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Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography☆

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Abstract

A method for separation and purification of plant hormones auxin and abscisic acid based on mixed mode reversed-phase anion-exchange solid phase extraction and two-dimensional HPLC was developed. Two-dimensional HPLC in "heart cutting" mode was very efficient in the purification of these two hormones. Its purification power is high enough to allow reliable on-line quantification of both hormones even with non-selective detectors.
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Keywords: Auxin; Abscisic acid; Solid phase extraction; 2D-HPLC

1. Introduction

Plant hormones are of vital importance for the normal functioning of plants. Their minute quantities trigger basic developmental processes such as cell division, enlargement and differentiation, organ formation, seed dormancy and germination, leaf and organ senescence and abscission [1]. Plant hormones are difficult to analyze because they occur in very low amounts in plant extracts which are very rich in interfering substances, especially secondary metabolites. To cope with this problem the plant extract must undergo several purification steps using unrelated separation mechanisms in order to increase orthogonality and purification efficiency. Common purification procedures such as column chromatography, solid phase extraction (SPE), liquid–liquid extraction, etc. are employed for plant hormone purification. However, these procedures usually require significant amounts of solvent, time and labor. Furthermore, they all are "off-line" procedures often requiring sample pre-treatment (e.g. preconcentration) when used in series.

"On-line" purification methods encompassing multidimensional HPLC have become increasingly popular. Separation of peptides by comprehensive two-dimensional high performance liquid chromatography (2D-HPLC) has appeared to be complementary to the traditional 2D-gel electrophoresis in the proteome analysis [2–4]. So called "Heart-cutting" 2D-HPLC, in which only a part of the first dimension run is "heart-cut" and introduced into the second dimension, is a very suitable purification technique when a limited number of substances have to be purified [5,6]. Features contributing to 2D-HPLC popularity are high purification potential, reproducibility, robustness, high throughput and unattended operation.

Auxin (indole-3-acetic acid, IAA) and abscisic acid (ABA) are plant hormones with contrasting biological functions. Whereas IAA stimulates growing processes such as cell elongation and division, ABA controls plant senescence and responses to stress [1]. However, IAA and ABA exhibit many similar chemical properties which can be exploited for their chromatographic purification. Both IAA and ABA are relatively hydrophobic compounds containing a carboxylic group

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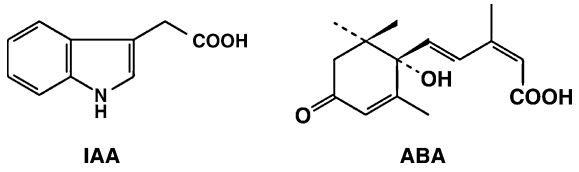

Fig. 1. Chemical structures of indole-3-acetic acid (IAA) and abscisic acid (ABA).

(Fig. 1). Therefore, when IAA and ABA are extracted and purified from plant material by common chromatographic techniques they very often end up in the same fraction.

IAA and ABA determination is usually preceded by extensive purification involving, for example liquid–liquid extraction [7–9], solid phase extraction [10], or HPLC purification [11]. Pre-purification of IAA and ABA on immuno-affinity columns was also reported [12]. Sample purification time can be reduced by application of a very specific separation and quantification technique that is able to distinguish the analyte of interest from the matrix. In the past, immunoassays as specific quantification methods were popular [13]. They excel in specificity due to the unique ligand–antibody binding. However, when applied to low purity extracts, quantification using immunoassays can be misleading due to the cross-reactivity of antibodies or their inhibition (or activation) by interfering substances. Capillary electrophoresis (CE) was also applied for the determination of IAA and ABA [8,14,15]. This technique is promising due to its high resolving power, excellent sensitivity and low sample consumption. Although good CE methods were developed for pure hormones, the complex nature of plant extracts presents the main challenge for CE. Mass spectrometric (MS) detection is another very specific technique for analyte determination. GC–MS is a well recognized technique for determination of IAA and ABA [10,16,17]. However, it also requires considerable sample purification as well as derivatization to run on GC. There have been reports on utilizing the selectivity of MS detection to measure hormones in crude extracts. Gómez-Cadenas et al. [18] analyzed ABA in crude citrus extracts using LC–MS. However, as the authors pointed out, the technique is reliable only when tandem MS (MS/MS) in selected reaction monitoring mode and internal standardization with heavy isotope labeled ABA are used. They were not successful in ABA quantification in single stage MS (selected ion monitoring mode) due to the high background. There are several other reports where brief sample pre-purification is followed by tandem MS. For example, several acidic phytohormones, including IAA and ABA, were analyzed on GC–MS/MS, preceded by purification on one or two microscale SPE columns [10]. Similarly, rapid extraction of ABA and its metabolites and purification through single polymeric SPE column followed by quantification on LC–MS/MS was reported [19]. In conclusion, there is a compromise between thorough pre-purification followed by lower specificity detection and brief pre-purification followed by very selective but expensive detection.

Recently we developed a procedure for fast and efficient extraction and purification of three groups of plant hormones, namely IAA, ABA and cytokinins [20]. It allowed us to prepare a very pure fraction of cytokinins. However, the fraction of co-eluting IAA and ABA contained relatively high amounts of UV-absorbing and fluorescing contaminants. This shortcoming complicated their quantification using HPLC coupled to fluorescence detection (IAA) and/or mass spectrometry (IAA and ABA). To overcome this problem we developed an additional SPE purification step, as well as a 2D-HPLC system that allowed us to obtain very pure separate fractions of IAA and ABA and to quantify these compounds with much higher reliability.

2. Experimental

2.1. Chemicals and materials

Unlabeled IAA and ABA were from Sigma, St. Louis, MO, USA. Radioactive [5-^{3}H]IAA (0.74 TBq mmol^{-1}) was from ARC Inc., St. Louis, MO, USA, and [G-^{3}H]ABA (1.74 TBq mmol^{-1}) was from Amersham Biosciences UK Ltd., Little Chalfont, UK. $^{13}C_6$-IAA was from CIL, Andover, MA, USA. Deuterated ABA ($^{2}H_6$-ABA) was synthesized by a modified procedure of Rivier et al. [21] which is based on base-induced hydrogen exchange as follows: A solution of NaO^{2}H was prepared by reaction of Na with $^{2}H_2O$ (99.9%, IsotopTech, Russia) under an argon atmosphere. ABA was dissolved into 0.3 N NaO^{2}H and kept at 65 °C for 1 h. After cooling, the solution was acidified to pH 2 with $^{2}H_3PO_4$ (prepared by reaction of P_2O_5 with $^{2}H_2O$). The resulting $^{2}H_8$-ABA was filtered, converted to $^{2}H_6$-ABA by washing with ice cold $^{1}H_2O$, dried and re-crystallized from a mixture of ethyl acetate and diethyl ether. The synthesized $^{2}H_6$-ABA had identical melting point and infra-red spectrum as the parent ABA. Mass spectrometric analysis (electro-spray, negative mode) revealed a molecular ion at *m*/*z* 269 with no detectable *m*/*z* 263 from the molecular ion of parent ABA confirming complete exchange of six deuterium atoms in the molecule. The synthesized $^{2}H_6$-ABA is stable, provided that pH is kept lower than pH ∼ 8.

1-Methyl-3-nitro-1-nitrosoguanidine (MNNG, 97%) was from Aldrich, Milwaukee, WI, USA. HPLC gradient grade methanol and acetonitrile were obtained from Merck KGaA, Darmstadt, Germany. Formic acid and ammonium hydroxide both of p.a. grade were from Lachema a.s., Neratovice, Czech Republic. Oasis MAX columns (150 mg/6 cc) were obtained from Waters, Milford, MA, USA.

2.2. Recoveries of standards of IAA and ABA on Oasis MAX columns

Tested compounds were dissolved in 5 ml of 1 M formic acid to give 0.5–1 AU and the actual absorbances of solutions were measured. The standard solution of a single compound

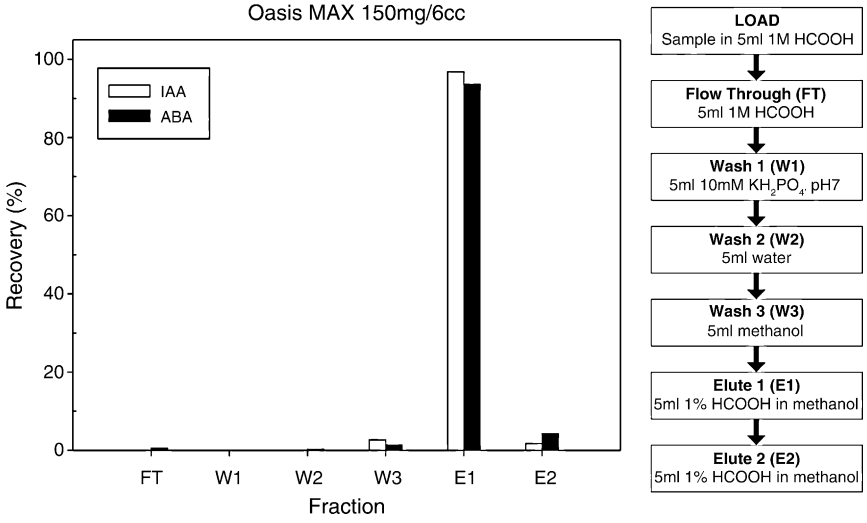

Fig. 2. Purification protocol and recoveries of IAA and ABA on Oasis MAX sorbent.

was applied to an Oasis MAX column, then the column was sequentially eluted as shown in the scheme in Fig. 2 and the absorbance of each eluate was measured. Absorbance readings were used directly for recovery calculations. Absorbances were measured on a 5625 UV–vis spectrophotometer (Unicam Ltd., Cambridge, UK) with 1 cm light path at wavelengths 280 nm for IAA and 260 nm for ABA.

2.3. 2D-HPLC instrumental set-up

The instrumental set-up consisted of a series 200 autosampler (Perkin Elmer, Norwalk, CT, USA), two HPLC gradient pump systems (first pump: ConstaMetric 3500 and 3200 with 500 μl mixer, TSP, Riviera Beach, FL, USA; second pump: Series 200 Quaternary Pump, Perkin Elmer), two columns (first column: ACE-3CN, 150 mm × 4.6 mm, 3 μm, ACT, Aberdeen, Scotland, UK; second column: Luna C18(2), 150 mm × 4.6 mm, 3 μm, Phenomenex, Torrance, CA, USA), one 2-position, fluid processor SelectPRO with 1 ml loop (Alltech, Deerfield, IL, USA), diode array- and fluorescence detectors (235C and LC 240, respectively, Perkin Elmer) and fraction collector FC 203B (Gilson, Middleton, WI, USA). The scheme of tubing connections of 2D-HPLC is presented in Fig. 3.A, B. The actions of the different components of the 2D-HPLC system described in Fig. 3.C were electronically synchronized and regulated via timed events and chromatographic software (Turbochrom 4, PE Nelson, San Jose, CA, USA) permitting unattended operation.

2.4. 2D-HPLC conditions

The flow rate (0.6 ml min^{-1}) and mobile phase (A: 40 mM formic acid adjusted to pH 3 with ammonium hydroxide and B: acetonitrile/methanol, 1/1, v/v) were the same for both dimensions. Samples dissolved into 20% methanol in water (v/v) were injected via the autosampler in volumes up to 100 μl. Columns were kept at 35 °C. The linear gradient in the first dimension was: 10–30% B in 10 min, 30–100% B in 2 min, 100% B for 5 min, 100–10% B in 1 min. The linear gradient in the second dimension was: 45–70% B in 5 min, 70–100% B in 2 min, 100% B for 2 min, 100–45% B in 1 min, 45% B for 5 min. Eluate from the second dimension was monitored on the diode array detector at λ 270 nm, and on the fluorescence detector with excitation at λ 270 nm and emission at λ 339 nm.

2.5. Plant material

Developing grains of field grown spring wheat (*Triticum aestivum* L. cv. Jara) were collected between 3 and 43 days after anthesis (DAA). Tobacco BY2 (*Nicotiana tabacum* L., cv. Bright Yellow 2) cell suspension was cultivated [22] for 5 days, filtered, and the cells collected. The aerial (rosette) parts of 3-week-old greenhouse cultivated *Arabidopsis thaliana* L. (ecotype Columbia) plants were collected. Leaves of different age [upper (young), middle, bottom (old)] were collected from greenhouse cultivated 3-month-old tobacco plants (*Nicotiana tabacum* L., cv. Wisconsin 38). Ten-day-old wheat plants (*Triticum aestivum* L. cv. Jara) cultivated in Perlite in a growth chamber (18 h day at 21 °C; 6 h night at 18 °C; 90% relative humidity) were cut into two parts – aerial part and roots. The collected plant material ranging from 0.3 to 2 g of fresh weight was frozen in liquid nitrogen and stored at −80 °C until analysis. Extraction and purification of plant material followed the procedure described in [20]. At the beginning of extraction, 1000 Bq of each [5-^{3}H]IAA and [G-^{3}H]ABA were added to monitor the losses during purification. The dried fraction containing IAA and ABA was dissolved in 100 μl 20% methanol (v/v) and an aliquot was injected into 2D-HPLC.

2.6. GC–MS

Known amounts of stable isotope labeled $^{13}C_6$-IAA and $^{2}H_6$-ABA were added to the 2D-HPLC purified samples prior to derivatization. The derivatization was carried out using diazomethane produced with MNNG diazomethane generator (cat. no. Z411736, Aldrich, Milwaukee, WI, USA) according to the manufacturer's instructions. The derivatized samples were dried under a nitrogen stream, redissolved in 50 μl methanol, and analyzed using a Polaris Q/Trace GC 2000 GC–MS system (Thermo Finnigan, Austin, TX, USA) equipped with Combi PAL au-

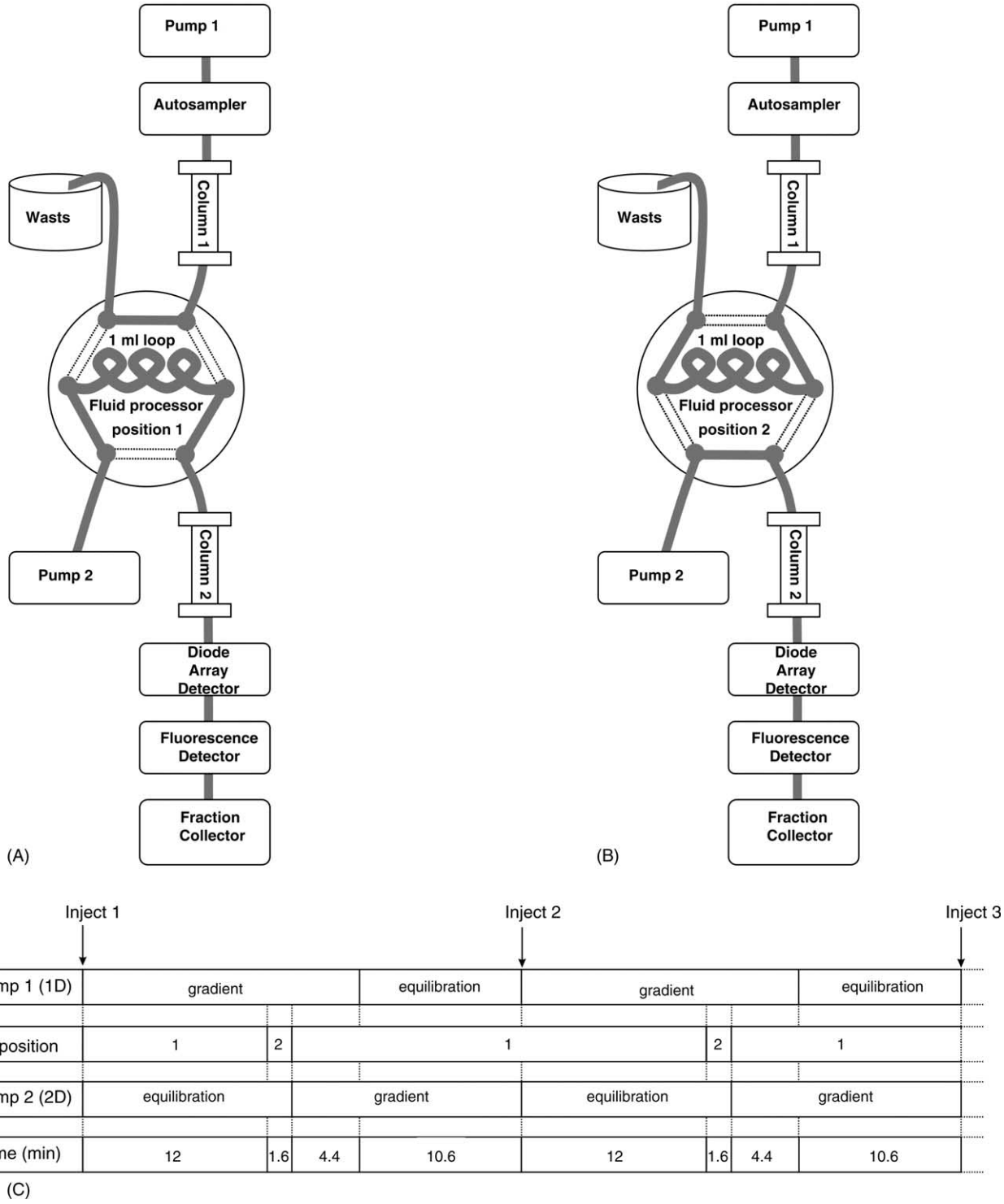

Fig. 3. Arrangement of connections of the instruments in 2D-HPLC set-up. (A) Fluid processor (FP) in position 1, (B) FP in position 2, (C) Scheme of the time-based instrument settings.

tosampler (CTC Analytics, Zwingen, Switzerland). The Rtx-5MS capillary column (Restek Corp., Bellefonte, PA, USA), (30 m × 0.25 mm × 0.25 μm df) was coupled directly to the ion source of the mass spectrometer with an interface heated to 250 °C. Flow rate of helium carrier gas (purity, 99.999%) was maintained at 1 ml min^{-1}. MS source temperature was 200 °C and the electron energy was 70 eV. The PTV injector working in split-less mode was tempered at 220 °C. After injection of sample (2 μl) the column oven was maintained at 60 °C for 1 min with the splitter closed, after which the splitter (30:1) was opened and the oven temperature was increased at 120 °C min^{-1} to 160 °C and then at 7 °C min^{-1} to a final temperature of 250 °C which was maintained for 5 min. The spectra were collected in centroid full scan mode (*m*/*z* 50–300) using the software Xcalibur 1.3 (Thermo Finnigan). The following fragment ions were used for quantification: *m*/*z* 134, 162 and 190 for ABA-Me and 138, 166 and 194 for [$^{2}H_{6}$]-ABA-Me; *m*/*z* 130 and 189 for IAA-Me and 136 and 195 for [$^{13}C_{6}$]-IAA-Me. The concentrations of IAA and ABA in sample were computed from the response ratio of target compound and the appropriate internal standard.

3. Results and discussion

As reported elsewhere we developed a protocol for extraction and purification of the plant hormones cytokinins IAA and ABA [20] using a mixed mode reversed-phase cation-exchange polymeric sorbent Oasis MCX (Waters). It allows retention of hydrophobic analytes, including weak carboxylic acids when their ionization is suppressed, as well as cationic species. The extract is applied in acidic solution to suppress ionization of the weak acids and to promote the protonization of bases for retention on the sorbent. Species retained only by reversed phase mechanism including the hydrophobic carboxylic acids, such as IAA and ABA can be eluted by pure methanol in the first elution step. The retained cationic species are then sequentially eluted by ammonia in water (polar analytes) and ammonia in methanol (hydrophobic analytes). The purified fraction containing IAA and ABA, however, was not clean enough for quantification by direct application to reversed phase HPLC coupled to UV and fluorescence detection or to GC–MS because of the bulky presence of co-eluting substances. This could be expected, because Oasis MCX methanolic eluate theoretically should contain not only hydrophobic carboxylic acids but also neutral hydrophobic substances. To address this problem we developed an additional purification step utilizing the Oasis MAX SPE support (Waters) which is based on a mixed mode reversed-phase anion-exchange polymeric sorbent. This SPE sorbent allows separation of the acidic analytes from the ones of neutral character. The sample is applied in acidified solution to promote retention of weak hydrophobic acids by reversed-phase mechanism. The column is washed with aqueous solution buffered at neutral pH, which enhances the electrostatic binding of weak acids to the resin's ion-exchange sites. The

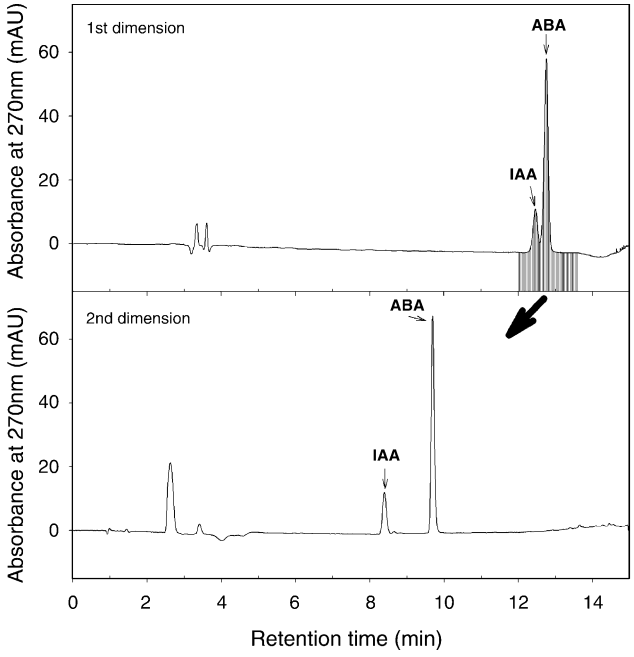

Fig. 4. 2D-HPLC chromatograms of standards of IAA and ABA, 250 pmol each. The dashed part of the chromatogram from the first HPLC dimension was directed to the second HPLC dimension. Note: The chromatogram of the first HPLC dimension was recorded from a separate run where the output of column 1 was connected to the UV detector.

subsequent methanolic wash removes the neutral hydrophobic analytes, leaving the acidic analytes bound to the sorbent. The acidic analytes are eluted with acidified methanol. The purification protocol based on Oasis MAX columns as well as recoveries of IAA and ABA standards in the individual elution steps are presented in Fig. 2. This protocol worked well with some plant tissues, however, it was not very efficient in removing UV and fluorescing interferences from other plant sources (data not shown).

Therefore, we developed 2D-HPLC as an alternative step for purification of the Oasis MCX column fraction containing IAA and ABA (Fig. 3). We used gradient elution in both dimensions, which improved resolution and sorbent regeneration. In the first dimension the sample was loaded into silica-cyanopropyl column. When run in reversed-phase mode the polar sorbent of this column allows the elution of IAA and ABA with relatively low proportion of organic solvent (about 35%, v/v). Furthermore, very close elution of IAA and ABA from the cyanopropyl column (see 1st dimension in Fig. 4) was advantageous since it permitted a narrow segment of the 1D-run containing a relatively clean fraction to be applied to the second HPLC dimension. Low concentration of organic solvent in the segment applied to the second dimension allowed concentrating IAA and ABA on the more hydrophobic column (silica-C18) used in the second dimension. Indeed, the narrow peaks of IAA and ABA in the second dimension indicated that the large volume loaded from the 1D-run (1 ml) did not influence the resolution and efficiency of the 2nd dimension (see 2nd dimension in Fig. 4). IAA and ABA were

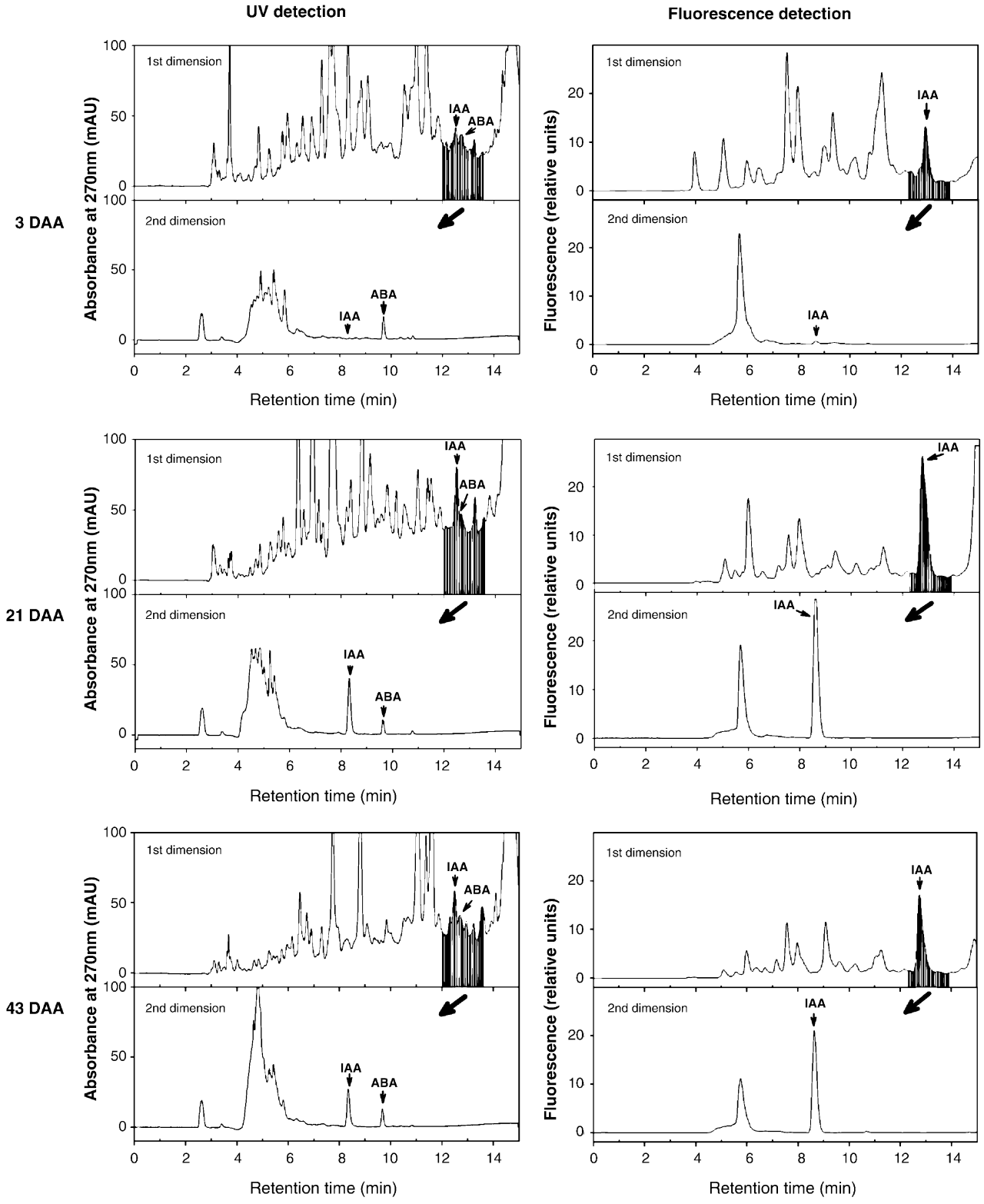

Fig. 5. 2D-HPLC chromatograms of extracts of wheat grains at different days after anthesis (DAA). The dashed parts of the chromatograms from the first HPLC dimension were directed to the second HPLC dimension. Note: The chromatograms of the first HPLC dimension were recorded from separate runs where the output of column 1 was connected to the UV and fluorescence detectors.

well retained and separated in the second HPLC dimension with capacity factor higher than 2 and resolution $Rs = 4$. The described 2D-HPLC method has relatively high throughput since the injection-to-injection cycle time is less than 30 min (Fig. 3C).

The reproducibility of the 2D-HPLC was comparable to that of single dimensional HPLC. The relative standard deviations of retention times and areas for both hormones were $\leq 0.27\%$ and $\leq 1.44\%$, respectively ($n = 10$). No hormone loss due to irreversible binding, or incorrectly timed "heart-cut" segment was observed. The recoveries of the analytes were > 95%.

The potential of 2D-HPLC for IAA and ABA quantification was demonstrated using connection of the 2D-HPLC system to non-destructive diode array (DAD) and fluorescence (FLD) detectors in series (Fig. 3). IAA, as naturally fluorescing compound, can be detected by FLD with low detection limit and high selectivity. The IAA quantification using 2D-HPLC-FLD is characterized by the following calibration parameters: $f(X) = X389.6 + 957.1$, $r^2 = 0.9997$, linear range 1.2–760 pmol, limits of detection at three times signal to noise (LOQ, $3 \times S/N$) = 0.4 pmol, limits of quantification at 10 times signal to noise (LOQ, $10 \times S/N$) = 1.2 pmol.

Both hormones were detected on DAD at λ 270 nm which is a compromise between the absorbance maxima of ABA (λ 260 nm) and IAA (λ 280 nm). Due to the high extinction coefficient of ABA [23] its detection by UV is very sensitive. However, unless the sample is very pure, UV detection is not suitable for ABA quantification due to its low selectivity. The purification potential of 2D-HPLC allowed quantifying ABA by UV. Scanning capabilities of some UV detectors such as DAD, e.g. measurement of peak purity index, can be an additional source of information of the homogeneity of the ABA peak. The ABA quantification using 2D-HPLC-UV is characterized by the following calibration parameters: $f(X) = X298.6 - 182.9$, $r^2 = 0.9999$, linear range 2.6–6700 pmol, LOD ($3 \times S/N$) = 1.2 pmol, LOQ ($10 \times S/N$) = 2.6 pmol.

The purification potential of 2D-HPLC was tested on plant extracts of developing wheat grains. As is evident from Fig. 5, IAA and ABA were easily detected in the second dimension without any visible co-eluting interferences.

To estimate the quantification capability of 2D-HPLC we compared the levels of ABA and IAA in developing wheat grains measured by 2D-HPLC and GC–MS (Fig. 6). The measured values of IAA and ABA by the two different methods were statistically compared using a paired *t*-test. The results show that quantification by 2D-HPLC with on-line UV (ABA) and FLD (IAA) detection are statistically identical (with 95% confidence) to the ones measured by GC–MS. In terms of the biology of developing grains, the measured values of IAA and ABA correspond well to the ones already published. IAA content is usually low at the initial stages of grain development, followed by a substantial increase about 20 days after anthesis and a decrease at the later grain maturation stage [24,25]. ABA content, when expressed per gram

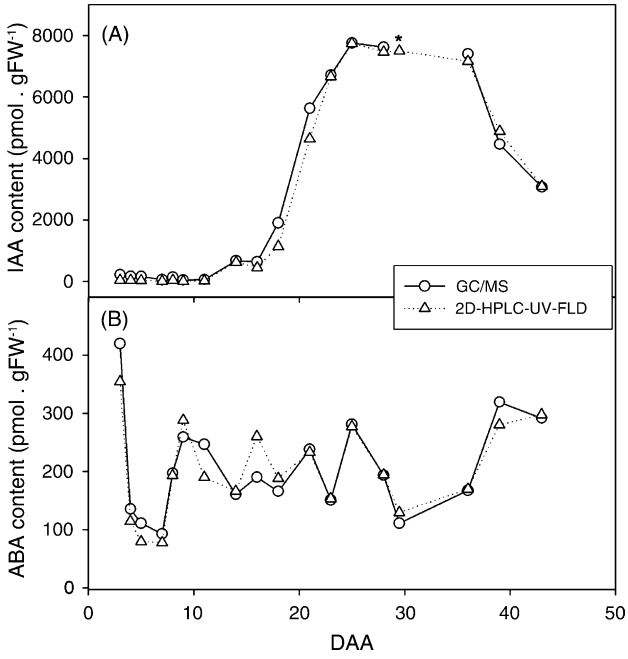

Fig. 6. Endogenous IAA (A) and ABA (B) content in wheat grains throughout their development (3–43 DAA) measured on 2D-HPLC-UV-FLD (triangles) and GC–MS (circles). * Missing value due to accidental sample loss.

of fresh weight, remains approximately constant, but when expressed on a per seed basis, ABA increases during grain development in parallel with increasing grain mass [25,26,27]. We also tested the quantification potential of 2D-HPLC-UV-FLD on various plant materials differing in species origin, age, content of pigments and cultivation conditions (Table 1). The measured values corresponded well with GC–MS measurements within the common statistical variation in plant hormone analysis [9–11,18,19].

The biochemical processes in living organisms are highly coordinated and influence each other. This is particularly true for plant hormones. Therefore, the recent trend when analyzing particular tissues is to measure an array of hormones [9,10,12,28]. Our previously developed extraction and purification method [20] is suitable for purification of cytokinins, IAA and ABA from a single extract. We presented here a reliable analytical method based on 2D-HPLC coupled with unspecific detectors (UV and FLD), which can directly quantify the Oasis MCX purified fraction of IAA and ABA. 2D-HPLC was not only a very powerful purification technique, but also, as shown in Fig. 6 and Table 1, a reliable quantification method. To our knowledge this is the first report using on-line "heart-cut" 2D-HPLC in plant hormone analysis. There have been a few other reports, however, dealing only with on-line pre-concentration techniques [8,28].

During our attempts to optimize 2D-HPLC we tried to shorten the run time per sample by isocratic running so that wash and equilibration steps necessary in gradient runs could be excluded (e.g. as in [29]). However, excluding the high concentration organic wash step after each sample led to faster plugging and ageing of the column in the first dimen-

Table 1
Levels of IAA and ABA in different plant extracts measured by 2D-HPLC-UV-FLD and GC–MS

Sample description	IAA (pmol gFW^{-1})		ABA (pmol gFW^{-1})	
	2D-HPLC	GC–MS	2D-HPLC	GC–MS
Cell suspension BY2	3.43 (±1.30)	2.85 (±1.05)	0.86 (±0.29)	0.86 (±0.49)
Arabidopsis thaliana, aerial part	10.52 (±1.27)	13.63 (±3.29)	3.61 (±0.96)	3.28 (±1.56)
Tobacco W38, top (young) leaves	54.97 (±13.45)	45.70 (±11.82)	255.89 (±41.33)	256.93 (±62.66)
Tobacco W38, middle leaves	7.93 (±1.57)	9.42 (±2.07)	533.28 (±36.13)	489.82 (±60.88)
Tobacco W38, bottom (mature) leaves	13.77 (±3.84)	12.54 (±4.62)	822.23 (±61.73)	812.39 (±120.46)
Wheat, aerial part	5.86 (±1.05)	8.05 (±1.53)	15.66 (±1.93)	16.77 (±2.01)
Wheat, roots	29.85 (±3.10)	37.84 (±5.02)	3.43 (±0.38)	3.57 (±1.00)

Values are means of four measurements with standard deviations in brackets.

sion. Furthermore, a gradient run in the first dimension allowed a narrower fraction containing both hormones to be directed into the second dimension. Therefore, the presented 2D-HPLC method, where gradient runs in both dimensions are applied, is a compromise with longer analysis times being offset by improved column life and sample purity.

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