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Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction☆

Petre Ivanov Dobrev, Miroslav Kamínek*

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, CZ-16502 Praha 6, Czech Republic

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Abstract

A method for separation of cytokinins from auxin and abscisic acid, which allows further separation of cytokinin ribotides from cytokinin bases, ribosides and glucosides and their purification on a single Oasis MCX column was developed. Due to the mixed reversed-phase and cation-exchange mode of the Oasis MCX sorbent the cationic cytokinin bases, ribosides and glucosides as well as the anionic auxin, abscisic acid and cytokinin ribotides are retained and can be sequentially eluted by solvents containing different concentrations of methanol and ammonium hydroxide. Characteristics of the method are high recoveries of analyzed phytohormones and their sufficient purity for quantification by HPLC–ELISA (RIA) or HPLC–MS.

Keywords: Solid-phase extraction; Cytokinins; Auxin; Abscisic acid; Plant hormones

1. Introduction

Cytokinins are a group of plant hormones defined by their ability when in the presence of an auxin to promote cell division in plant tissues cultured in vitro and to control a number of other physiological processes including apical dominance and senescence [1]. Cytokinins occur in the free state in plants and as constituents of certain tRNAs in almost all living organisms (for reviews see Refs. [2,3]). The difficulty of cytokinin analysis arises from their presence in minute quantities among the bulk of other substances in plant extracts [4]. Therefore, the determination of cytokinins in biological material requires extensive purification prior to final quantification step.

The naturally occurring cytokinins are N^6-substituted adenines with an isoprenoid or benzyl side-chain. The side chain can be hydroxylated to yield zeatin (*cis*- and *trans*-zeatin, [Z]) and the isoprene double bond of Z reduced to yield dihydrozeatin (DHZ). Cytokinins occur as free bases, nucleosides, nucleotides, and as a number of sugar conjugates substituted at position N7 and N9 of the purine ring (7*N*-, 9*N*-glucosides) or at the hydroxylated side chain (*O*-glucosides and *O*-xylosides). Structures and

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*Corresponding author. Tel.: +420-2-2039-0445; fax: +420-2-2039-0446.

E-mail address: kaminek@ueb.cas.cz (M. Kamínek).

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abbreviations of cytokinins analyzed in this report are summarized in Table 1.

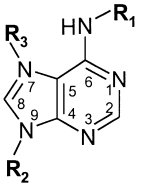
All cytokinins are able to ionize after alteration of pH [5]. Thus, the exocyclic amino group at the sixth position of purine has $pK_a \approx 4$, giving the positive charge of cytokinins at pH<3. On other hand the NH group of the imidazole ring (N9) of cytokinin bases has $pK_a \approx 10$ and at pH>11 is predominantly negatively ionized. Cytokinin nucleotides bear a phosphate group, which is doubly negatively ionized with pK_as of ≈1 and ≈6. The N^6-side chain gives the hydrophobic character of cytokinins as compared to adenine.

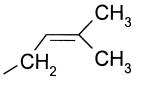
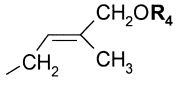
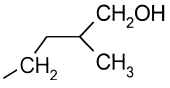
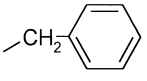
These properties of cytokinins are very advantageous with respect to their purification and separation. Many purification protocols include reversed-phase-, cation-exchange- and anion-exchange-chromatography as well as liquid–liquid partition [5–7]. Some of these protocols indeed remove most of the bulk of plant extract but at the price of tedious manipulations, low recoveries and using large volumes of toxic solvents.

A straightforward cytokinin purification method is immunoaffinity chromatography (IAC), giving cytokinin fractions essentially clear of contaminants [6,8]. However, this purification is not widely used due to the limited availability of IAC columns and low or total absence of their affinity for certain cytokinins, namely to cytokinin N7- and *O*-glucosides, which are the main cytokinins in some plants. Moreover, the common quantification procedures for plant hormones, e.g. radio-immuno assay (RIA), enzyme-linked immuno-sorbent assay (ELISA), gas chromatography–mass spectrometry (GC–MS) or high-performance liquid chromatography–mass spectrometry (HPLC–MS) are highly selective and can be successfully applied to partially purified samples.

Solid-phase extraction (SPE) using C_{18} bound

Table 1
Structures and abbreviations of investigated cytokinins



R_1	R_2	R_3	R_4	Common name	Abbreviation
	H	–	–	N^6-Δ^2-(isopentenyl)adenine	iP
	R	–	–	N^6-Δ^2-(isopentenyl)adenosine	iPR
	RP	–	–	N^6-Δ^2-(isopentenyl)adenosine-5′-monophosphate	iPRMP
	G	–	–	N^6-Δ^2-(isopentenyl)adenine-9-glucoside	iP9G
	H	–	H	*trans*-zeatin	Z
	R	–	H	*trans*-zeatin riboside	ZR
	RP	–	H	*trans*-zeatin riboside-5′-monophosphate	ZRMP
	–	G	H	*trans*-zeatin-7-glucoside	Z7G
	G	–	H	*trans*-zeatin-9-glucoside	Z9G
	H	–	G	*trans*-zeatin-*O*-glucoside	ZOG
	R	–	G	*trans*-zeatin riboside-*O*-glucoside	ZROG
	H	–	–	Dihydrozeatin	DHZ
	R	–	–	dihydrozeatin riboside	DHZR
	RP	–	–	dihydrozeatin riboside-5′-monophosphate	DHZRMP
	H	–	–	N^6-benzyladenine	BA

H, hydrogen; R, β-D-ribofuranosyl; RP, β-D-ribofuranosyl-5′-monophosphate; G, β-D-glucopyranosyl.

silica has been frequently used as an efficient method for pre-purification of cytokinins and some other plant hormones on the basis of reversed-phase interactions [9,10]. However, cytokinins cannot be separated from auxin and abscisic acid (ABA) and cytokinin ribotides are poorly retained on this sorbent [10]. Moreover, this single step is not sufficient for purification of samples for HPLC fractionation and another purification step(s), mostly ion-exchage- or immunoaffinity chromatography is (are) required prior to cytokinin determination by HPLC–MS or HPLC–ELISA (RIA) [7].

Recent availability of SPE sorbents bearing both the reversed-phase and the cation-exchange characters has offered a possibility to reduce the multiple step purification to one step and to increase retention of a wide spectrum of cytokinins and to improve sample recoveries. Moreover, resistance of some of these materials to solutions with extreme pH values allows efficient separation of cytokinin bases and ribosides from corresponding ribotides and other plant hormones of acidic character (auxins, abscisic acid) under conditions which inhibit activity of metabolic enzymes and increase efficiency of removal of impurities.

We present here an efficient method that allows simple and fast separation of cytokinins from auxin and abscisic acid after their simultaneous extraction, and further purification and separation of cytokinin ribotides from cytokinin bases, ribosides and glucosides. The method exploits the advantages of recently developed reversed-phase-ion-exchange supports and is characterized by high recoveries, easy manipulation and an acceptable degree of purification of cytokinins for HPLC fractionation prior to their quantitative determination.

2. Experimental

2.1. Chemicals and materials

Cytokinins (purity ≥97%) were from Apex Organics (Honiton, Devon, UK). [^{3}H]-*trans*-zeatin (1.3 TBq mmol^{-1}, radiochemical purity >97%) was obtained from the Isotope Laboratory of the Institute of Experimental Botany, Prague, Czech Republic. Adenine (Ade), adenosine (Ado), AMP, ADP, indole-3-acetic acid (IAA) and abscisic acid (ABA) of purities >95% were products of Sigma (St. Louis, USA). Methanol, formic acid, ammonium hydroxide, all of analytical grade, were from Lachema a.s. (Neratovice, Czech Republic).

The following SPE products were obtained from Waters Co. (Milford, MA, USA):

(1) Sep-Pak Plus †C_{18} cartridges containing 400 mg sorbent of C_{18} phase bonded on silicagel matrix. The manufacturer uses † symbol for silica-based bonded phase with strong hydrophobicity due to trifunctional bonding chemistry, which gives it an increased hydrolytic stability over common C_{18} [11].
(2) Oasis MCX columns (65×13 mm, 4 mm bed height) containing 150 mg sorbent of a sulfonated poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) copolymer. The hydrophobic part of the copolymer (divinylbenzene) gives the sorbent its reversed-phase character, while the hydrophilic part (*N*-vinylpyrrolidone) increases water wettability allowing the sorbent to retain its capacity even when it runs dry [12]. The sorbent has cation-exchange capacity of 1 meq g^{-1}, specific surface area 810 m^2 g^{-1}, average pore diameter of 8 nm, total pore volume of 1.3 cm^3 g^{-1} and particle diameter of 30 μm. Thus, the Oasis MCX sorbent has mixed mode reversed-phase and cation-exchange characteristics. In comparison with the silica-SPE materials, Oasis MCX has the advantage of stability over the pH range 0–14, two to three times higher capacity due to its higher surface area and to its water wettability [11].

2.2. Cytokinin binding capacity of Sep-Pak Plus †C_{18} and Oasis MCX sorbents

Washing with 5 ml of methanol followed by 5 ml 1 *M* formic acid was used for pre-conditioning of Oasis MCX column and Sep-Pak Plus †C_{18} cartridge. To test sorbent capacity, benzyladenine (BA) was dissolved either in water or in formic acid (40 m*M* or 1 *M*). Solutions were continuously supplied to the Sep-Pak Plus †C_{18} and Oasis MCX sorbents at flow-rate ~5 ml min^{-1} and fractions of 5 ml were collected. The cumulative BA mass at which the UV

reading reached 10% of maximum absorbance (0.3 AU_{270}) was taken as the dynamic capacity of each column.

2.3. Recoveries of standards of cytokinins and other compounds on Oasis MCX columns

Tested compounds were dissolved in 5 ml of 1 *M* formic acid to give 0.5–1 AU (20–50 μ*M*) and the actual absorbances of solutions were measured. The standard solution of a single compound was applied to an Oasis MCX column, then the column was sequentially eluted as shown in Fig. 2 and absorbance of each eluate was measured. Since preliminary measurements revealed that the molar absorption coefficients of tested compounds in the different solvents used differed by less than 5%, absorbance readings were directly used for recovery calculations.

Absorbances were measured on a 5625 UV–Vis spectrophotometer (Unicam Ltd., Cambridge, UK) with 1 cm light path at wavelengths 270 nm for cytokinins, 280 nm for IAA and 260 nm for adenylates and ABA.

2.4. Recovery of [^{3}H]-trans-zeatin from different plant extracts

Two plant materials were chosen to test recoveries of cytokinins from plant extracts following their solid-phase extraction on Oasis MCX columns: 7-day-old in vitro dark-grown suspension-cultured BY-2 tobacco cells and 20-day-old light-grown radish seedlings. The plant material was homogenized in liquid nitrogen, extracted and purified according to the protocol shown in Fig. 2. Before application to Oasis MCX columns, different amounts of plant extracts were spiked with 4000 Bq of [^{3}H]-Z (1.3 TBq mmol^{-1}). The radioactivity of an aliquot of each elution step of Oasis MCX column was measured after addition of BCS liquid scintillation cocktail (Amersham Co., Arlington Heights, USA) on a 2500TR liquid scintillation analyzer (Packard Inst. Co., Meriden, USA).

2.5. Reduction of plant extract dry mass residue at different purification steps

Samples of 10–15 g of plant material were extracted and purified as shown in Fig. 2. In order to avoid capacity break of Oasis MCX columns due to mass overload, aliquots of plant extract corresponding to 2.5 gFW of plant material were applied per column (150 mg sorbent). Eluate from each purification step was evaporated at 40 °C under vacuum to dryness and the dry residue was weighed. The dry mass of plant material was determined after drying at 80 °C for 16 h.

3. Results and discussion

The method for separation of cytokinins from other plants hormones (auxin and abscisic acid) and their further purification described here is based on the application of acidic plant extract on Oasis MCX sorbent exhibiting reversed-phase and cation-exchange functionalities. These properties of the sorbent allow step-wise elution of auxin and abscisic acid followed by cytokinin ribotides and by other cytokinins (cytokinin bases, ribosides and glucosides) using solvents containing different concentrations of methanol and NH_4OH.

A widely used extraction solvent for cytokinins is a mixture of methanol/chloroform/water/formic acid (12/5/2/1, v/v/v/v) [13] since it prevents degradation of cytokinin nucleotides by deactivating the endogenous phosphatases. The presence of chloroform in the mixture, however, causes problems associated with extraction of large amount of lipid material. Therefore, the chloroform is often omitted [14,15]. In the highly acidic extraction mixture, cytokinins should be predominantly positively charged. This inspired us to test their purification by direct application of the acidic plant extract on Oasis MCX columns. Although the recoveries for more hydrophobic cytokinins were acceptable (e.g. 70% for iP) the polar cytokinin glucosides were mostly not retained on the column (data not shown). This problem was solved by removal of the organic constituent of the extract by evaporation prior to application to the column.

We compared the capacity of Oasis MCX columns (150 mg sorbent) and silica Sep-Pak Plus †C_{18} cartridges (400 mg sorbent) by frontal analysis of a cytokinin standard (BA) in different solvents. Oasis MCX sorbent had the highest capacity for BA in

acidic aqueous solutions. The concentration of formic acid between 0.04 *M* and 1 *M* had no influence on capacity, which was about 15 mg BA per 150 mg sorbent (Fig. 1A). The lower capacity for BA dissolved in neutral water suggests that ion-exchange retention is mainly responsible for the capacity of Oasis MCX sorbent for BA. On the other hand, the highest capacity of silica Sep-Pak Plus †C_{18} cartridges was recorded for BA dissolved in water and reached 25 mg per 400 mg sorbent (Fig. 1B). When capacities of both materials are compared in favorable solvents, the Oasis MCX sorbent has about 1.6 times higher capacity (Oasis MCX: 100 mg g^{-1} sorbent in acidified water; Sep-Pak Plus †C_{18}: 63 mg g^{-1} sorbent in water).

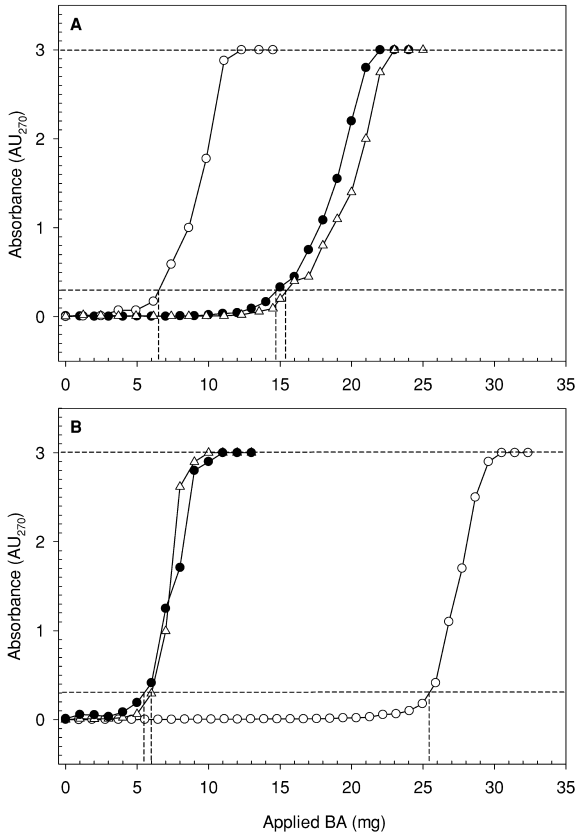


Fig. 1. Actual binding capacities of Sep-Pak Plus †C_{18} and Oasis MCX sorbents for N^6-benzyladenine (BA). N^6-benzyladenine (2 mg 10 ml^{-1}) dissolved in water (– ○ –), 40 m*M* formic acid (– △ –) and 1 *M* formic acid (– ● –) was applied to Oasis MCX column (A) and Sep-Pak Plus †C_{18} cartridge (B) containing 150 mg and 400 mg of corresponding sorbents, respectively, at flow-rate ~5 ml min^{-1}. Fractions of 5 ml were collected until three consecutive readings showed maximum absorbance (AU_{270}) of ≥3.0. The actual binding capacity was taken as the cumulative BA mass at which the AU_{270} reached 10% of its maximum, i.e. 0.3 AU_{270} (marked by the lower dotted lines).

The protocol which was developed for extraction and purification of cytokinins and their separation from auxin and ABA is shown in Fig. 2. This protocol was tested with standards of cytokinins, IAA, ABA and adenylates (Fig. 3). All the tested compounds with exception of ADP, and portion of AMP, were completely retained on Oasis MCX matrix. This corresponds to the theoretical expectations that on Oasis MCX the cations as well as hydrophobic compounds should be retained. In 1 *M* formic acid (pH 1.4) cytokinins are positively charged. The mechanism of retention of cytokinin nucleotides, which have one positive and one negative charge and are relatively polar, is not clear. IAA and ABA are hydrophobic carboxylic acids that lose their negative charge at pH≤3 and are evidently retained by the reversed-phase mechanism.

Subsequent column elution with methanol (W2) washed out the neutral hydrophobic compounds including IAA (103%) and ABA (95%) (Fig. 3). Surprisingly, the zwitterionic cytokinin nucleotides were not eluted, suggesting that they are retained by electrostatic forces. Next elution with 0.35 *M* NH_4OH (E1) drastically increased the pH to 11, establishing favorable conditions for elution of cytokinins. The E1 eluate contained cytokinin nucleotides, adenine and the remaining AMP. In total 87% of ZRMP and DHZRMP and 74% of iPRMP were recovered in E1. The rest of iPRMP was eluted in E2 step. Small amounts of ZOG and Z7G were also detected here. The cytokinin bases, ribosides and glucosides were subsequently eluted with 0.35 *M* NH_4OH in 60% (v/v) methanol (E2, Fig. 3). The cytokinins in E2 eluate showed recoveries well above 85%, with exception of ZOG (77%) and Z7G (82%), which were partially eluted in the previous E1 elution. The most hydrophobic 2-methylthio cytokinins had lower recoveries in E2 (~40–50%), however, they are completely eluted in the final elution step (0.7 *M* NH_4OH in methanol, E3) (data not shown).

Procedures so far used separate anionic auxin, ABA and cytokinin nucleotides from cationic cyto-

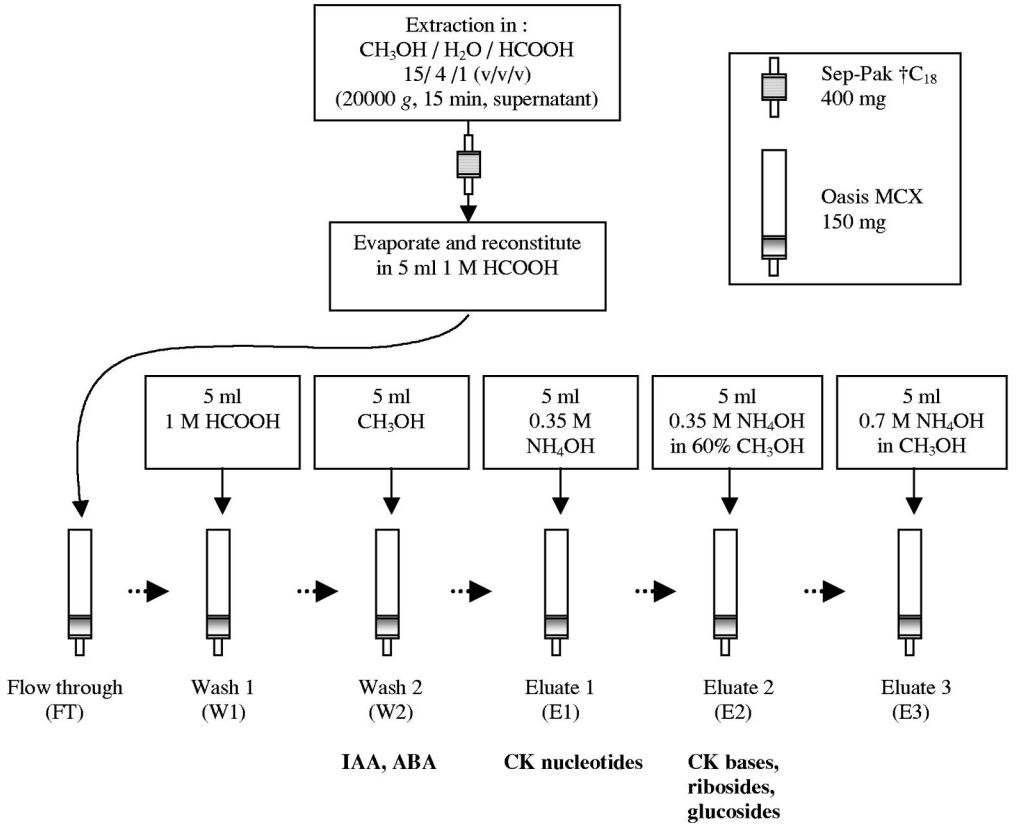


Fig. 2. Extraction and purification protocol for cytokinins (CK), auxin (IAA) and abscisic acid (ABA). Plant material is homogenized in liquid nitrogen and dropped in cold (−20 °C) extraction mixture of methanol/water/formic acid (15/4/1, v/v/v) at 5 ml g^{-1} fresh weight containing labeled internal standards. After overnight extraction at −20 °C solids are separated by centrifugation (20 000 *g*, 15 min) and re-extracted for 30 min in additional 5 ml gFW^{-1} of extraction mixture at −20 °C. Pooled supernatants are passed through Sep-Pak Plus $\dagger C_{18}$ cartridge to remove lipids and part of plant pigments and evaporated either to near dryness or until methanol is removed. The residue is dissolved in 5 ml 1 *M* formic acid and applied to Oasis MCX column pre-conditioned with 5 ml of methanol followed by 5 ml of 1 *M* formic acid. The column is washed and eluted with indicated order of solutions. After passing of each solvent the columns are purged briefly with air. Solvents are evaporated at 40 °C under vacuum.

kinin bases, ribosides and glucosides on anion-exchange sorbents such as DEAE–cellulose [6] and DEAE–Sephadex [16] or cation-exchangers like phosphocellulose [17,18]. Alternatively auxin, ABA and cytokinins can be separated on immunoaffinity columns specific to the corresponding phytohormones [16]. According to our knowledge separation of anionic auxin and abscisic acid from anionic cytokinin nucleotides has not been achieved on any other sorbent with ion-exchange functionality.

The capacity of the Oasis MCX column to isolate cytokinins from plant extracts was tested. Two plant materials differing in complexity and cultivation conditions were chosen. Whereas tobacco BY-2 cell suspension consists of non-differentiated cells growing freely in liquid medium in dark, the 20-day-old radish seedlings are fully developed plants with differentiated tissues and green pigmentation. Increasing amounts of plant extracts were loaded onto a single Oasis MCX column (150 mg sorbent) and the recovery of spiked [^{3}H]-Z was measured (Fig. 4). More than 80% of [^{3}H]-Z was recovered in E2 from both plant sources when 2 g or less of plant material was extracted and purified on a single Oasis MCX column. Further increases in the amount of extracted plant material per column decreased the recovered

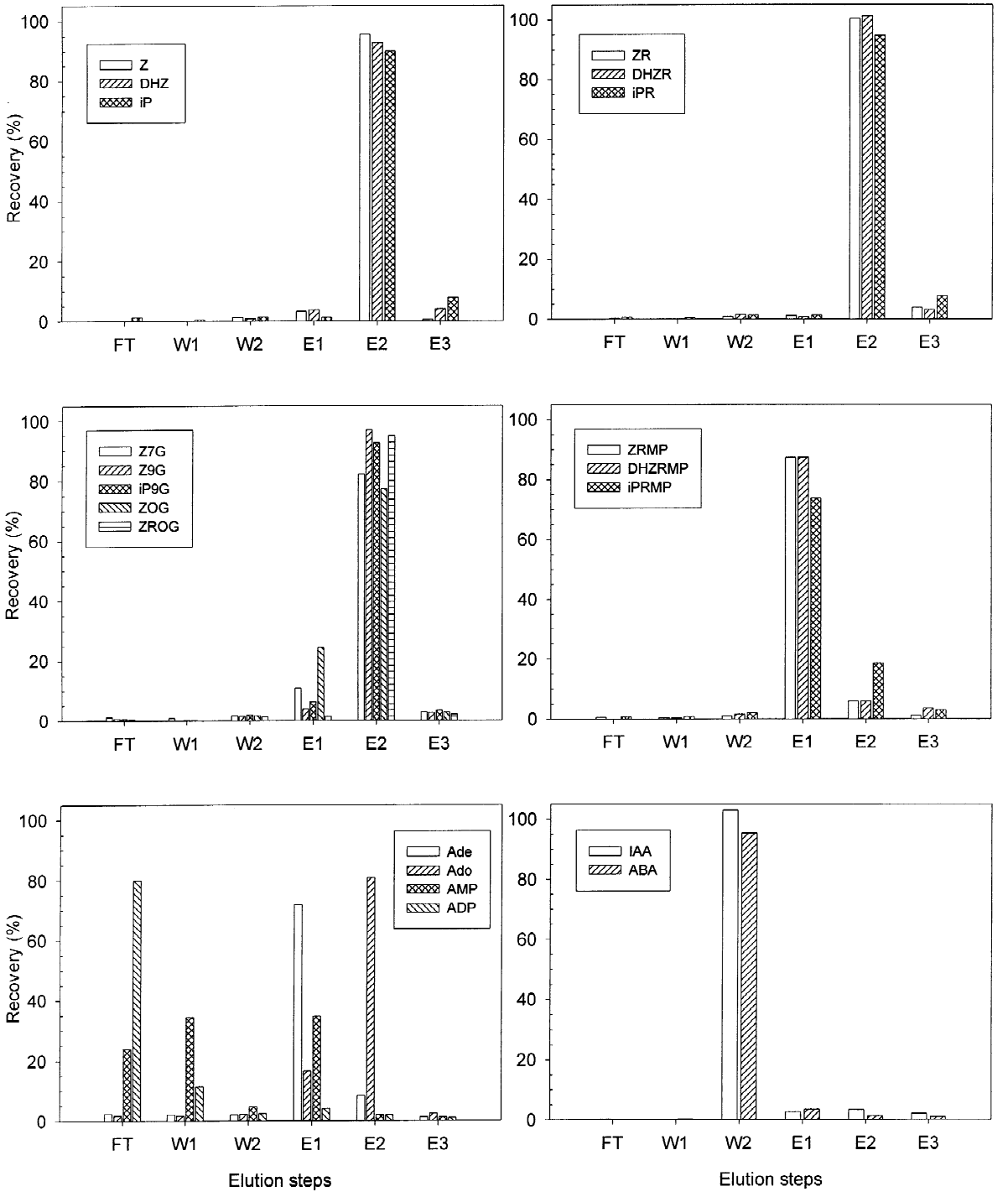


Fig. 3. Recoveries of cytokinins, adenylates, auxin and abscisic acid at different steps of purification protocol described in Fig. 2. Compounds were dissolved in 5 ml of 1 *M* formic acid at final concentration of 30–50 μ*M* and were applied on columns containing 150 mg of Oasis MCX sorbent. Columns were stepwise eluted as shown in Fig. 2. Recoveries were determined on the basis of measurement of absorbances at 270 nm (cytokinins), 280 nm (auxin), and 260 nm (adenylates and ABA).

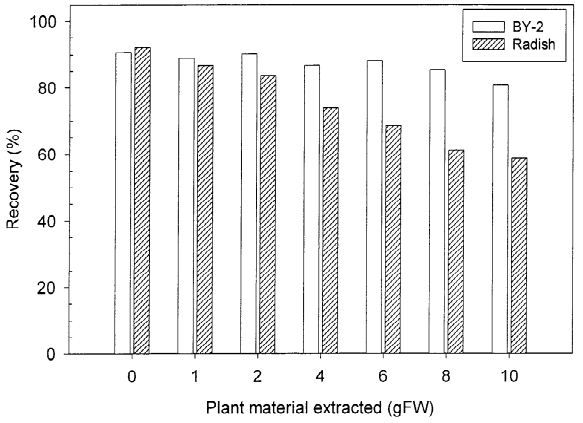


Fig. 4. Recoveries of [^{3}H]-*trans*-zeatin spiked with different amounts of extracts of tobacco BY-2 cells and radish seedlings. Extracts corresponding to 0–10 g fresh weight of plant material were supplied with 4000 Bq (1.3 TBq mmol^{-1}) of [^{3}H]-*trans*-zeatin and purified as shown in Fig. 2. The recovery of radioactivity was determined in the E2 eluate by scintillation counting.

radioactivity. The recoveries decreased with sample size to a lesser extent in case of tobacco cells than in radish seedlings. Thus, when 10 g of fresh plant material was extracted 81 and 59% of [^{3}H]-Z were recovered from tobacco cells and radish plant extracts, respectively. The remaining radioactivity was found in the FT eluate. It should be mentioned that most analyses of plant material by immuno- or MS methods for final quantification of cytokinins require less than 2 g of plant material.

Quantification of cytokinins is usually preceded by HPLC separation on analytical or (when an MS detector is used) narrowbore and microbore columns. These separations, especially the latter ones, are sensitive to mass overload. Therefore, we tested how our purification method reduced the dry residue of plant extracts (Table 2). The majority of the dry mass was present in FT and W1 eluates of Oasis MCX columns. The methanolic wash (W2), where IAA and ABA are eluted, contained 1.6–1.8% of the extracted dry residue and relatively high amounts of UV-absorbing material (data not shown). The fraction E1, where cytokinin nucleotides were present, contained 5–7% of the extracted dry material while in the eluate E2, where cytokinin bases, ribosides and glucosides were found, the dry mass residue was reduced to 0.4–1.3%, equivalent to a 70–250-fold decrease of dry mass.

The extraction and purification protocol described here solves several drawbacks of the commonly used procedures:

- The separation of cytokinins from auxin and ABA and their purification is achieved on a single column using step elution with different solvents which can be removed by evaporation.
- The column may be allowed to run dry with no loss of capacity. This allows high sample throughput and potential automation.
- The plant extract is separated into fractions containing: (1) IAA and ABA; (2) cytokinin nucleotides; and (3) cytokinin bases, ribosides and glucosides.

Table 2
Reduction of plant extract dry residue during purification of 10 g of 7-day-old dark-grown BY-2 tobacco cells and 20-day-old light-grown radish seedlings using purification protocol described in Fig. 2

	Tobacco BY-2 (mg)		Radish (mg)	
Fresh mass	10 000		10 000	
Dry mass	361		405	
Dry residue in:		% of extract		% of extract
Extract	221	100	224	100
Si–C_{18} purified extract	191	87	222	99
Oasis MCX purified extract:				
FT	128	58	170	76
W1	32	14	26	12
W2	3.5	1.6	4.1	1.8
E1	15.9	7.2	10.9	4.9
E2	2.8	1.3	0.9	0.4
E3	3.5	1.6	1.8	0.8

- There are high recoveries of analyzed hormones (>80%).
- The purified cytokinin-containing fraction has a considerably reduced dry mass and a very low level of interference from other UV-absorbing compounds. This allows direct use of the fraction for quantification of cytokinins by methods such as HPLC–ELISA or HPLC–MS without the need for further purification.

Acknowledgements

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