination of IAA in plant tissues by CE with fluorimetric detection is demonstrated. An extraction procedure for IAA prior to CE was developed. The suitability of this method is shown for the analyses of leaves of *Kalanchoë daigremontiana*, stems of *Ricinus communis* and etiolated shoots of *Zea mays*.

2. Experimental

2.1. Apparatus

Electrophoresis was carried out on a laboratory built CE machine, equipped with a high voltage power supply, HCN 6 M-30000 (FUG, Rosenheim, Germany) and a fluorescence detector, JASCO 821-FP (JASCO, Groß-Umstadt, Germany). For all measurements, untreated fused-silica capillaries with an I.D. of 75 μm (TSP 075375, Polymicro Technologies, Phoenix, AZ, USA) and a total length of 95 cm (74 cm to the detection window) were used.

2.2. Chemicals and solutions

The electrolyte for the determination of IAA contained 10 m*M* Na_2HPO_4 (Fluka, Buchs, Switzerland) and 10 m*M* NaH_2PO_4 (Merck, Darmstadt, Germany), natural pH=6.6. Methanol, of LiChrosolv gradient grade quality, was obtained from Merck. All standard substances were purchased as analytical-reagent p.a. quality from Sigma (St. Louis, MO, USA) and were used without further purification. All electrolytes and standard chemicals were dissolved in Milli-Q water and filtered through a 0.45-μm filter.

2.3. Plant material and extraction procedure

Vegetative tissue (10 g fresh weight) of *Kalanchoë daigremontiana*, *Ricinus communis* or *Zea mays* was frozen in liquid nitrogen and homogenized by a micro-dismembrator (Braun, Melsungen, Germany). The homogenized plant tissue was extracted at 0°C for 90 min with 10 ml of methanol containing 10 mg of butylhydroxytoluene (BHT), as a non-fluorescent antioxidation agent. After centrifugation (4900 *g*/10 min) of the methanolic solution, indole-3-butyric acid (IBA) was added as an internal standard. The solution was evaporated in a vacuum rotary evaporator up to the aqueous phase and stored at −18°C. The solution was adjusted to pH 3.5 with acetic acid and applied to an SPE C_{18} Bakerbond cartridge (J.T. Baker, Phillipsburg, NJ, USA). Cartridges were preconditioned by washing them with 1 ml of methanol followed by 1.5 ml of 50 m*M* acetic acid (pH 3.5). The methanolic plant extract was loaded on the cartridge and washed with 2 ml of 50 m*M* acetic acid and with 1 ml of water. The IAA was eluted with 1 ml of methanol and was concentrated in a flow of nitrogen to a final volume of 100 μl.

2.4. Preconditioning of the CE capillary

Before each run, the capillary was preconditioned by rinsing it with methanol (1 min), 100 m*M* sodium hydroxide (2 min), water (1 min) and electrolyte (3 min), at a pressure of 50 kPa. This procedure is necessary to eliminate adsorbed matrix compounds from the capillary surface in order to achieve good reproducibility.

3. Results and discussion

3.1. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is advantageous for the determination of IAA in plant

samples compared to chromatographic methods for different reasons. After the extraction procedure, a large variety of fluorescent non-polar compounds still remain in the solution, which can lead to co-elution of matrix compounds with IAA in the chromatography step. In CZE, these compounds are transported by electroosmotic flow and are observed as a single broad intensive signal. As IAA provides electrophoretic mobility, due to the carboxylate function, no co-elution of matrix compounds is expected and a more selective determination of the counter-electroosmotically migrating IAA should be achieved.

Fig. 1 shows the separation of IAA and IBA (as internal standard) by CZE using a phosphate electrolyte. The optimum electrolyte pH was found to be 6.6, because the fluorescence intensity of IAA with an excitation at 283 nm and emission at 350 nm is maximum around that value. Additionally, at this pH, no co-migration was observed with charged matrix compounds.

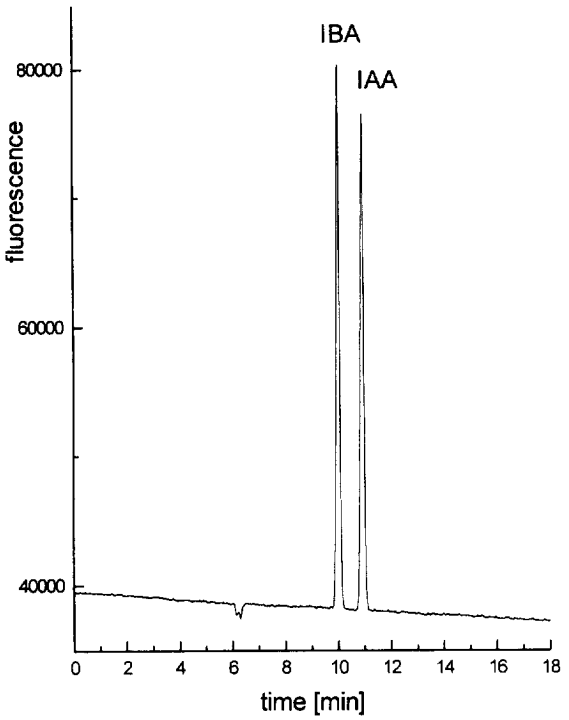

Fig. 1. Determination of a 20 μ*M* standard solution of IAA and IBA. Electrolyte: 10 m*M* Na_2HPO_4, 10 m*M* NaH_2PO_4, pH 6.6. Capillary: 74 cm (53 cm to detector)×75 μm I.D. Conditions: voltage, 25 kV; current, 17 μA; injection, hydrostatically, 10 cm, 60 s. Detection: fluorescence, excitation wavelength, 283 nm and emission wavelength, 350 nm.

Using this system, the determination of IAA is achieved within 12 min. The limit of detection was found to be 0.5 μ*M*. The calibration curve was linear between 2 and 350 μ*M* for IAA and IBA.

3.2. Extraction

Various extraction procedures have been published for IAA and other plant growth regulators [31]. For the extraction of IAA from the plant material, methanol was found to be the most effective solvent. As IAA is sensitive to oxidation, the addition of an antioxidation agent was necessary. Malsy et al. [33] described the use of BHT for this purpose, which seemed to be advantageous because it does not interfere with the IAA signal nor does it cause any disturbance of the fluorescence detection. For the solid-phase extraction, the methanolic plant extract was adjusted to pH 3.5. At this pH, IAA is uncharged and is retarded by the C_{18} cartridge material. After washing with diluted acetic acid and water, IAA was eluted using methanol. The eluted methanolic IAA solution was concentrated in a flow of nitrogen to a final volume of 100 μl, corresponding to a 100-fold enrichment. Evaporation of the methanol to dryness lead to a serious loss of IAA, presumably due to oxidation.

The IAA homologue, IBA, was employed as an internal standard, as no IBA signals were observed in the extracts of *Ricinus* and *Kalanchoë* tissues. It was added after homogenisation of the plant tissue. The recovery for a standard solution of IAA was 99%. Further recovery experiments in spiked plant extracts showed a recovery of 85%. The recovery for plant tissue extracts spiked with IBA was found to be 87%. This value is close to the recovery for IAA and proves the suitability of IBA as an internal standard for the employed plant materials.

For etiolated maize shoots, a different type and concentration of matrix compounds occurs, but this extraction procedure was also found to be useful in

this case. For other plants, the suitability of the extraction procedure has to be further investigated.

3.3. Determination of IAA in plant material

The described extraction procedure allows 100-fold enrichment of IAA and, therefore, the determination of nanomolar concentrations of IAA in the plant material used. Three different plant samples were examined by this approach: Mesophyll of *Kalanchoë daigremontiana*, stem of *Ricinus communis* and etiolated shoots of *Zea mays*.

The determination of IAA in such samples is shown in Fig. 2 for a leaf sample of *Kalanchoë* and in Fig. 3 for a *Ricinus* stem sample. The observed concentrations and recoveries of IAA in these samples and in etiolated maize shoots are listed in Table 1, together with corresponding values obtained by other groups with different methods. The measured concentrations are of a similar order of magnitude as the literature values, indicating the suitability of this method for the quantitative determination of IAA in plant tissues.

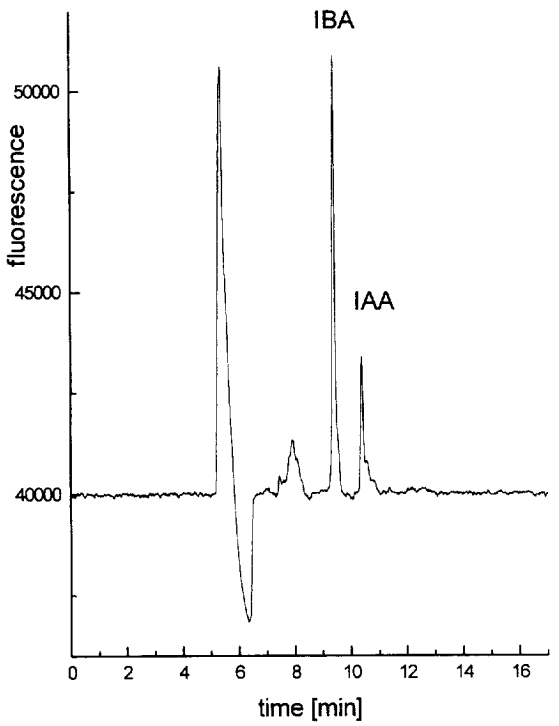

Fig. 2. Determination of IAA in a *Kalanchoë daigremontiana* leaf, CZE conditions as in Fig. 1.

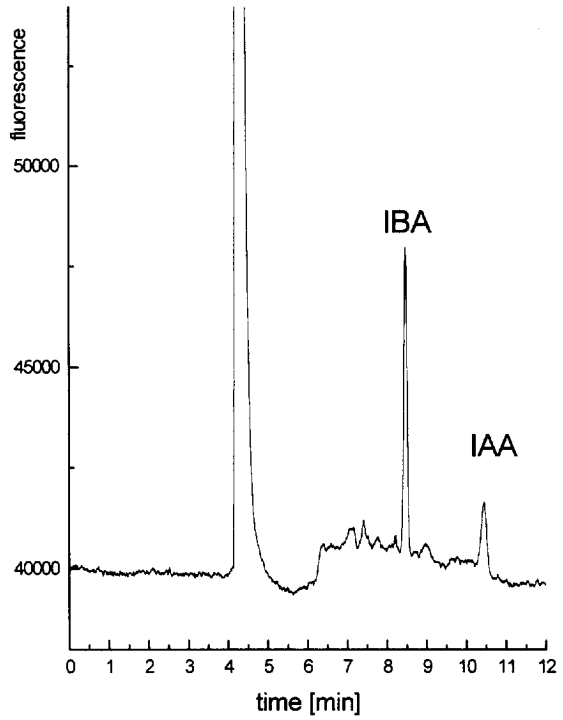

Fig. 3. Determination of IAA in a stem of *Ricinus communis*, CZE conditions as in Fig. 1.

4. Conclusion

The combination of capillary zone electrophoresis with direct fluorimetric detection provides for the fast quantitative determination of the plant growth regulator, IAA, with adequate precision. The extraction procedure prior to CE is easy and, in contrast to most ELISA determinations, no derivatization is necessary. Nevertheless, it should be noticed that the suitability of the extraction procedure for plants other than those used here has to be examined in future work.

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Table 1
Concentrations of apparent free IAA in three different plant samples, as measured by CE in comparison to literature values, determined by pyrone fluorimetry[a], ELISA[b], colorimetry[c], GC–MS[d], and bioassay[e]

Sample	Measured concentration of IAA by CE (pmol/g FW)	Recovery (%)	Concentration of IAA from reference (pmol/g FW)
Kalanchoë mesophyll	95	55	3–12[a] [32]; 55[b] [33]
Ricinus stem	92	36	63[d] [34]; 300[c] [35];
Zea mays etiolated shoot, 3 to 5 d old	1120	96	250[d] [36]; 1030[b] [37]; 2000[d,e] [38,39]

FW=fresh weight.

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