Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 847 (2007) 162–173
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Immunoaffinity chromatography of abscisic acid combined with electrospray liquid chromatography–mass spectrometry

Veronika Hradecká [a,*], Ondřej Novák [a], Libor Havlíček [b], Miroslav Strnad [a]

[a] *Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany ASCR, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*
[b] *Isotope Laboratory, Institute of Experimental Botany ASCR, Vídeňská 1083, 142 20 Praha, Czech Republic*

Received 6 February 2006; accepted 25 September 2006
Available online 24 October 2006

Abstract

Polyclonal antibodies with high specificity for C1-immobilised (+)-*cis*,*trans*-abscisic acid (ABA) were raised, characterised by enzyme-linked immunosorbent assay (ELISA) and used for preparation of an immunoaffinity chromatography (IAC) gel. The detection limit of the ELISA was approximately 4.6×10^{-10} mol/L. Sensitive electrospray liquid chromatography–mass spectrometry (LC–ESI-MS) methods were also developed with detection limits below 0.1×10^{-12} mol. The IAC allowed quick, single-step processing of samples prior to the analyses. The LC–ESI-MS and LC–ELISA techniques were used for comparative estimation of endogenous ABA levels in immunoaffinity purified extracts of normal and water-stressed *Nicotiana tabacum* L. leaves. The analytical approaches were validated using deuterium- and tritium-labelled internal standards, respectively. The IAC method was found to be highly effective, sensitive and convenient for isolating the target analyte from plant material.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Abscisic acid; Immunoaffinity chromatography; Liquid chromatography–mass spectrometry

1. Introduction

(+)-*cis*,*trans*-Abscisic acid (ABA) is an important plant growth substance with established roles in the regulation of transpiration, stress responses, seed germination and embryogenesis. It appears to act as a signal of reduced water availability [1–3], and most effects of ABA seem to be adaptive responses that promote water conservation in plants by reducing water losses and slowing growth. However, ABA influences most aspects of plant growth and development to some extent—partly through interactions with other phytohormones [4,5].

Quantitative measurement of the endogenous levels of ABA is extremely difficult because of its instability and the low concentration of the hormone in plants, which are generally in the ng/g fresh weight (f.w.) range, although concentrations can increase several-fold in mature seeds and stressed plants [6,7]. A wide range of methods are currently employed for the determination and quantification of ABA in plants, including bioassays [8], diverse chromatographic procedures, radioimmunoassays [9–11] and enzyme-linked immunosorbent assays (ELISAs) based on competitive binding between free and alkaline phosphatase-labelled ABA [12,13]. The chromatographic techniques include liquid chromatography (LC) [14–16] and gas chromatography coupled to mass spectrometry (MS) in single ion monitoring mode [17–20] with electron impact [21], or chemical ionisation [22]. Alternatively, combinations of LC and ELISA [23], or LC and MS with ion-spray, plasma-spray [24] or electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) [25–29] may be used.

In recent years LC–ESI-MS based methods have become increasingly popular for analysing various plant hormones, including indole-3-acetic acid and cytokinins as well as ABA [30–33]. However, the sensitivity limits of this approach for determining ABA in plant tissues are relatively high, around 50 pmol/g dry weight, probably because a large number of background contaminants are present at much higher concentrations in most samples [25]. Thus, in many cases tedious

Abbreviations: ABA, (+)-*cis*,*trans*-abscisic acid; ABAMe, (+)-*cis*,*trans*-abscisic acid methyl ester; BSA, bovine serum albumin; DDC, sodium diethyldithiocarbamate; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionisation; f.w., fresh weight; IAC, immunoaffinity chromatography; LC, liquid chromatography; MS, mass spectrometry; RT, retention time

* Corresponding author. Tel.: +420 5856348750; fax: +420 5856348750.
E-mail addresses: hradeckav@post.cz (V. Hradecká), strnad@prfholnt.upol.cz (M. Strnad).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.034

purification steps are required prior to the chromatographic separation [34,35].

Therefore, to improve the rapidity, throughput and convenience of ABA purification, we have developed an immunoaffinity chromatography (IAC) method using rabbit polyclonal antibodies that we raised against C1-ABA and characterised by ELISA. As reported here, highly purified ABA preparations containing only traces of other UV-absorbing material could be obtained in this way. We also report the successful combination of the IAC with sensitive LC–ESI-MS for determining endogenous ABA levels and compare the results obtained with this approach and LC–ELISA. To demonstrate the potential of the technique, we isolated ABA from normal and water stressed tobacco leaf tissues and analysed them by LC–ESI-MS.

2. Experimental

2.1. Chemicals

(+)-*cis*,*trans*-ABA, (±)-*cis*, *trans*-ABA, (−)-*cis*,*trans*-ABA, (±)-*cis*,*trans*-ABA methyl ester, farnesol (mixed isomers), *trans*,*trans*-farnesol, bovine serum albumin (BSA) and tritium-labelled (±)-*cis*,*trans*-abscisic acid ([^{3}H](±)ABA, 50–100 Ci/mmol) were obtained from Sigma (St. Louis, MO, USA). (−)-Phaseic acid, (−)-dihydrophaseic acid, (±)-abscisylaldehyde, (±)-abscisylalcohol, (+)-neophaseic acid, (±)-7′OH-ABA and (+)-9′OH-ABA were kindly provided by S. Abrams (National Research Council, Saskatoon, Canada). (+)-*cis*,*trans*-ABA methyl ester (ABAMe) and (±)-abscisyl-β-D-glucosylester were generous gifts of Olchemim Ltd. (Czech Republic).

(+)-*cis*,*trans*-ABA was photoisomerized to (+)-*trans*,*trans*-ABA (t-ABA) by UV light giving a mixture of ABA (≅60%) and t-ABA (≅40%) as detected by TLC (toluene/ethyl acetate/acetic acid; 25:15:2, v/v). t-ABA was isolated by preparative HPLC (Beckman Instr., CA, USA). The reverse phase column was a Synergi (250 mm × 10 mm, 4 μm; Phenomenex, CA, USA). The column was equilibrated with a solution of 70% A:30% B (where A was 10% MeOH with 0.05% CH_3COOH and B was MeOH with 0.05% CH_3COOH). Initial conditions were 30% A; then a linear gradient to 50% A for 12 min; a linear gradient to 80% A for 13 min and 100% B for 2 min. Flow rate was 3.5 mL/min. Purity of the t-ABA was determined by HPLC/MS (99.5%) and half of the sample was subsequently methylated by ethereal diazomethane to give t-ABA methylester.

Alkaline phosphatase for the enzyme immunoassays (2500 U/mg) and *p*-nitrophenylphosphate were from Roche (Mannheim, Germany). Deuterium labelled (+)-*cis*,*trans*-abscisic acid ((+)-[3′,5′,5′,7′,7′,7′-$^2H_6$]ABA) was synthesised by the method of Prinsen et al. [36]. It was subsequently methylated by ethereal diazomethane and recrystallised from diethylether-cyclohexane to give (+)-[3′,5′,5′,7′,7′,7′-$^2H_6$]ABAMe (LC purity: 99.5%, λ_{max} = 265 nm), an important standard for quantification. Affi-Gel 10 was obtained from Bio-Rad Laboratories (USA). MilliQ water (Millipore, Bedford, USA) was used throughout. Other solvents and reagents were provided either by Sigma or Lachema (Brno, Czech Republic).

2.2. Preparation of antibodies and the enzyme-linked immunosorbent assay (ELISA)

Polyclonal antibodies were raised in rabbits against ABA conjugated to BSA through its carboxyl group (C1) according to Weiler [10]. The immunisation schedule and purification of antibodies followed the procedures described by Strnad et al. [37,38]. Anti-ABA antibodies isolated from the rabbit serum by ammonium sulphate precipitation were lyophilised, stored at −20 °C and used in ELISAs as follows.

A 96-well microtitre plate (GAMA, České Budějovice, Czech Republic) was coated with 150 μL per well of rabbit anti-ABA antibodies (4.06–4.67 μg/mL) in 50 mM $NaHCO_3$ (pH 9.6) and incubated at 4 °C overnight. The wells were washed twice with H_2O, filled with 200 μL BSA solution (0.2 g/L) in Tris-buffered saline (TBS; 50 mM Tris, 10 mM NaCl, 1 mM $MgCl_2$, 0.1% NaN_3, pH 7.5), and the plate was incubated at 25 °C for 1 h. The wells were then washed twice with H_2O and subsequently filled with 100 μL TBS, 50 μL of standard or sample in TBS and 50 μL ABA-alkaline phosphatase tracer (0.02% BSA in TBS). After 2 min shaking and 1 h at 25 °C, the plate was decanted, washed four times with H_2O, filled with 150 μL per well of *p*-nitrophenylphosphate solution (1 mg/mL) in 50 mM $NaHCO_3$ (pH 9.6) and incubated for 1 h at 25 °C. The reaction was stopped by adding 50 μL per well of 0.5 M NaOH and absorbance was measured at 405 nm using a Labsystem Multiscan®PLUS photometer (Labsystem, Finland). The resulting data were processed by the ImmunoRustregAnalyser computer program (Laboratory of Growth Regulators, Olomouc, Czech Republic).

2.3. Preparation and use of immunoaffinity columns

The IgG fraction (including anti-ABA antibodies) was isolated from rabbit serum using a column of Protein A-sepharose beads (supplied by Sigma) following the manufacturer's recommended protocol and concentrated by ammonium sulphate precipitation. The antibodies were dialysed against 0.1 M MOPS (3-[N-morpholino]propanesulfonic acid, Sigma; pH 8.0) and coupled to *N*-hydroxysuccinimide-ester activated agarose (30 mg IgG/mL Affi-Gel 10, Bio-Rad, Hercules, USA). IAC columns were then created by dispensing 0.6 mL portions of the immunoaffinity gel into 3 mL polypropylene syringes and stored in phosphate-buffered saline (PBS; 50 mM NaH_2PO_4, 15 mM NaCl, 0.1% NaN_3, pH 7.2) at 4 °C. A precolumn containing 0.5 mL of rabbit IgG immobilised on Affi-Gel was coupled to the anti-ABA column (to eliminate compounds that unselectively bind to the Affi-Gel), and the combined set was conditioned in PBS. Immediately after elution of each sample, both gels were regenerated in preparation for the next series of samples by 9 mL H_2O, 3 mL MeOH, 9 mL H_2O, and finally re-conditioned by 9 mL PBS. Methanol was removed from the eluates by a nitrogen stream.

2.4. Plant material and sample preparation

To test the suitability of the developed methods for analysing endogenous ABA levels in plants, *Nicotiana tabacum* L. plants were cultivated at +20 °C under 12-h dark/12-h light cycles in a greenhouse and leaves were harvested from well-watered 8-week-old plants and corresponding plants that had not been watered for 1, 2, 3, 4 and 5 days. The leaf samples were immediately frozen in liquid nitrogen and then stored at −80 °C until used. Two sample processing procedures were employed.

In the first procedure, frozen plant material (1 g samples) was homogenised in ice cold 70% ethanol containing DDC (sodium diethyldithiocarbamate, 400 μg/g f.w.) as an antioxidant. To check recovery during purification and to validate quantification, either deuterium-labelled ABA (100 pmol $[^2H_6]$(+)ABA for LC–ESI-MS) or (71,000 dpm, 1183 Bq, $[^3H](\pm)$ABA for LC–ELISA) was added to the samples as an internal standard. After a 3 h extraction, the homogenate was centrifuged (15,000 rpm, 20 min, 4 °C) and the pellets were extracted again in the same way. The combined extracts were transferred by pipette onto a 3 mL C_{18} column (reverse phase), which was washed twice with 4 mL 80% methanol and the combined methanolic samples were concentrated to approximately 1.0 mL by rotary evaporation at 35 °C. The samples were diluted to 20 mL in 40 mM ammonium acetate buffer (pH 6.5) and passed through two columns in tandem: a DEAE Sephadex A-25 (Sigma; 1 mL/g fresh weight) column coupled to a Sep-Pak C_{18} cartridge (Waters, Ireland). After loading the samples the columns were washed with 15 mL of 40 mM CH_3COONH_4 (pH 6.5). ABA retained on the Sep-Pak C_{18} was eluted with 5 mL 80% MeOH (pH 3.6; −20 °C) and the eluates were dried under vacuum. Before use, the Sep-Pak C_{18} cartridges were activated with 10 mL MeOH, 5 mL H_2O, and 5 mL 40 mM CH_3COONH_4 (pH 6.5). The ABA fractions were dissolved in 100 μL MeOH, then 300 μL of ethereal diazomethane was added to methylate the ABA, and after 10 min incubation at room temperature, the samples were evaporated under a nitrogen stream.

The purified samples from the DEAE-C_{18} columns were dissolved in 100 μL of 70% EtOH and 400 μL PBS and passed through the IAC columns five times. After washing with 9 mL H_2O, the IAC columns were eluted with 3 mL 80% MeOH (pH 3.0, −20 °C) into silanized glass tubes. After evaporation of the samples under a nitrogen stream, the residues were reconstituted in 100% MeOH and 10 mM HCOOH (30:70) or in 100% MeOH and 0.1% CH_3COOH (25:75) and analysed by either LC–ESI-MS or LC–ELISA, respectively.

The second procedure was a very simple method for extracting ABA from the plant material. The frozen tobacco leaf tissue (0.1–0.25 g f.w.) was homogenised by a vortex mixer (Maxi Mix II, Cole-Parmer, IL, USA) and extracted in 5 mL of one of the following cold extraction buffers: MilliQ water, 40% ethanol, 70% ethanol or 80% methanol, either unadjusted (neutral) or acidified to pH 3.5 with CH_3COOH. Each of the eight extraction media contained DDC (400 μg/g f.w.) and 100 pmol $[^2H_6]$ABA. After 1 h extraction at 4 °C the samples were centrifuged (15,000 rpm, 20 min, 4 °C), filtered centrifugally (4500 rpm, 5 min, 4 °C) through a 0.45 μm PP filter (Whatman, UK) and the supernatants were dried under vacuum. The extracts were methylated, processed by IAC and analysed by LC–ESI-MS as described above.

All extraction and purification steps were validated by processing a control sample containing internal labelled standards in the extraction buffer to demonstrate the occurrence of any ABA remaining adsorbed on different column materials after washing and before re-using them.

2.5. LC–ELISA analysis

Immuno-purified samples containing $[^3H](\pm)$ABA as a recovery marker were analysed by LC–ELISA. The equipment used consisted of a 2690 Separations Module solvent delivery system coupled to a 996 photodiode array detector (UV-PDA, scanning range 200–400 nm) and a Symmetry C_{18} reversed-phase column (150 mm × 2.1 mm, 5 μm), all from Waters, Milford, USA. The column was equilibrated with a 25% A:75% B solution (where A was 100% MeOH and B was 0.1% CH_3COOH) before each sample was applied, and analytes were eluted using a linear gradient of 75–50% B over 15 min, followed by a linear gradient to 37% B at 24 min (the balance provided by A throughout). The column was then washed with 100% MeOH for 2 min, and regenerated using 75% B over 8 min before applying the next sample. The flow rate was 0.3 mL/min throughout and the injection volume was 50 μL. Timed fractions (0.5 min) were collected by a FRAC 100 fraction collector (Pharmacia, Uppsala, Sweden), dried under a nitrogen stream and redissolved in 400 μL TBS buffer. Aliquots of 0.15 mL fractions were investigated in duplicate by scintillation counting (LS 6500, Beckman Instruments, Fullerton, USA) and C1ABA2 ELISA. The ABAMe contents in the immunoreactive fractions were expressed as ABA equivalents, using signal to equivalent concentration ratios obtained from a series of ELISAs including dilution analyses and internal standardisation [13,39,40]. The values were corrected for recovery of $[^3H](\pm)$ABA.

2.6. LC–ESI-MS analysis

LC–ESI-MS analyses were performed using the 2690 Separations Module (Waters, Milford, MA, USA) linked to a ZMD 2000 single-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface and a 996 PDA photodiode array detector (Waters, Milford, USA). Samples containing ABA were injected onto a Symmetry C_{18} reversed-phase column (150 mm × 2.1 mm, 5 μm; Waters, Milford, USA) which was eluted with a linear gradient of 30–60% A (0–12 min) followed by 60–80% A (12–20 min), with a final 1 min at 80% A, where again A was 100% methanol and B 10 mM HCOOH. A second separation clearly distinguishing all ABA isomers was based on an elution with a mixture of solvents A (100% acetonitrile) and B (15 mM $HCOONH_4$, pH 4.0) in which A changed linearly from 10 to 50% (0–25 min). The column temperature was set to 30 °C, the flow rate to 0.25 mL/min and the volume of standard or sample injections to 30 μL. The eluent was introduced into the electrospray interface using a postcolumn split of 1:1, (capillary voltage +3.5 kV, desolvation gas temperature 250 °C, source temperature 100 °C, cone

Table 1
Parameters of the enzyme-linked immunosorbent assay using different polyclonal anti-C1-ABA antibodies

	Antibody no.		
	C1ABA1	C1ABA2	C1ABA3
Unspecific binding (%)	0.3	1.2	2.3
Detection limit (mol/L)	1×10^{-9}	4.6×10^{-10}	4.6×10^{-10}
Midrange (mol/L)	7×10^{-9}	4×10^{-9}	4×10^{-9}
Linear average of log/logit plot (mol/L)	1×10^{-9} to 3.8×10^{-8}	4.6×10^{-10} to 3.8×10^{-8}	4.6×10^{-10} to 3.8×10^{-8}
Intraassay variance (%)	1.7	0.66	0.67
Interassay variance (%)	2.24	2.28	1.41
Amount of tracer/assay (ng)	8	8	8

voltage +20 V). The detector parameters were: dwell time 1.20 s, interchannel delay 0.05 s, span size ± 0.50 *m*/*z*. The dwell time was calculated to give 16 scan points per peak and quantification was done by SIM analysis of quasi-molecular ions of $[M+H]^+$. The PDA detector was used in the scanning range of 200–400 nm and the eluate was monitored at 265 nm.

The data were processed by Masslynx software (Version 4.0, Micromass, Manchester, UK) using a standard isotope dilution method. The ratio of endogenous ABA (ABAMe) to deuterium-labelled ABA (ABAMe) standard was determined and further used to quantity the level of endogenous ABA in the original extract, based on the known quantity of added $[^2H_6]$ABA.

3. Results and discussion

3.1. Anti-ABA antibody characteristics

All immunised rabbits produced antisera to C1-ABA protein conjugate, but serum titres differed considerably, reflecting differences in the responses between the animals. The antibodies designated C1ABA2, which showed high specificity for ABA, were selected and routinely used for ABA ELISA. In ELISA assays, the antibodies were used in conjunction with ABA-alkaline phosphatase conjugate as a tracer and ABAMe as a reference standard. Selected assay parameters are summarised in Table 1. The mean standard curve obtained for the C1ABA2 ELISA and the corresponding log/logit plot are shown in Fig. 1. As little as 0.1×10^{-12} mol could be detected by the ELISA. Within the measuring range, the standard curves were almost linear over three orders of magnitude, with only small inter- and intra-assay variations. The inset shows the linearised curve, providing a measuring range between 4.6×10^{-10} and 3.8×10^{-8} mol/L. The measuring range of the assay compares favourably with those of other published ABA immunoassays [10–13,35,40].

The specificity of the antibodies was determined by cross-reactivity studies and the results are shown in Table 2. The polyclonal antibodies reacted most strongly with ABAMe

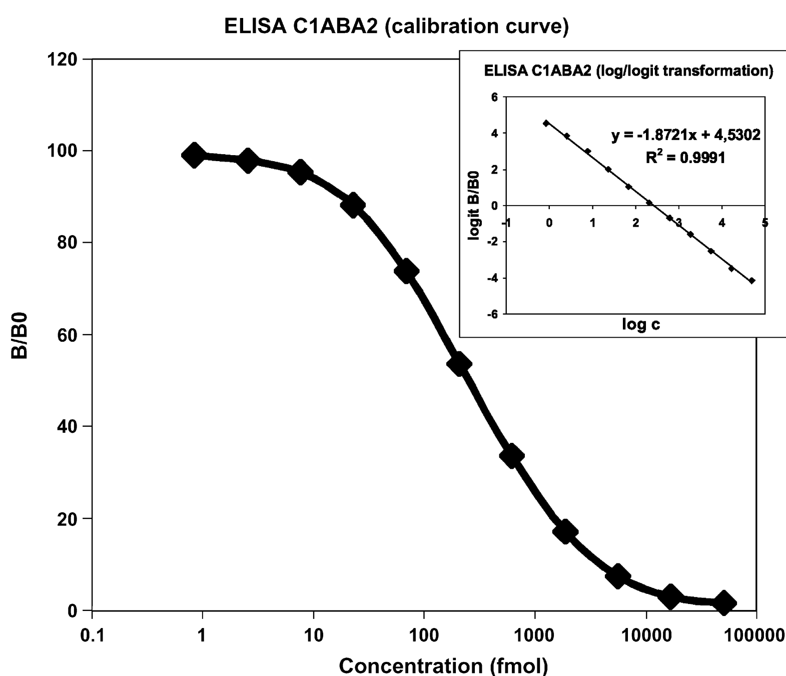

Fig. 1. Typical standard curve obtained in enzyme-linked immunosorbent assays with C1ABA2 antibody. B/B_0 is the relative binding (%) of tracer (ABA-alkaline phosphatase conjugate) in the presence (B) relative to the absence (B_0) of respective ABAMe standards. Bars indicate standard deviations of duplicates ($n = 20$). The inset shows the linearized log/logit plot of the same data (logit $B/B_0 = \ln[B/B_0{:}\ 100 - B/B_0)]$). Intrassay variation was 0.66–1.7% for the linear measuring range of the assay.

Table 2
Molar cross-reactivities of ABA and structurally related compounds

	C1ABA1	C1ABA2	C1ABA3
(+)-*cis*,*trans* Abscisic acid methyl ester	100	100	100
(±)-*cis*,*trans* Abscisic acid methyl ester	29.80	26	46
(−)-*cis*,*trans* Abscisic acid methyl ester	0.90	0.72	1.52
(±)-*cis*,*trans* Abscisic acid glucosylester	40.08	35.60	29.50
(+)-*trans*,*trans* Abscisic acid methyl ester	0.93	0.76	0.36
(+)-*trans*,*trans* Abscisic acid	0.04	0.09	<0.01
(+)-*cis*,*trans* Abscisic acid	4.24	4.01	2.61
(−)-*cis*,*trans* Abscisic acid	0	0.02	0
(±)-*cis*,*trans* Abscisic acid	1.77	2.47	2.38
(±)-*cis*,*trans*-7′OH ABA	0	0.14	<0.01
(±)-*cis*,*trans* Abscisic acid aldehyde	11.87	1.62	2.73
(±)-*cis*,*trans* Abscisic acid alcohol	6.48	2.23	2.60
(+)-*cis*,*trans*-9′OH ABA	0	0	0
(−)-Phaseic acid	<0.01	0	0
(−)-Dihydrophaseic acid	0	0	0
(+)-Neophaseic acid	0	<0.01	<0.01
Farnesol	0	0	0
trans,*trans*-Farnesol	0	0	0

Data presented are expressed as percentage ratio of molar concentration of ABAMe and competitor giving 50% binding. Varying concentrations of the compounds were tested in ELISA for their ability to displace the alkaline phosphatase labelled ABA from the antibodies.

(100%) and relatively well with (±)-*cis*,*trans*-ABA glucosylester (29–40%) and (±)-*cis*,*trans*-ABAMe (26–46%). (±)-*cis*,*trans*-ABA alcohol (2.2–6.5%), (±)-*cis*,*trans*-ABA aldehyde (1.6–12%), and natural ABA (2.6–4.2%, (+)-*cis*,*trans* enantiomer) were much less imunoreactive. (±)-*cis*,*trans*-ABA (1.7–8.5%) was even less reactive, and immunoreactivity to the (−)-enantiomer was close to zero. The (+)-*trans*,*trans*-ABA isomer and its methylester, which have been reported to occur in some plant extracts probably because of an enzymatic reaction or as a result of phoitoisomerization during extraction [41,42,43], were almost nonreactive. Cross-reactivity with the ABA-catabolites (−)-phaseic and (−)-dihydrophaseic acid, and several ABA-related compounds, such as (+)-*cis*,*trans*-9′OH ABA, (±)-*cis*,*trans*-7′OH ABA, farnesol and *trans*,*trans*-farnesol was negligible. Weiler [13] also produced antiserum using this type of (+)-*cis*,*trans*-ABA protein conjugate. The antibodies they prepared were more reactive towards (+)-ABA than to (−)-ABA and had high affinity for both the free acid and C1 derivatives. Less well characterised antibodies to ABA, but of the same quality, have also been raised by other groups [35,44]. In contrast to the ELISA described by Weiler [13], in our case methylation of standards and samples resulted in a marked increase (20–50-fold) in assay sensitivity. The results contrast with all known reports of ABA antibodies prepared against carboxyl-linked haptens, which usually show equally high affinity for ABA and ABAMe. Our results are, however, closely analogous to those obtained in immunological studies of indole-3-acetic acid and the gibberellins, in which carboxyl-linked haptens have also yielded antibodies that do not bind well the free carboxylate [45,46].

3.2. Immunoaffinity chromatography

A mixture of the high affinity antibodies C1ABA1 and C1ABA2 was employed to prepare the immunoaffinity gel. The IAC was done using coupled pre-immune and immune cartridges, each containing 0.6 mL of the gel. Measurement of ABA retention at various applied concentrations of ABAMe with the tritium-labelled tracer confirmed that ABAMe is not retained on pre-immune columns with rabbit IgG (data not shown). The immunoaffinity-bound ABA can be eluted with a variety of disruptive agents, but the most convenient for subsequent steps appears to be acidified 80% methanol (pH 3.0). The immobilised antibodies showed unexpected stability, losing activity very slowly during repeated elution with methanol (<5% per 10 samples) and gave the highest recoveries in this solvent. Indications that significant losses tend to occur in the capacity of IAC columns with repeated use have been reported [47], but no such indications were found in our study.

The most important IAC parameter is capacity because the recoveries are no longer constant and the calibration range no longer linear when IAC columns are overloaded. The sorbent capacity is defined as the maximum amount of the analyte that can be pre-concentrated by a given volume of immunosorbent [48,49]. When testing our dynamic column capacities, amounts of ABA and ABAMe standards ranging from 10 pmol to 10 nmol were applied to 0.6 mL of the gel and the binding capacities and recovery were determined by LC. The capacities of the IAC for (+)-*cis*,*trans*-ABA and (+)-*cis*,*trans*-ABAMe were about 1.7 ± 0.2 and 8 ± 0.9 nmol/mL, respectively. In comparison, Crozier et al. [50] found 1.7 g of affinity support to bind only 80 ng of indole-3-acetic acid and Nicander et al. [51] found 1 mL of affinity support to bind around 1 nmol of various cytokinins. Thus, the capacity of our IAC columns is slightly higher than those of polyclonal immunocolumns prepared for other phytohormones [50,51]. However, monoclonal antibody-based columns have greater capacities [52]. For this reason it would be of great interest to develop monoclonal anti-ABA IAC materials in the near future. Recoveries when 50 pmol/mL to 6.5 nmol/mL ABAMe was applied were higher than 90%,

Table 3
Capacity of 600 μL of the immunoaffinity gel with mixed immobilised C1ABA1 and C1ABA2 antibodies, and analyte recoveries, observed in tests with (A) 1–10 nmol of (+)-*cis*,*trans*-abscisic acid methyl ester (ABAMe) and (B) 0.025–10 nmol of (+)-*cis*,*trans*-abscisic acid (ABA)

	ABAMe (nmol)									
	1	2	3	4	5	6	7	8	9	10
Part A										
Gel capacity (pmol/0.6 mL)	1048.4	2018.4	3000.8	3845	4366.4	4804.1	4796.9	4894.4	4793.6	4981.6
Recovery (%)	104.8	100.9	100	96.1	87.3	80.1	68.5	61.2	53.3	49.8
S.D. (%)	0.2	3.4	4.8	2.4	0.7	2.3	4.2	4.7	3.7	7.5

	ABA (nmol)						
	0.025	0.1	0.5	1	2	5	10
Part B							
Gel capacity (pmol/0.6 mL)	21.7	72.9	333.4	564.3	948.2	1122.7	1012.5
Recovery (%)	86.6	72.9	66.7	56.4	47.4	22.5	10.1
S.D. (%)	3.3	3.6	6.3	7.4	5.9	1.1	1.4

and recoveries were still higher than 50% even when up to 15 nmol/mL of the standard was applied (Table 3).

The effectiveness of the IAC for purifying ABA from plant tissue extracts was evaluated using an LC equipped with a photodiode array detector (PDA). In tobacco leaf extracts large numbers of UV-absorbing substances are present even after ion-exchange (DEAE-Sephadex A-25) and reversed phase (Sep-Pak C_{18}) chromatographic steps. Water extracts without IAC were even more heavily contaminated (data not shown). Comparison of LC chromatograms before (0.2–0.6 AU) and after (0.001–0.006 AU) IAC (Fig. 2) show that considerable purification was achieved by this step. The absence of interfering peaks in the vicinity of the ABAMe peak even allowed very precise quantification on the basis of UV peak area measurements. IAC has been used in one previous study to isolate ABA from plant extracts [52] and in another for the enantiomeric separation of commercially available racemic $[^3H](\pm)$ABA [53]. The advantages of using IAC prior to LC–ESI-MS have not previously been shown, but the LC–ESI-MS chromatograms of partially purified (C_{18}, DEAE-C_{18}) and immunopurified plant extracts showed that the immunoaffinity purification can also strongly increase the selectivity and sensitivity (more then 10-fold) of LC–ESI-MS analysis. The potential of the method is clearly illustrated by the data in Fig. 3, showing that immunopurified samples give a strong signal in single ion recording mode (SIR) at *m/z* 279.2 (corresponding to ABAMe). The traces of internal deuterium standard at *m/z* 271.2 and 285.2 were even cleaner of contaminants (data not shown). In contrast, the partially purified (C_{18}, DEAE-C_{18}) extracts of the same sample were contaminated by many impurities and the signal was, consequently, weaker and more difficult to resolve.

3.3. LC–ESI-MS

(+)-*cis*,*trans*-Abscisic acid yielded quasi-molecular ions of $[M-H]^-$ in electrospray-negative mode (ESI−, *m/z* 263) and $[M+H]^+$ in electrospray-positive mode (ESI+, *m/z* 265). In positive ion mode, *m/z* 287 ions corresponding to $[M+Na]^+$ and the dehydrated protonated molecule $[M+H-H_2O]^+$ (*m/z* 247) have also been detected in a previous investigation [29]. We further investigated the effects of various parameters (e.g. desolvation temperature, capillary and cone voltage) on the sensitivity of mass spectrometric detection and the optimal values found were used for the LC–ESI-MS method (see Fig. 2). Due to the presence of a carboxyl group, very sensitive electrospray MS measurements could be obtained using negative ionisation [25,27,29]. However, under the conditions tested, specific $[ABA+H]^+$ ions were also observed, which are not usually used in ABA analyses. The best results were obtained for ABAMe in ESI+ mode. Compared to previously described procedures for ABA analyses (with reported sensitivities of 0.76 pmol g^{-1} DW, 3.8 pmol g^{-1} FW and 50 pmol g^{-1} FW; [25,27,29]) this approach exhibits ca. 10-fold higher sensitivity. Prinsen et al. [30] also obtained the best sensitivity for auxin methylesters. Although methylation is not an absolute prerequisite for the ABA analysis, the derivatization of samples before IAC and/or LC–ESI-MS analyses allowed us to improve not only the response to ABA, but also the affinity and capacity of the IAC, and ABA stability, in the extracts. Thus, methylation of samples and standards may be regarded as advantageous in this context since it improves the stability, extraction yield, recovery, sensitivity and reliability of ABA analyses.

Quantification was performed using the standard isotope dilution method. Final concentrations were calculated from areas of the *m/z* 271.2 (285.2) peak for labelled and *m/z* 265.2 (279.2) peak for authentic ABA (ABAMe), in the SIM chromatograms. Under the LC separation conditions the limits of detection (LOD), retention time stability, linear ranges and linear regression equations found for standard compounds are given in Table 4. LODs (defined as a signal to noise ratio of 3) range from 100 to 250 fmol. The data shown were obtained by analysing each analyte at nine different concentrations, ranging between 0.1 and 1000 pmol per injection. After log-transformation, a linear regression function adequately described the ratios between concentration and integrated area units of the corresponding peak signals within the concentration range. The results clearly show that the method gives good linear correlation, with correlation coefficients ranging from 0.9990 to 0.9993.

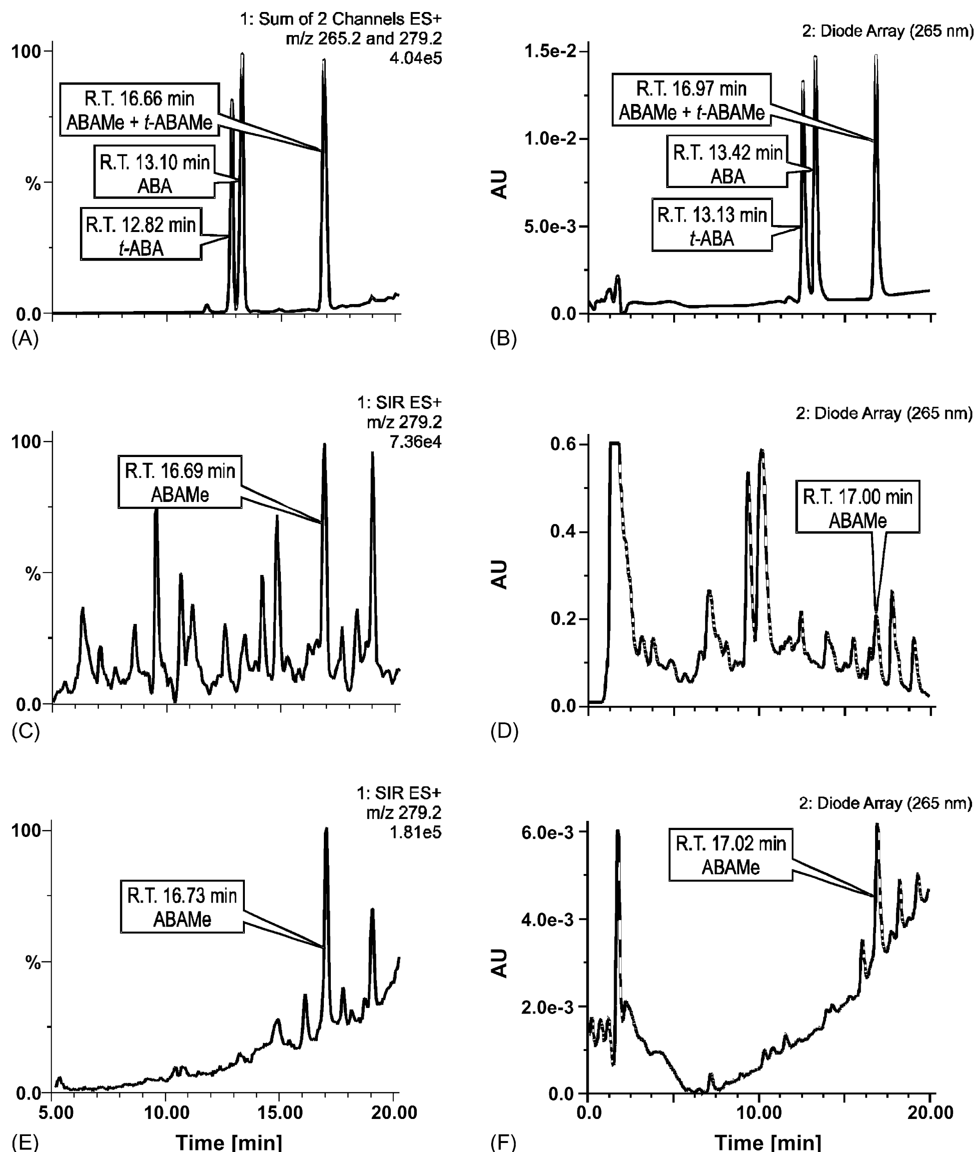

Fig. 2. Effect of immunoaffinity chromatography on subsequent analysis of ABA in tobacco leaf extracts by reversed-phase LC followed by ESI-MS (A, C, E) and PDA (B, D, F); the injected amount (30 μL) corresponds to an extract from 0.33 g f.w. tissue. ABA, t-ABA and ABAMe standards (A and B) and ethanolic extract before (C and D) and after IAC (E and F). LC conditions as follows: a Symmetry C_{18} column (150 mm × 2.1 mm, 5 μm) eluted with a linear gradient of 30–60% A (0–12 min) followed by 60–80% A (12–20 min), with a final 1 min at 80% A, where A was 100% methanol and B 10 mM HCOOH, column temperature: 30 °C, flow rate: 0.25 mL/min.

The linear ranges found for ABA (0.5–1000 pmol) and ABAMe (0.25–1000 pmol) are also in good agreement with previously published data for auxin methylesters [30]. Prinsen et al. [30] did not investigate the sensitivity and linear range of 2 mm i.d. columns, but our sensitivity results are intermediate between the results they obtained for conventional (4.6 mm i.d.) and micro (1 mm i.d.) columns.

3.4. Optimization and validation of the procedure

ABA is readily soluble in a variety of solvents and therefore has been traditionally extracted by relatively cheap, non-hazardous solvents such as aqueous methanol (80–90%) [54–57] or acidified (0.5–2%) acetone (70–100%). It has been recently shown that water [25] and 40% ethanol can also be effective solvents. Milborrow and Mallaby [58] have presented indications that use of alkaline or even neutral methanol can lead to the breakdown of ABA conjugates found in plants to ABAMe [59,60]. To test the efficiency of our extraction method, its compatibility with IAC, the possibility that it may cause the conversion of ABA conjugates to ABAMe and the possible effects of such conversion on the analytical results, we compared the results obtained with several extraction and purification methods for profiling ABA. A first series of samples was extracted by the classical purification method currently used by several laboratories in plant hormone analyses (i.e. each sample was extracted twice in 70% ethanol for 3 h) and the resulting extracts were purified by several different chromatographic steps (using a C_{18} column, DEAE Sephadex coupled with Sep-Pak C_{18} cartridges and IAC). In a second series of experiments we tried to accelerate

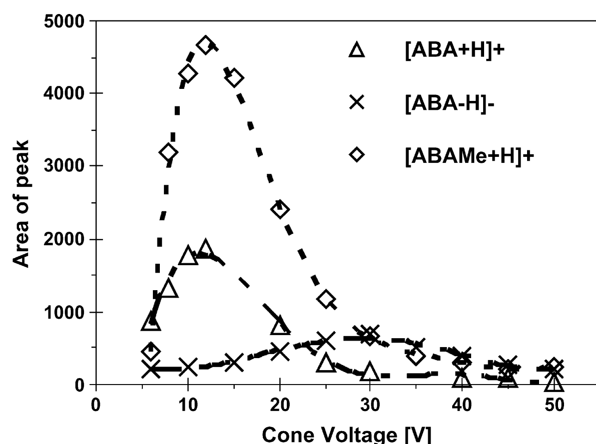

Fig. 3. Sensitivity test of LC–ESI-MS analyses in SIM mode of quasi-molecular positive $[M+H]^+$ (*m/z* 265) and negative $[M-H]^-$ (*m/z* 263) ions of ABA and its methyl ester (*m/z* 279) in relation to cone voltage (5 pmol of each compound per injection).

this procedure and replaced the complex purification procedure by single-step IAC. In this series tobacco leaf samples were extracted with MilliQ water, 40% ethanol, 70% ethanol or 80% methanol for 1 h followed by the IAC. All extraction solvents used gave quite consistent results (Table 5). The ABA levels in neutral tobacco leaf extracts were only 6.5–8.5% higher than in acid organic extracts. Thus, if the use of neutral solvents leads to the formation of ABAMe from natural ABA conjugates, as suggested for neutral methanol (see above), then the amounts involved appear to be minor. The lowest levels were obtained after water extraction, but still the estimated endogenous concentrations in tobacco leaves were only ca. 12% lower than those obtained after organic solvent extraction.

The classical purification procedure yielded 20–50% recoveries (Table 6), similar to the values obtained for cytokinins [33]. In the second series, based on simple IAC purification, about 80–90% of the ABA was recovered (Table 5), showing much higher yields than the classical approach, probably due to the greatly simplified purification procedure retaining more of the extracted ABA. The IAC–LC–ESI-MS also has additional advantages originating from the immediate stabilization of ABA by methylation shortly after the extraction. In the classical approach ABA is methylated later, often after several chromatographic steps, and can thus be more easily degraded. Furthermore, it is highly likely that calculated concentrations obtained using the simplified procedure (see Table 5 compared to the value 2261 ± 53 pmol/g f.w. of the same samples purified by classical procedure) will be almost identical to those obtained using more complex extraction and purification approaches (provided that it is well standardised). The IAC avoids the use of time-consuming purification steps and numerous samples can be processed in parallel. With the present procedure more than 50 samples can be processed in less than 3 days using this approach.

The method was first evaluated using regular calibration data obtained with tissue-free samples. However, since plant tissue samples contain numerous compounds (including endogenous ABA) the method of standard additions was also used to evaluate performance in the presence of matrix suppresion and the results can be seen in Fig. 4. For experiments shown in Fig. 4, dose-response curves were determined using aliquots of tobacco leaf extracts each corresponding to 100 mg of fresh weight extracted in 5 mL cold 80% methanol (pH 3.0), for which the endogenous ABA levels had been determined by the standard procedure. Portions (5 mL) of these extracts were spiked with 0, 50, 250, 450 and 950 pmol ABA and 100 pmol $[^2H_6]$ABA, they were then purified by IAC and analyzed by LC–ESI-MS. As shown in Fig. 5, 1:1 linear relationships between the added and detected amounts of ABA were also obtained in the presence of matrix effects, while a larger intercept reflected the presence of endogenous ABA after spiking with internal standard [28]. The very small standard deviations of the mean values (<5% in Fig. 4; blank samples consistently exhibited zero responses) demonstrate that the reproducibility between repeated experiments was much higher when the IAC–LC–ESI-MS approach was used

Table 4
Dynamic range, detection limit, retention time stability and linearity (linear regression and correlation coefficients) for ABA and ABAMe quantifications using LC–ESI-MS.

Compound	Detection limit[a] (fmol)	Dynamic range (pmol)	Retention time stability (min)	Linear regression equation	ρ^2
ABA	250	0.5–1000	13.53 ± 0.03	$y = 1.00(\pm 0.02)x + 0.33(\pm 0.05)$	0.9990
ABAMe	100	0.25–1000	17.01 ± 0.04	$y = 1.03(\pm 0.01)x + 0.17(\pm 0.04)$	0.9993

All measurements (five replicates) were made using 30 μL injections.
[a] Based on a signal to noise ratio of 3:1.

Table 5
Evaluation of methods used to extract ABA from leaves of *Nicotiana tabacum* L. plants that had been water-stressed for 4 days

Extracting buffer (neutral)	LC–ESI-MS (pmol/g f.w.)	Recovery (%)	Extracting buffer (acidified)	LC–ESI-MS (pmol/g f.w.)	Recovery (%)
MiliQ water	2440 ± 8	72 ± 5	MiliQ water	2179 ± 6	84 ± 3
40% Ethanol	2525 ± 9	76 ± 1	40% Ethanol	2370 ± 41	78 ± 5
70% Ethanol	2447 ± 62	70 ± 1	70% Ethanol	2292 ± 78	87 ± 9
80% Methanol	2404 ± 43	82 ± 3	80% Methanol	2213 ± 155	85 ± 7

Plant tissues were extracted with different extracting buffers for 1 h, methylated, purified by immunoaffinity chromatography and analysed by LC–ESI-MS. Data shown are means ± S.E. of 3 replicates.

Table 6
Endogenous ABA levels in control and water-stressed tobacco plants

Sample collection Time (h)	Endogenous ABA levels (pmol/g f.w.)					
	$[^2H_6]$ABA		$[^3H]$ABA		$[^3H]$ABA	
	LC–ESI-MS	Recovery (%)	PDA-LC	Recovery (%)	LC–ELISA	Recovery (%)
Control plants						
0	71 ± 10	40 ± 0.9	66 ± 32	39 ± 2	45 ± 12	27 ± 0.6
Water-stressed plants						
24	648 ± 5	41 ± 1	536 ± 12	36 ± 1	484 ± 22	24 ± 2
48	633 ± 86	45 ± 0.7	587 ± 6	43 ± 3	510 ± 36	20 ± 5
72	567 ± 14	54 ± 8	555 ± 22	41 ± 0.7	490 ± 35	29 ± 1
120	529 ± 98	42 ± 1	410 ± 6	42 ± 2	410 ± 42	29 ± 0.9

Two triplicate series of the same samples were extracted in 80% methanol and purified using a C_{18} column, a coupled DEAE Sephadex and C_{18} Sep-Pack system, and (after methylation) immunoaffinity chromatography. ABAMe was then analyzed by LC–ESI-MS (*m/z* 279.2), LC with a photodiode array detector (265 nm) and immunoassays (C1ABA2 ELISA) of 0.15 ml LC fractions. The values have been corrected for the recovery of internal $[^2H_6]$- or $[^3H]$-ABA standards added during extraction.

compared to results from LC–MS–MS analyses reported by other laboratories [25–29].

To further validate the method for analyses of plant samples the LC–ESI-MS based approach was compared with LC–ELISA analysis. Using the highly specific anti-C1ABA2 antibodies, half-minute HPLC fractions of real samples were tested by ELISA. Fig. 5 shows an immunohistogram and mass chromatogram of one of the immunopurified samples, illustrating clearly a *m/z* 279.2 ($[M+H]^+$) peak at a retention time of 16.80 min. C1ABA2 ELISA of LC fractions of the same sample also detected immunoreactivity at a retention time corresponding to ABAMe (20.50 min). Furthermore, LC–ELISA detected another, more polar immunoactive peak which was putatively attributed to ABA-β-D-glucosylester based on its co-chromatography with an authentic standard (UV-absorbing peak at λ_{max} = 274 nm, retention time 8.5 min). The immunoreactive conjugate found in significant amount was identified as abscisyl-β-D-glucosylester by LC–MS/MS and by exact mass determination using Q-TOF mass spectrometry (data not shown). Since the electrospray mass spectrometer is a concentration-selective detector [61,62], the flux from the chromatography column can be split without consequent loss of sensitivity. In the present method 50% of the flow was introduced into the PDA, in order to obtain additional spectral information. This approach allowed us to detect other ABA-like UV-absorbing peaks (λ_{max} = 265–270 nm) in the immunopurified extracts (see also Fig. 2), whose retention times do not correspond with those of known ABA metabolites. For example, the occurrence of (+)-*trans*,*trans*-ABA has been reported in a few tissues [63], but in low amounts unlikely to interfere with the ABA purification because of the very low cross-reactivity of antibodies for t-ABAMe (C1ABA2: 0.76%). For example, if 10% of the total free ABA is present as the *trans*-isomer, the value obtained for t-ABAMe will be below 0.1 pmol when competing with 100 pmol ABAMe. Thus, the IAC should lead to almost complete removal of t-ABA. The photoisomerization of ABAMe to t-ABAMe after IAC is unlikely as the methylester of natural ABA is very stable against UV radiation and thus additional increase in t-ABAMe is impossible (data not shown). The effectiveness of the IAC to separate both isomers was checked by both UV and MS following a different LC system. Both isomers can also be readily separated by a second LC run in an acetonitrile gradient, as shown on UV traces in Fig. 6. The UV profile for the extract after IAC indicated excellent purification of ABA with only a minor interfering peak of t-ABAMe (RT: 22.85 min, λ_{max} = 265 nm, t-ABAMe content <1.0% of the ABAMe peak).

Given the high specificity of the antibodies, the detection of other peaks with appropriate UV and MS characteristics strongly indicates the potential existence of previously unknown ABA-like molecules in plants. The high purity of the immunopurified samples could facilitate structural elucidation of the compounds, which is currently in progress.

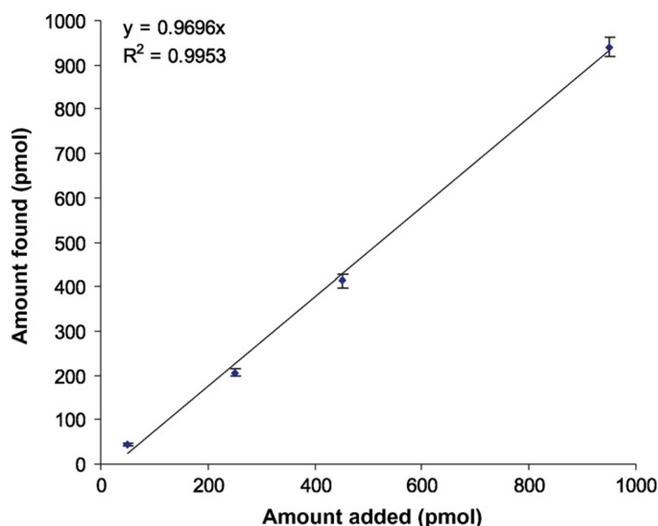

Fig. 4. Accuracy of the LC–ESI-MS analysis of endogenous ABA levels. Water-stressed *Nicotiana tabacum* L. leaves (3 days) corresponding to 100 mg FW were extracted (5 mL extract volume). At the beginning of the extraction, 0, 50, 250, 450 or 950 pmol of ABA together with 100 pmol $[^2H_6]$ ABA were added, and the endogenous amount of the hormone was quantitatively determined. Results of two independent experiments are shown. R^2, correlation coefficient; the slope of each graph indicates the accuracy of the analysis.

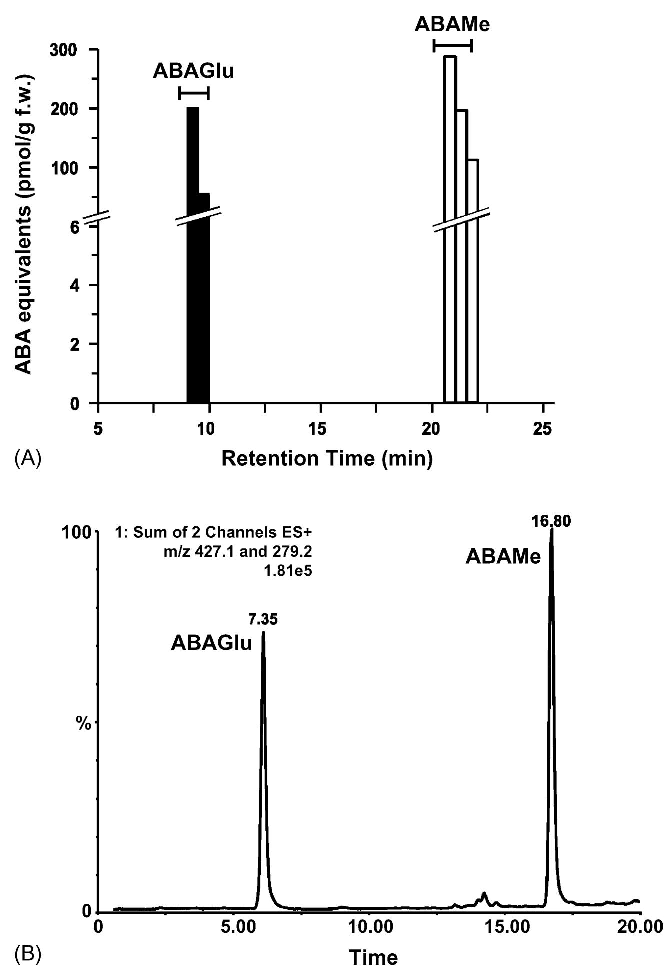

Fig. 5. Distribution of immunoreactivity in LC fractions (0.5 min) (A) and SIM ion chromatogram (B) of natural methylated ABA and ABA-β-D-glucosylester in the immunoaffinity purified extracts of drought-stressed tobacco plants (3 days). Immunoreactive regions (white columns) correspond to UV-traces (265 nm) of the cold and labelled standards. Different chromatographic conditions were used: LC–ELISA, a Symmetry C_{18} reversed-phase column (150 mm × 2.1 mm, 5 μm), the column was equilibrated with a 25% A:75% B solution (where A was 100% MeOH and B was 0.1% CH_3COOH) and eluted using a linear gradient of 75–50% B over 15 min, followed by a linear gradient to 37% B at 24 min, flow rate 0.3 mL/min, injection volume 50 μL; LC–ESI-MS, a Symmetry C_{18} column (150 × 2.1 mm, 5 μm) eluted with a linear gradient of 30–60% A (0–12 min) followed by 60–80% A (12–20 min), with a final 1 min at 80% A, where A was 100% methanol and B 10 mM HCOOH, column temperature 30 °C, flow rate 0.25 mL/min, injection volume 30 μL.

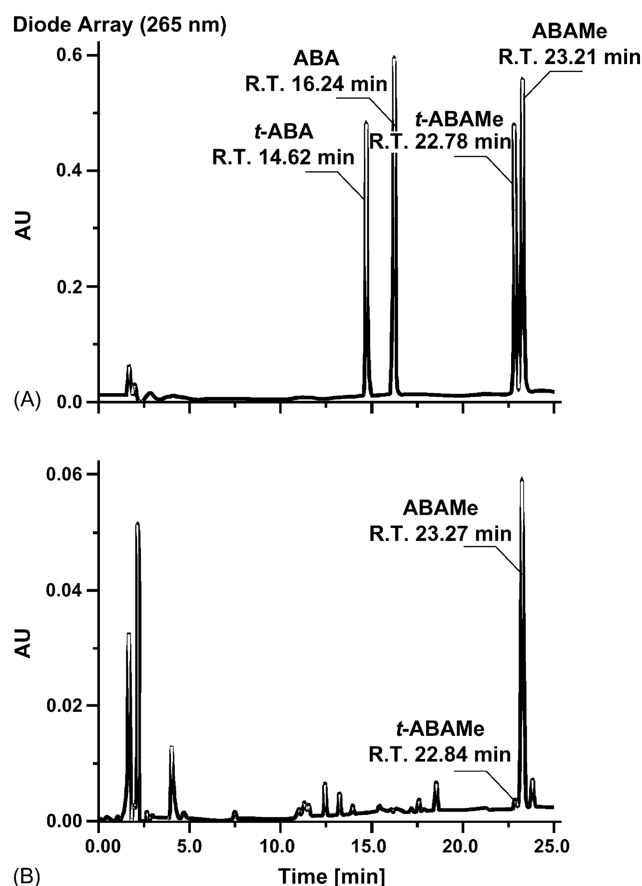

Fig. 6. LC-PDA chromatograms of ABA, t-ABA, ABAMe and t-ABAMe standards (A) and immunoaffinity purified extracts of drought-stressed tobacco plants (3 days) (B). LC conditions as follows: a Symmetry C_{18} column (150 mm × 2.1 mm, 5 μm) eluted with a linear gradient of 10–50% A (0–25 min) where A was 100% acetonitril and B 15 mM $HCOONH_4$ (pH 4.0), column temperature 30 °C, flow rate 0.25 mL/min.

3.5. Quantification of endogenous abscisic acid levels in extracts of normal and water-stressed N. tabacum L.

To illustrate the applicability of the new method, we analysed the ABA levels in water-stressed tobacco plants (Table 5). Quantification was performed using the standard isotope dilution method. Final concentrations were calculated from the areas under peaks in the SIM chromatograms. Ratios of endogenous ABA to appropriate labelled standards were determined and further used to quantify the level of endogenous compound in the original extracts, based on the known quantities of added internal standard. Well-watered tobacco plants showed relatively constant, low levels of endogenous ABA, but stressed plants had amounts of ABA that were 5–7-fold higher, and highest after 24 h of the water stress. The ABA levels were strongly dependent on the duration of the water stress applied to the plants. The total ABA content slowly declined, after peaking at 24 h, decreasing to around 500 pmol/g f.w. at the end of the drought period. Other workers have also reported increases in the ABA content of water-stressed plants [25,29]. Cramer [64] suggests that this increase in ABA may be responsible for growth inhibition of the plants.

In addition, the LC–ELISA and LC–ESI-MS approaches are based on the co-chromatography of UV, mass and immunoactivity traces with signals of authentic deuterium and tritium labelled standards, respectively. The applied [^{3}H]- and [^{2}H]-ABA co-eluted with the authentic natural compound, according to its retention time. The addition of labelled derivatives to the extracts further facilitated detection of natural ABA, giving better resolution of immuno- and mass-traces, as well as a measure of the recovery percentages throughout the purification process. Both of the above mentioned methods were applicable for estimating endogenous ABA levels. We established that the absolute levels of ABA determined by the two methods are practically identical (Table 6) although the ELISA values tend to be a little

lower than the LC–ESI-MS values. The comparatively poor recoveries were probably due to the relatively complex C_{18}-DEAE-C_{18}-IAC purification procedure applied to this series of samples.

4. Conclusions

C1-abscisic acid (ABA)-specific antibodies were raised and used to develop a highly efficient immunoaffinity chromatography (IAC) technique. The immunoaffinity columns were successfully used for single-step purification of ABA from tobacco leaf tissues. The method, which showed high selectivity and sensitivity, is based on ABA-specific IAC purification, and co-chromatography of UV, mass and immunoactivity traces with signals of authentic tritium and deuterium labelled standards. The results show that the IAC purification of plant extracts enhances the reliability and sensitivity of subsequent LC–ESI-MS (or LC–ELISA) measurement of endogenous ABA levels. Due to the application of different principles of enzyme immunoassay and mass spectrometry, together with the use of two different chromatographic systems, the identity of the quantified ABA was well verified. The results also show that as well as facilitating reliable measurement of ABA levels, the simple IAC technique is convenient for the detection, identification and quantification of cross-reacting ABA-like substances. Potential applications of this approach in analyses of ABA together with its metabolites in single IAC/LC–ESI-MS/MS runs are under development.

Acknowledgements

This work was supported by the Ministry of Education, Youth, and Sports of the Czech Republic, Grants No. 6198959216 and LC06034, the Grant Agency of the Czech Academy of Sciences, Grant No. IBS5038351, and by Grant FRVS, Grant No. 2543/2005G6. We would like to thank Dr. John Blackwell for linguistic correction of the text.

References

[1] J.A.D. Zeevaart, R.A. Creelman, Annu. Rev. Plant Physiol. Plant Mol. Biol. 39 (1988) 439.
[2] E.A. Bray, Plant Cell Environ. 25 (2002) 153.
[3] R.R. Finkelstein, S.S. Gampala, C.D. Rock, Plant Cell 14 (2002) S15.
[4] P.M. Hasegawa, R.A. Bressan, J.K. Zhu, H.J. Bohnert, Annu. Rev. Plant Physiol. Plant Mol. Biol. 51 (2000) 463.
[5] L. Xion, J.K. Zhu, Plant Physiol. 133 (2003) 75.
[6] D. Walton, Y. Li, in: P.J. Davies (Ed.), Plant Hormones, Physiology, Biochemistry and Molecular Biology, Kluwer Academic Publisher, Dordrecht, 1995, p. 140.
[7] A. Goméz-Cadenas, J. Mehouachi, F.R. Tadeo, E. Primo-Mill, M. Talon, Planta 210 (2000) 636.
[8] G. Sembdner, G. Schneider, K. Schreiber, Methoden zur Pflanzenhormonanalyse, Veb Gustav Fischer Verlag, Jena, 1988, p. 176.
[9] E.W. Weiler, Planta 144 (1979) 255.
[10] E.W. Weiler, Planta 148 (1980) 262.
[11] D. Walton, W. Dashek, E. Galson, Planta 146 (1979) 139.
[12] J. Daie, R. Wyse, Anal. Biochem. 119 (1982) 365.
[13] E.W. Weiler, Physiol. Plant 54 (1982) 510.
[14] P.B. Sweetser, A. Vatvars, Anal. Biochem. (1975) 68.
[15] N.L. Gargile, R. Borchert, J.D. McChesney, Anal. Biochem. 97 (1979) 331.
[16] G. Guinn, D.L. Brummett, R.C. Beier, Plant Physiol. 81 (1986) 997.
[17] T. Subbaiah, L. Powell, Physiol. Plant. 71 (1987) 203.
[18] J.R. Dunlap, G. Guinn, Plant Physiol. 90 (1989) 197.
[19] X. Li, C.E. La Motte, C.R. Stewart, N.P. Cloud, S.W. Bagnall, C.Z. Jiang, J. Plant Growth Regul. 11 (1992) 55.
[20] J. Kamboy, G. Browning, P.S. Blake, J.D. Quinlan, D.A. Baker, Plant Growth Regul. 28 (1999) 21.
[21] R.T. Gray, R. Mallaby, G. Ryback, V.P. Williams, J. Chem. Soc., Perkin Trans. 2 (1974) 919.
[22] A.G. Netting, B.V. Milborrow, A.M. Duffield, Phytochemistry 21 (1982) 385.
[23] S. Marx, W. Grosse, H.A.W. Schneider-Poetsch, J. Plant Physiol. 133 (1988) 475.
[24] L.R. Hogge, G.D. Abrams, S.R. Abrams, P. Thibault, S. Pleasance, J. Chromatogr. A 623 (1992) 255.
[25] A. Gómes-Cadenas, O.J. Pozo, P.G. Augustín, J.V. Sancho, Phytochem. Anal. 13 (2002) 228.
[26] S.D.S. Chiwocha, S.R. Abrams, S.J. Ambrose, A.J. Cutler, M. Loewen, A.R.S. Ross, A.R. Kermode, Plant J. 35 (2003) 405.
[27] R. Zhou, T.M. Squires, S.J. Ambrose, S.A. Abrams, A.R.S. Ross, A.J. Cutler, J. Chromatogr. A 1010 (2003) 75.
[28] A.R.S. Ross, S.J. Ambrose, A.J. Cutler, J.A. Feurdato, A.R. Kermode, K. Nelson, R. Zhou, S.R. Abrams, Anal. Biochem. 329 (2004) 324.
[29] M. López-Carbonell, O. Jáuregui, Plant Physiol. Biochem. 43 (2005) 407.
[30] E. Prinsen, W. Van Dongen, E.L. Esmans, J. Mass Spectrom. 32 (1997) 12.
[31] E. Prinsen, W. Van Dongen, E.L. Esmans, J. Chromatogr. A 826 (1998) 25.
[32] E. Witters, K. Vanhoutte, W. Dewitte, I. Machackova, E. Benkova, W. Van Dongen, E.L. Esmans, H.A. van Onckelen, Phytochem. Anal. 10 (1999) 143.
[33] O. Novák, P. Tarkowski, D. Tarkowská, K. Doležal, R. Lenobel, M. Strnad, Anal. Chem. Acta 480 (2003) 207.
[34] M. Okamoto, K.I. Takahashi, T. Doi, Rapid Commun. Mass. Spectrom. 7 (1993) 1067.
[35] P.H. Rosher, H.G. Jones, P. Hedden, Planta 165 (1985) 91.
[36] E. Prinsen, P. Reding, M. Strnad, I. Galís, W. Van Dongen, H. Van Onkelen, Methods Mol. Biol. 44 (1995) 245.
[37] M. Strnad, T. Vaněk, P. Binarová, M. Kamínek, J. Hanuš, in: M. Kutáček, M.C. Elliott, I. Macháčková (Eds.), Molecular Aspects of Hormonal Regulation of Plant Development, SPB Academic, Hague, 1990, p. 41.
[38] M. Strnad, W. Peters, E. Beck, M. Kamínek, Plant Physiol. 99 (1992) 74.
[39] J. Badenoch-Jones, D.S. Letham, C.W. Parker, B.G. Rolfe, Plant Physiol. 75 (1984) 11175.
[40] R. Mertens, B. Deus-Neumann, E.W. Weiler, FEBS 160 (1983) 269.
[41] B.V. Milborrow, J. Exp. Bot. 21 (1970) 17.
[42] L. Rivier, P.E. Pilet, in: H.L. Schmidt, H. Forstel, K. Heinzinger (Eds.), Stable Isotopes, Elsevier, Amsterdam, 1982, p. 535.
[43] W.W. Jones, C.W. Coggins, T.W. Embleton, Plant Physiol. 58 (1976) 681.
[44] R. Maldiney, B. Leroux, I. Sabbagh, B. Sotta, L. Sossountzov, E. Miginiac, J. Immunol. Methods 90 (1986) 151.
[45] E.W. Weiler, P.S. Jourdan, W. Conrad, Planta 153 (1981) 561.
[46] R. Atzorn, E.W. Weiler, Planta 159 (1983) 1.
[47] E.M.S. MacDonald, R.O. Morris, Methods Enzymol. 110 (1985) 347.
[48] W. Haasnoot, A. Kemmers-Voncken, D. Samson, Analyst 127 (2002) 87.
[49] N. Delaunay-Bertoncini, M.C. Hennion, J. Pharmaceut. Biomed. 34 (2004) 717.
[50] A. Crozier, G. Sanberg, A.M. Monterio, B. Sanberg, Plant Growth Substances, Springer-Verlag, Heidelberg, 1985, p. 13.
[51] B. Nicander, V. Stahl, P.O. Byorkhan, E. Tillberg, Planta 189 (1993) 312.
[52] T. Kannangara, A. Wieczorek, D.P. Lavender, Physiol. Plant. 75 (1989) 369.
[53] R. Mertens, M. Stuning, E.W. Weiler, Naturissenschaften 69 (1982) 595.
[54] L.R. Hogge, G.D. Abrams, S.R. Abrams, P. Thibault, S. Pleasance, J. Chromatogr. 623 (1992) 255.
[55] M. Okamoto, K. Takahashi, T. Doi, Rapid. Commun. Mass Spectrom. 7 (1993) 1067.
[56] M. López-Carbonell, L. Alegre, A. Pastor, E. Prinsen, H.V. Onckelen, Plant Growth Regul. 20 (1996) 271.

[57] K. Cornish, J.A.D. Zeevaart, Plant Physiol. 76 (1984) 1029.
[58] B.V. Milborrow, R. Mallaby, J. Exp. Bot. 26 (1975) 741.
[59] A.G. Netting, R.D. Willows, B.V. Milborrow, Plant Growth Regul. 11 (1992) 327.
[60] P.H. Duffield, A.G. Netting, Anal. Biochem. 289 (2001) 251.
[61] P. Kebarle, Y. Ho, in: R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry, Wiley, New York, 1997, p. 3.
[62] G. Wang, R.B. Cole, in: R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry, Wiley, New York, 1997, p. 137.
[63] S.H. Schwartz, J.A.D. Zeevaart, in: P.J. Davies (Ed.), Plant Hormones, Biosynthesis, Signal Transduction, Action!, Kluwer Academic Publishers, Dordrecht, 2004, p. 137.
[64] R. Cramer, Funct. Plant Biol. 29 (2002) 561.