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Journal of Chromatography A, 824 (1998) 231–239

JOURNAL OF CHROMATOGRAPHY A

Determination of indole-3-acetic acid and indole-3-acetylaspartic acid in pea plant with capillary electrophoresis and fluorescence detection

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Received 29 June 1998; received in revised form 20 August 1998; accepted 21 August 1998

Abstract

A method for the simultaneous separation and determination of indole-3-acetic acid (IAA) and its primary metabolite, indole-3-acetylaspartic acid (IAAsp), in an extract from pea (*Pisum sativum*), is described. The technique used is capillary electrophoresis with fluorescence detection, the finally selected analytical parameters being: buffer 30 m*M* acetate, pH 4.5, injection volume 26 nl, high voltage 30 kV and excitation and emission wavelengths of 254 and 360 nm, respectively. The detection limits obtained were 18 n*M* (0.39 fmol) for IAA and 28 n*M* (0.73 fmol) for IAAsp. Sample preparation procedures have also been examined. The amounts of IAA and IAAsp in shoot apices of pea were determined to $1.9 \cdot 10^{-10}$ and $1.1 \cdot 10^{-10}$ mol/g fresh mass, respectively, corresponding to 33 ng/g fresh mass for both, using indole-3-butyric acid as an internal standard.

Keywords: Plant hormones; Indoleacetic acid; Indoleacetylaspartic acid; Organic acids

1. Introduction

Substances, produced at low concentrations in plants and regulating their growth and development, are collectively referred to as plant hormones. In the group of hormones termed auxins, indole-3-acetic acid (IAA) is regarded as the principal native hormone, and is known to regulate processes such as division, elongation and differentiation of cells. The concentration range for IAA, is 10–100 ng/g fresh mass in most plant tissues. Other metabolites in plants are usually found at 1000-fold higher levels. Biosynthesis, transportation, degradation and conjugation with other small biomolecules maintain specific concentration levels of IAA [1]. Consequently, a given tissue not only contains IAA, but also a whole spectrum of IAA related compounds [2]. The involvement of IAA in different growth processes may be reflected as alterations of the level of IAA metabolites, rather than of the level of IAA itself. Furthermore, a change in turnover rate of IAA and/or its metabolites is usually observed during certain growth phases [3]. Although IAA is the active hormone, it may thus, in addition, be desirable to simultaneously determine its metabolites. The primary IAA metabolite is indole-3-acetylaspartic acid (IAAsp) [3].

Extensive reviews dealing with currently used

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00694-3

analytical methods for plant indoles have been presented [2,4–6]. Today, gas chromatography–mass spectrometry (GC–MS) is the most reliable technique due to the structural information gained and the possibility to use stable isotopes as internal standards [7–10]. Other chromatographic methods such as high-performance liquid chromatography (HPLC) with fluorimetric detection [11–14], as well as immunoassay methods [15], are also frequently described in the literature. However, disadvantages with most of these methods are the high demands placed on sample purity, and, in the case of GC–MS, the derivatisation requirements, making the use of several preparation steps inevitable. Besides being time consuming, these steps also introduce errors and discrimination in the analysis.

Recently, a few papers evaluating capillary electrophoresis (CE) as a tool for the determination of indolic compounds have been published, detection principles being UV [16], amperometric [17], lamp-based fluorescence [18] and laser-induced fluorescence (LIF) [19]. Obvious advantages with CE are the small sample volumes required and the high separation efficiency.

In this paper, we describe a method for the determination of IAA and IAAsp in pea plant (*Pisum sativum* L. cv. Marma) employing CE equipped with a lamp-based fluorescence detector. The detector is designed to overcome the sensitivity problem usually associated with utilisation of small I.D. (50–75 μm) capillaries. Automation is possible since the detector is compatible with a commercial CE instrument (HP-3D-CE). Indole-3-butyric acid (IBA) is used as an internal standard.

2. Experimental

2.1. Capillary electrophoresis system

Untreated fused-silica capillaries, 75 cm×75 μm I.D.×375 μm O.D., were obtained from Polymicro Technologies (Phoenix, AZ, USA). Before use, the capillaries were preconditioned with 0.1 *M* NaOH for 20 min, distilled water for 10 min and run buffer for 20 min. In order to achieve repeatable migration times, the capillary was rinsed with the separation buffer for 3 min before each run. Further rinsing involved 0.1 *M* NaOH (2 min), water (1 min) and electrolyte (5 min) between every tenth run.

Two different CE instruments were used: (1) HP-3D-CE (Hewlett-Packard, Palo Alto, CA, USA) and (2) a laboratory-built CE apparatus. The input of the high-voltage power supply, ±0–30 kV, (Brandenburg, Thornton Heath, UK) together with the injection end of the capillary, were placed in a plexiglass box with an interlock on the access door for protection. Rinsing of the capillary was accomplished by applying vacuum to the detection end vial. Sample injections were made hydrodynamically by raising the injection end of the capillary 2–5 cm for 30–60 s.

2.2. The detector

The fluorescence detector (Fig. 1), developed by Camedi (Täby, Sweden) and marketed by Flux Instruments (Karlskoga, Sweden), applies a novel technique to minimise the stray light. The fused-silica capillary (D) is illuminated laterally by the excitation from a combined Xe–Hg high-pressure lamp (A). Suitable excitation wavelengths are selected by interference bandpass filters. The sample molecules fluoresce when passing the excitation light beam. Approximately 12% of the emitted fluorescence is transported by total reflection inside the capillary, like in a light guide, and can then be picked up at a suitable point by a transparent body (B) brought into optical contact with the capillary wall (C). Thereby the emitted light leaves the excitation zone before detection, thus minimising the effect of the great amount of stray light produced when the excitation beam hits the wall of the

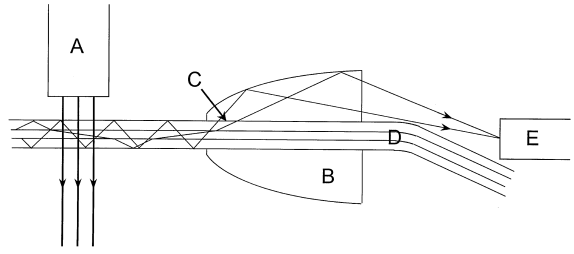

Fig. 1. Schematic diagram of the fluorescence detector. A= Excitation light guide; B=transparent elliptic body; C=optical contact; D=fused-silica capillary; E=emission light guide.

capillary. In the HP-3D-CE, the detector head is screwed into a modified capillary cassette, while in the laboratory built equipment, the detector head is fastened to a stand. Data collection was accomplished with a personal computer equipped with ELDS 900 (Chromatography Data System, Kungshög, Sweden) software.

2.3. Chemicals and reagents

All chemicals were of analytical grade and solutions were prepared with water from an Elgastat UHQII (Elga, High Wycombe, UK). The run buffers were degassed and filtered before use. Standard stock solutions (1 m*M* in methanol) were prepared from the following indoles obtained from Sigma (St. Louis, MO, USA): IAA; indole-3-propionic acid (IPrA); IBA and indole-3-L-acetyl aspartic acid (IAAsp). Working standard solutions were prepared daily by mixing and diluting the stock solutions with methanol. The stock solutions were stored at −20°C when not in use.

2.4. Sample preparation procedures

Fig. 2 presents a summary of the sample preparation procedures examined in the present work for the determination of IAA and related indoles in pea. The purification has previously been used for quantitative determination of IAA by HPLC and CE [3,17,20].

Seeds of pea (*Pisum sativum* L. cv. Marma) were soaked in tap water for 6 h and germinated in trays with vermiculite in a growth chamber. After 10 days, shoot apices were collected and homogenised in liquid nitrogen.

2.4.1. Extraction and filtering No. 1

The resulting powder (3–10 g) was extracted in 5 m*M* phosphate buffer, pH 7.0 (2 ml buffer/g plant material), for 1 h at 4°C in darkness. To prevent oxidation of the sample, butylhydroxytoluene (BHT), a non-fluorescent antioxidation agent, was added (1 mg/g plant material). IBA was used as an internal standard. The extract was filtered through a glass microfibre filter, GF/C 1.2 μm (Whatman, Maidstone, UK).

2.4.2. Extraction and filtering No. 2

The resulting powder was treated as described in Section 2.4.1, except that the extraction medium was 100% methanol instead of phosphate buffer. The resulting extract was filtered as above and stored at −20°C.

2.4.3. Purification via steps 1→3, giving sample A

A 0.5 g Bond Elut C_{18} column (Isolute, Mid Glamorgan, UK) was conditioned with 2×1 ml methanol and 2×1 ml 5 m*M* phosphate buffer, pH 2.42. The extract was pH adjusted to 2.5 using phosphoric acid, and was allowed to run through the column at a flow-rate of 8–12 ml/h. The column was then washed with 2×1 ml 5 m*M* phosphate buffer, pH 2.42, and 1 ml water. At this pH the analyte species are uncharged and will be retained in the column, while the species that are charged will be washed out. Elution of the analyte species from the column was made with 1 ml 100% methanol.

2.4.4. Purification via steps 1→4→6, giving sample B

A 0.5 g Bond Elut C_{18} column was conditioned with 2×1 ml methanol and 2×1 ml 5 m*M* phosphate buffer, pH 6.6. Extract No. 1 was then allowed to run through the column at a flow-rate of 8–12 ml/h. At this pH, the analyte species are negatively charged and will pass through the column. The resulting eluate, thus containing the analyte species, was collected, pH adjusted to 2.5 using phosphoric acid, and purified through a second solid-phase extraction, see Fig. 2.

2.4.5. Purification via steps 2→5, giving sample C

The cold methanolic extract was ultrafiltrated through a Centricon filter (Amicon, Beverly, MA, USA) with a molecular mass cut-off at 30 000. By this procedure, considerable amounts of the proteins in the extract are retained on the filter, while small molecules, such as the analyte species, are filtered through. The capillary lifetime is thereby prolonged due to reduction in protein adsorption on the capillary surface.

2.5. Selection of wavelengths

The peak wavelengths for IAA excitation and

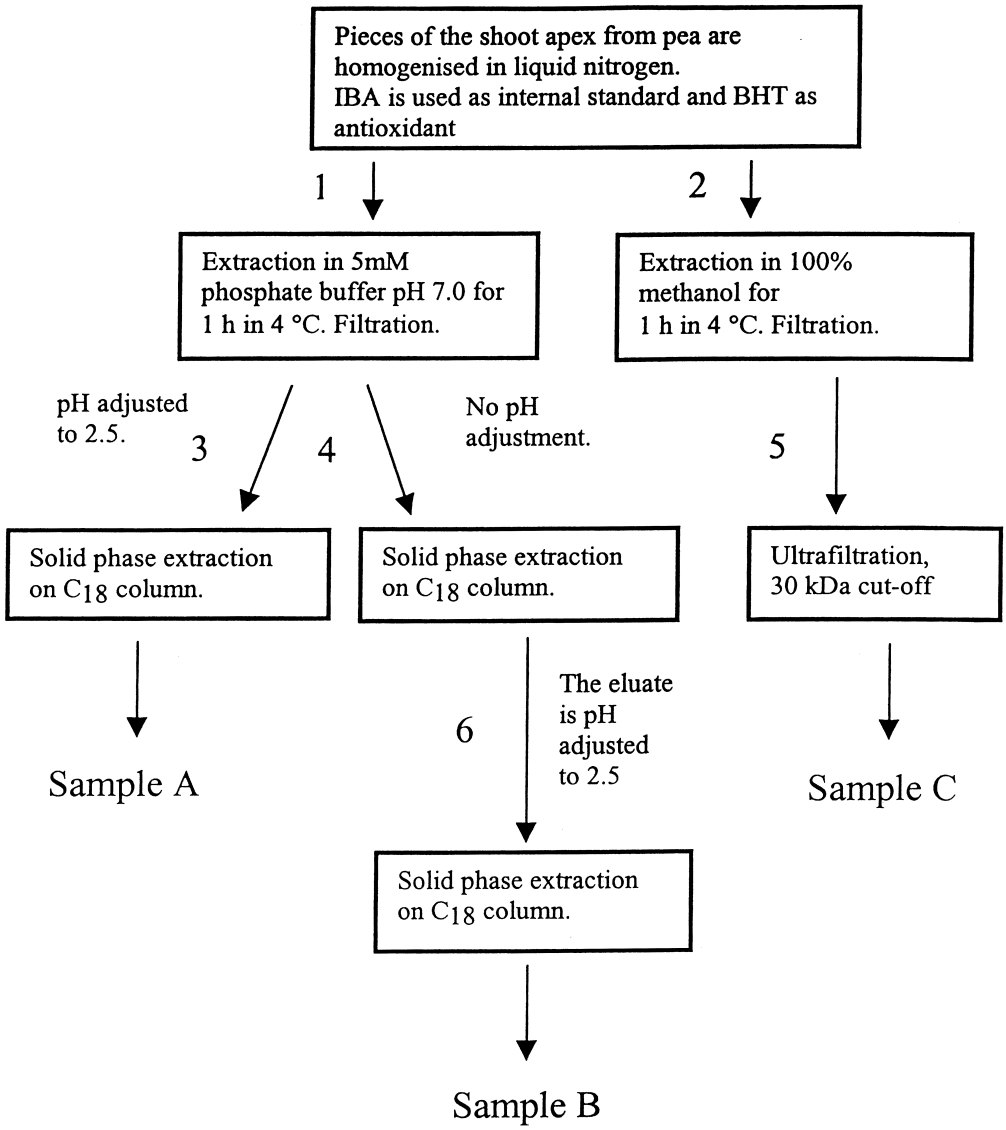

Fig. 2. Protocol for the different extractions and purifications examined.

emission are 280 nm and 350 nm, respectively [21]. Bandpass filters of 253.7 nm (HW 10 nm) and 280 nm (HW 10 nm) for the excitation and no filter, 330 nm (HW 10 nm), 360 nm (HW 10 nm), and longpass 389 nm for the emitted light were examined. The filters were obtained from Melles Griot (Irvine, CA, USA). The aim was to identify filters for the excitation and emission wavelengths that gave the best signal to noise ratios for an indole standard mixture. It was also desirable to use wavelengths at which interfering peaks in a sample electropherogram could be minimised. As an example, the combination 254 nm for excitation and no filter for emission gave a very high signal-to-noise ratio for an indole standard mixture, but the lack of selectivity resulted in a very complex electropherogram when used for a real sample. The combination of 254 nm for excitation and 360 nm for emitted fluorescence was finally selected due to its relatively good signal-to-noise ratios and to the selectivity obtained.

2.6. Selection of buffer

The composition, concentration and pH of the run buffer are important parameters in adjusting separation, migration times, selectivity and peak shape in CE. Using a separation buffer of 30 m*M* acetate, pH 4.5, Olsson et al. managed to separate plant indoles by CE with electrochemical detection [17]. The pK_a value of IAA is 4.7 [22]. It is beneficial for the separation to select a buffer pH in this range, since the differences between the analytes electrophoretic mobilities will exhibit a maximum. Therefore, acetate buffer solutions, 30 m*M*, with pH 4.5, 4.7 and 5.0, were evaluated for the separation of IAA and related indoles in the pea extracts. The pH 4.5 buffer was selected since the best separation of the peaks of interest in a sample electropherogram was obtained.

Experiments were also performed with addition of 10% ethanol to the 30 m*M* acetate buffer, pH 4.5, see Fig. 3. The resolution between IAAsp and the interfering matrix peak increased from 0.93 to 1.1. However, the gain in accuracy of the area measurement was not significant. Also, the migration time for IAAsp was increased from 17.7 to 28.7 min. Therefore acetate buffer, pH 4.5, without addition of ethanol, was utilised in all subsequent experiments.

2.7. Examination of injection volumes

When the experiments above (ethanol and pH) were performed, the injection volume was 52 nl (50 mbar, 10 s, 75 cm×75 μm capillary). This is a relatively large volume, constituting 1.6% of the capillary length. With the 30 m*M* acetate buffer, the maximum injection volume was 26 nl for sample A, before additional band broadening appeared.

2.8. Summary: selection of analytical parameters

The conditions selected for the study of IAA and related indoles in pea plant in the present work were:

Buffer	30 m*M* acetate, pH 4.5
Injection volume	26 nl
High voltage	30 kV
λ_{ex} and λ_{em}	254 nm and 360 nm

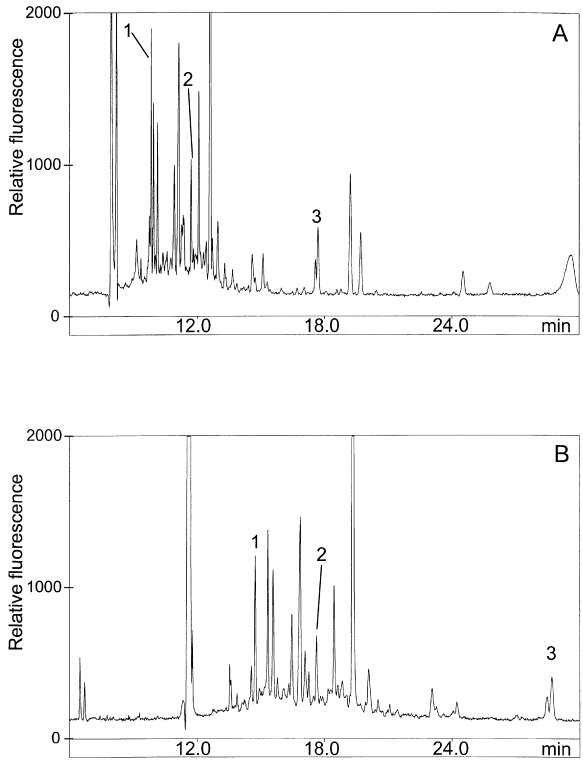

Fig. 3. Electropherograms of sample A obtained with (A) 30 m*M* acetate buffer, pH 4.5 and (B) 30 m*M* acetate, 10% (v/v) ethanol. Capillary: 75 cm (60 cm to detector)×75 μm I.D.; applied voltage: 30 kV; hydrodynamic injection: 52 nl; detection: fluorescence, λ_{ex}=254 nm, λ_{em}=360 nm. Peak identification: 1=IBA, 2=IAA, 3=IAAsp.

3. Results and discussion

3.1. Separation of indoles in a standard mixture

Shown in Fig. 4A is an electropherogram recorded for a laboratory-prepared mixture containing four different indoles. The migration order in all the tested buffer systems was: IBA, IPrA, IAA and, finally, IAAsp. When 30 m*M* acetate buffer, pH 4.5, was used as electrolyte the analysis time was approximately 20 min. Calibration data can be found in Table 1. The limit of detection (LOD, three-times the noise level) ranged from 15 to 28 n*M*. The LOD values were obtained with an injection volume of 26 nl (10 s, 25 mbar, 75 cm×75 μm capillary). For IBA, IAA and IAAsp the calibration curves were linear between 20 n*M* and 10 μ*M* (r^2=0.999).

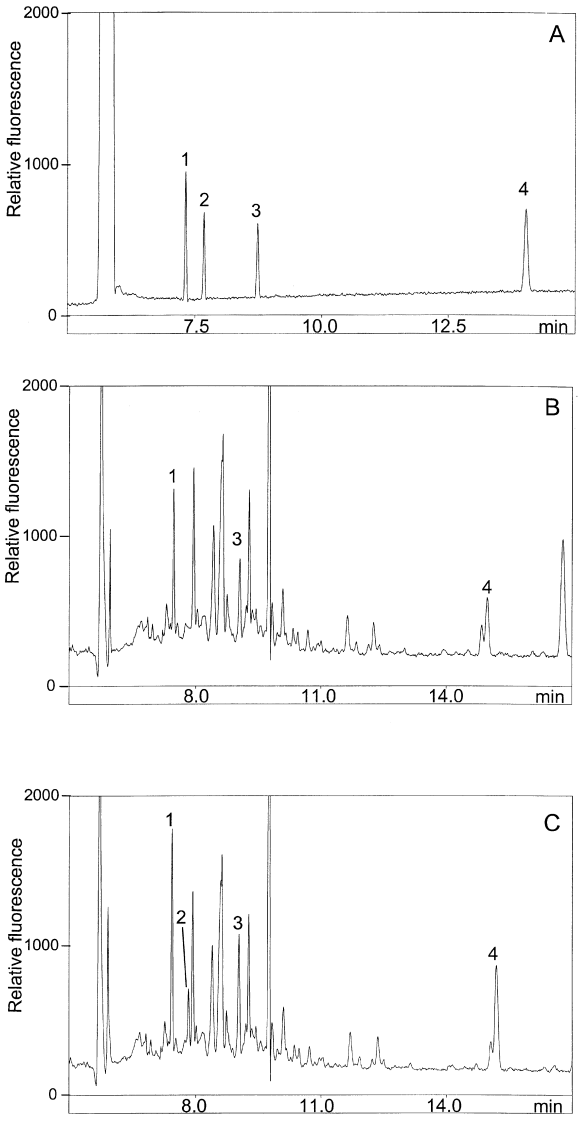

Fig. 4. Electropherograms of: (A) an indole standard mixture that contains 140 n*M* IBA, 100 n*M* IPrA, 100 n*M* IAA and 130 n*M* IAAsp; (B) an unspiked plant extract; (C) a plant extract spiked with 100 n*M* IBA, 73 n*M* IPrA, 69 n*M* IAA and 93 n*M* IAAsp. Buffer: 30 m*M* acetate, pH 4.5; capillary: 75 cm (60 cm to detector)×75 μm I.D.; applied voltage: 30 kV; hydrodynamic injection: 26 nl; detection: fluorescence, λ_{ex}=254 nm, λ_{em}=360 nm. Peak identification: 1=IBA, 2=IPrA, 3=IAA, 4=IAAsp.

3.2. Separation of indoles in real samples

3.2.1. Sample A

Electropherograms of unspiked and spiked extracts from pea plants (sample A) are shown in Fig. 4B and C. It can be seen that the sample still has a complex matrix, containing many compounds exhibiting fluorescence under the selected conditions. The most interesting peak, the IAA peak, is well separated and easily identified, and so are the IPrA and the internal standard, IBA, peaks. IAAsp is not entirely separated from an interfering, unknown peak, but can still be identified. The relative response factors were identical in sample and standard. The concentrations of IAA and IAAsp were determined to be 33 ng/g fresh mass, both, using IBA as internal standard. This is in accordance with results previously reported in the literature [3]. R.S.D. data for seven repeated injections of the same sample are shown in Table 1. Although the repeatability was acceptable, the electroosmotic flow (EOF) could vary substantially between different capillaries. The total recovery of IBA and IAA for the extraction and purification procedure was found to be 37% while it was 34% for IAAsp.

IBA has been proven to be a suitable internal standard for the determination of IAA in plants [18], and was consequently used also in this work. However, no signal from IPrA can be observed in the electropherogram of the unspiked sample (Fig. 4B). Thus, IPrA could serve as an alternative to IBA as an internal standard when determining IAA and related indoles in pea by CE with fluorescence detection. In conformity with the results obtained in this work, IPrA may be a better alternative since it is more similar to IAA with respect to carbon chain length and it elutes closer to IAA.

3.2.2. Sample B

Samples A and B were compared. It was found that there was no significant improvement in the appearance of the peaks of interest for sample B. It can thus be concluded that the additional purification step, subjected to sample B, is superfluous.

3.2.3. Sample C

Electropherograms of spiked and unspiked methanolic extracts of pea (sample C) are depicted in Fig. 5. The only purification step applied to sample C is filtration. After extraction, the remaining plant debris was filtered away, and then the sample was ultrafiltrated through a Centricon filter. The Centricon filter

Table 1
Calibration data

Analyte	Migration time (min)[a]	R.S.D. (time) (%)[a]	R.S.D. (area) (%)[a]	Concentration range (n*M*)[b]	LOD (n*M*)[b]	LOD (fmol)[b]	r^{2b}
IBA	10.45	0.17	2.7	20–10 000	15	0.39	0.999
IAA	12.38	0.57	3.7	20–10 000	18	0.47	0.999
IAAsp	19.37	0.21	1.6	20–10 000	28	0.73	0.999

Conditions as in Fig. 4.
[a] Migration times and relative standard deviations calculated from seven 26-nl injections of sample A.
[b] Concentration range, limit of detection (LOD) and r^2 are calculated from data for nine standard solutions. Injected volume: 26 nl.

has a molecular mass cut-off at 30 000 and was used in order to remove proteins from the sample. If compared to samples A and B, sample C is less concentrated and the signal-to-noise ratio is thereby lower. The concentration of IAA was determined, using the internal standard IBA, to be 33 ng/g fresh mass, i.e., exactly the same value as the one obtained for sample A. The IAAsp peak is below the limit of detection. The recovery for the extraction and purification was calculated to be 35% for IBA. Experiments comprising partial evaporation of methanol, in order to enhance the analyte concentrations, were not successful. Large matrix effects were observed in the electropherograms. This can be explained by the fact that the methanolic extraction is more efficient, so that hydrophobic compounds are coextracted.

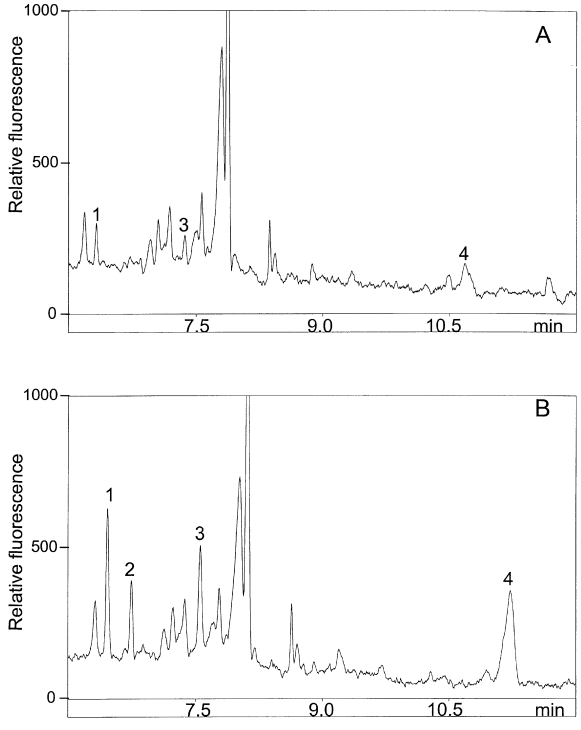

Fig. 5. Electropherogram of sample C, (A) unspiked, and (B) spiked with 100 n*M* IBA, 73 n*M* IPrA, 69 n*M* IAA and 93 n*M* IAAsp. Buffer: 30 m*M* acetate, pH 4.5. Conditions as for Fig. 4, except hydrodynamic injection of 34 nl. Peak identification: 1=IBA, 2=IPrA, 3=IAA, 4=IAAsp.

Since the analyte concentrations are lower in sample C than in samples A and B, somewhat larger volumes can be injected into the CE system without impairing the separation. This pretreatment method is suitable when the plant material contains concentrations of IAA and IAAsp above 100 ng/g. The method should be easy to miniaturise by using small filters for ultrafiltration where the sample volume required is only 50 μl. Thereby, it should be possible to reduce the quantity of plant material to approximately 20 μg.

3.3. Comparison of different detection techniques for indoles in CE

Due to the physical properties of indoles, several different detection principles can be applied for their determination, as evident from the comparison presented in Table 2.

Indoles can be detected by UV [16], but the short path length in CE leads to poor limits of detection, regarding both amount and concentration. Further, the lack of selectivity is a great disadvantage in applications where the sample matrix is complex. Extensive sample pre-treatment would be required if the detector were to be practically useful.

LIF detection is both selective and sensitive. Chan et al. have used a pulsed Kr–F laser with an excitation wavelength of 248 nm for the determi-

Table 2
Comparison of LODs obtained with different detection principles

Detection principle	Capillary I.D. (μm)	Injection volume (nl)	Injection plug length (%)	LOD (amol)			LOD (n*M*)		
				IBA	IAA	IAAsp	IBA	IAA	IAAsp
Fluorescence[a]	75	26	0.8	390	470	730	15	18	28
Fluorescence [18]	75	72	2	36 000	36 000	–	500	500	–
UV (230 nm) [17]	50	1	0.06	8800	15 000	8800	8800	15 000	8800
LIF [19]	50	4.6	0.3	–	25	–	–	5.5	–
Amperometric [17]	20	0.4	0.2	5.6	5.2	110	14	13	270

[a] The present work.

nation of indoles in urine and serum samples [19]. Even though lasers providing an excitation wavelength in the low UV region are extremely expensive, it would be interesting to apply this technique to plant extracts.

Amperometric detection of indoles, as performed by Olsson et al. [17], is very attractive, especially regarding the minimum detectable amount. Further advantages are the small sample volumes required and the fact that the amperometric detector is selective towards electroactive compounds, such as indoles. Unfortunately, the instrumentation is rather difficult to handle.

Lamp-based fluorescence detection has the selectivity of LIF detection but not the corresponding sensitivity. However, Brüns et al. have determined IAA in plant extracts using CE with fluorescence detection [18]. To overcome the problem with the poor limit of detection (500 n*M*), they had to both concentrate the sample extracts by evaporation and use a relatively large injection volume. In the present work, the special properties of the detector lead to minimum detectable amounts that are approximately 75-times better than that of Brüns et al. LODs are excellent and on the same order of magnitude as those obtained with both amperometric and LIF detection.

4. Conclusions

In this work, a method for the determination of nanomolar concentrations of IAA and IAAsp in pea plant extracts has been developed, using CE with fluorescence detection.

(1) The system is robust and easy to handle, due to the fusion of the fluorescence detector with the automated HP-3D-CE instrument. This, in combination with the relatively short total analysis time and simultaneous determination of IAA and IAAsp, makes the method suitable for routine screening analysis of a large amount of samples.

(2) Due to the selectivity of the detector, and the selectivity and high efficiency of the separation, only one simple purification step is necessary. Homogenised plant material is extracted in phosphate buffer and is then acidified and purified by solid-phase extraction. An even simpler method can be employed with samples containing indole concentrations above 100 ng/g. Homogenised plant material is then extracted in methanol and merely ultrafiltrated.

(3) The developed method requires small sample volumes, in the nanoliter range. Consequently, miniaturisation of the sample preparation method should be possible.

(4) IPrA is an alternative internal standard to IBA.

Acknowledgements

Part of this work was supported by the Swedish Council for Forestry and Agricultural Research. We are grateful to Mr. Leon Carlsson for providing the fluorescence detector, and for his skilful assistance.

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