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Efficiency of different methods of extraction and purification of cytokinins

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Abstract

The increasing use of advanced methods, such as mass spectrometry, for the determination of cytokinins has raised special requirements for the extraction and purification of this class of plant hormones. Extraction of *Arabidopsis thaliana* plants with three different solvents, [80% (v/v) MeOH, Bieleski's MCF-7, and modified Bieleski's] provided similar yields of most analyzed cytokinins determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS). However, the extraction with a modified Bieleski's solvent ($MeOH–HCO_2H–H_2O$ [15:1:4, v/v/v]) gave the highest responses of deuterated cytokinins (used as test compounds) in plant extracts as compared to the responses of pure deuterated standards (relative internal standard response, RISR). Purification of cytokinins using Oasis MCX sorbent with reversed-phase and cation-exchange characteristics, in comparison to the DEAE Sephadex RP-C_{18} method, provided higher levels of zeatin riboside monophosphate and similar levels of cytokinin bases, ribosides and glucosides. Using this method the content of UV-absorbing contaminates was decreased by about 90% and the RISR values of all tested cytokinin standards but riboside monophosphates were increased about two-fold. The former method provided preparations more suitable for HPLC/MS/MS analysis with respect to simplicity and sample purity.

Keywords: *Arabidopsis thaliana*; Cytokinin extraction; Cytokinin purification; Plant hormones; Solid-phase extraction; HPLC/MS/MS

1. Introduction

Cytokinins are a class of plant hormones that in cooperation with auxin play unique role in the control of developmental processes in plants such as cell division and differentiation, formation and growth of roots and shoots, apical dominance and senescence. Natural cytokinins are 6-*N*-substituted purine derivatives. Those which occur in plants as free bases are supposed to be the biologically active compounds. Glycosidic conjugates of cytokinins are transport, storage or inactivated forms of cytokinins; while cytokinin riboside phosphates predominantly represent the primary products of cytokinin biosynthesis. More than 40 natural cytokinins have been identified in plant tissues (Zažímalová et al., 1999). The structures of those cytokinins analyzed in the present report are shown in Fig. 1. Their occurrence in minute amounts in non-transformed plants ($<10^{-8}$ M), in the presence of structurally related compounds and enzymes catalyzing metabolic conversions and the degradation of cytokinins (Mok and Mok, 2001), complicates both their purification and determination (Horgan and Scott, 1987; Jones et al., 1996). Increasing use of mass spectrometry for the detection and determination of a wide spectrum of cytokinins requires both the operational as well as dependable extraction and purification techniques which prevent metabolic conversions during extraction, and also provide samples of sufficient purity for mass spectrometry.

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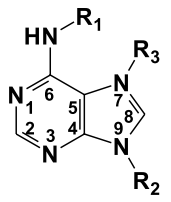
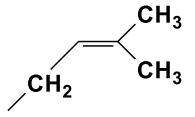
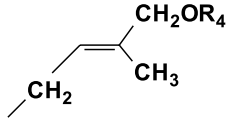


R_1	R_2	R_3	R_4	Common name	Abbreviation
	H	-	-	6-*N*-(2-isopentenyl)adenine	iP
	R	-	-	6-*N*-(2-isopentenyl)adenosine	iPR
	RP	-	-	6-*N*-(2-isopentenyl)adenosine 5′-monophosphate	iPRMP
	-	G	-	6-*N*-(2-isopentenyl)adenine 7-glucoside	iP7G
	G	-	-	6-*N*-(2-isopentenyl)adenine 9-glucoside	iP9G
	H	-	H	*trans*-zeatin	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, hydrogen; R, ß-D-ribofuranosyl; RP, ß-D-ribofuranosyl-5′-monophosphate; G,ß-D-glucopyranosyl.

Fig. 1. Structures, names and abbreviations of cytokinins investigated.

A wide array of solvents, particularly MeOH, EtOH, perchloric acid and their mixtures, have been used for the extraction of cytokinins (Laloue et al., 1974; Horgan and Scott, 1987; Crouch and Van Staden, 1992). Attention has been paid to the inactivation of phosphatases catalyzing the hydrolysis of cytokinin riboside phosphates during extraction. Bieleski (1964) developed MCF-7 solvent consisting of MeOH–$CHCl_3$–HCO_2H–H_2O (12:5:1:2, v/v/v/v) that was found to inactivate phosphatases in various plant materials. This solvent has also been widely used for the extraction of cytokinins.

As was shown in the model experiments of Horgan and Scott (1987), the extraction of soybean callus tissue with Bieleski's solvent minimizes hydrolysis of AMP. However, preservation of cytokinin riboside phosphates during extraction of the plant material with this solvent has not been directly tested yet. Analyzing soybean callus tissue after feeding with [^{14}C]benzyladenine, which was metabolically converted in vivo to the corresponding riboside monophosphate, Crouch and Van Staden (1992) found no substantial differences in the proportions of riboside monophosphate and the corresponding dephosphorylated product (riboside), when freeze-dried tissue was extracted with either 80% (v/v) EtOH or with Bieleski's solvent. Moreover, the presence of $CHCl_3$ in the Bieleski's solvent was reported to increase the extraction of lipophilic material that complicates further purification (Laloue et al., 1974; Horgan and Scott, 1987). The present availability of labeled cytokinin riboside monophosphates may allow accurate comparisons of different solvents, with respect to protection of cytokinin riboside phosphates.

Anion-exchange column chromatography provides an efficient step for the separation of cytokinin riboside phosphates from cytokinin bases, ribosides and glucosides. DEAE-cellulose and DEAE-Sephadex have been successfully used for this purpose. Cytokinin riboside phosphates, applied in neutral aqueous solution, are retained on such sorbents and can be eluted with an ammonium salt (~1 M) or 6% formic acid. Cytokinin bases, ribosides and glucosides, which are not bound to anion-exchange sorbents, can be retained either on a reversed-phase (RP)-C_{18} or on a cation-exchange column, attached in tandem with the anion-exchange column (MacDonald and Morris, 1985; Redig et al., 1996; Prinsen et al., 1995).

New solid-phase extraction (SPE) sorbents bearing both the RP and cation-exchange characteristics greatly increase and simplify the purification of cytokinins and allow separation of different plant hormones (auxin, abscisic acid, and cytokinins) on a single column, after their simultaneous extraction. Moreover, using MCX RP cation-exchange sorbent, cytokinin riboside monophosphates were separated from cytokinin bases, ribosides and glucosides at high recoveries by simple step elution, with solvents of increasing pH and concentration of MeOH (Dobrev and Kamínek, 2002). Another method using mixed mode reversed-phase anion-exchange SPE prior to two-dimensional HPLC was recently developed for purification of auxin and abscisic acid (Dobrev et al., 2005).

Purification of cytokinins using immunoaffinity chromatography allows very powerful purification of most cytokinins (MacDonald and Morris, 1985; Vaňková et al., 1998; Corbesier et al., 2003). Antibodies of low specificity allowing binding of a wide spectrum of cytokinins have been

successfully used for this purpose. However, even these antibodies do not bind cytokinin *N*-7- and *O*-glucosides (Banowetz, 1994; Jordi et al., 2000) that need to be analyzed separately (Corbesier et al., 2003). Cytokinin *O*-glucosides washed out from immunoaffinity columns can be purified on the same type of column after their hydrolysis by β-glucosidase (Werner et al., 2003; Novák et al., 2003).

The identification and determination of cytokinins by high-performance liquid chromatography-mass spectrometry (HPLC/MS/MS) is increasingly used in plant hormone research. In addition to the more rigorous identification of different cytokinins, these methods also allow the simultaneous quantification of a very wide spectrum of cytokinins in a single run. For these and other reasons, they are becoming a standard in the analysis of cytokinins in plant materials (Redig et al., 1996; Prinsen et al., 1998; Nordström et al., 2004). In contrast to immunological methods (RIA and ELISA), mass spectrometry is also suitable for the determination of cytokinin *N*7- and *O*-glucosides which are not well recognized by antibodies raised against the corresponding cytokinin ribosides (compare Sáenz et al., 2003 and Novák et al., 2003). Because of the high selectivity of mass spectrometry, different cytokinins can be detected and determined in partially purified samples.

The aim of this paper is to compare the efficiency of different methods of cytokinin extraction and purification, with respect to both the prevention of hydrolysis of the cytokinin riboside phosphates, as well as sufficient sample purity for cytokinin determination using HPLC/MS/MS.

2. Results and discussion

The comparison of efficiencies of the different extraction and purification methods is based on the determination of cytokinin levels and relative internal standard responses (RISR) of deuterated cytokinins, expressing the ratio of HPLC/MS/MS chromatogram peak areas of deuterated cytokinins recorded in plants extracts with those of the corresponding pure deuterated standards. The RISR characterizes both the losses during the extraction and purification, as well as the matrix effects of the mass spectrometric analysis.

2.1. Testing of different extraction solvents

In comparing three different solvents (a) 80% (v/v) MeOH, (b) Bieleski's MCF-7, and (c) modified Bieleski's, the deuterated cytokinins were added at the beginning of the extraction, and the extracts were purified using the mixed-mode-SPE as described in Section 4.3. As shown in Fig. 2A, the tested solvents gave similar yields of analyzed cytokinins. Small differences in levels of endogenous cytokinins confirm the efficacy of the internal standard calibration method. However, the modified Bieleski's solvent provided the highest RISR values for almost all cytokinins tested, but riboside monophosphates that were determined after enzymatic hydrolysis as corresponding ribosides. As described in Section 4.6. the hydrolysate was purified on Sep-Pak Plus †C_{18} column where, evidently, contaminants affecting RISR were removed. The results indicate that the solvents differ in their extraction efficiency of compounds other than cytokinins, which are not sufficiently removed during purification and may influence the sensitivity, accuracy and reliability of the determination of cytokinins by HPLC/MS/MS.

Differences in RISR values, between the extractions with Bieleski's and modified Bieleski's solvent, show that $CHCl_3$ in the Bieleski's solvent significantly enhances the extraction of compounds, decreasing the RISR of all tested cytokinins but Z where the difference was not statistically significant. The fact that differences in RISR values in plant material extracted with 80% MeOH and modified Bieleski's solvent are statistically significant only for few cytokinins (iP, iPR and iP7G) supports such opinion. The RISR values exceeding 1.0 (extraction of iPR with 80% MeOH and modified Bieleski's) indicate an increase of the signal due to the matrix effect.

Several authors used Bieleski's solvent without $CHCl_3$ to reduce extraction of contaminants, avoid the problems of handling and disposal of $CHCl_3$-containing extracts (Wang et al., 1995; Jones et al., 1996; Lighfoot et al., 1997). However, the effect of this on the extraction and determination of cytokinins has not been assessed. According to the results presented here, the modified Bieleski's solvent appeared to be the most suitable of the tested solvents for the extraction of cytokinins, prior to their determination using HPLC/MS/MS.

Cytokinin riboside phosphates can be hydrolyzed to the corresponding ribosides by phosphatases during plant tissue extraction (Horgan and Scott, 1987; Crouch and Van Staden, 1992; Redig et al., 1996). Conversions of ZRMP and iPRMP to the corresponding ribosides during extraction with 80% MeOH, Bieleski's MCF-7 and modified Bieleski's solvents were tested. Two hundred picomol of [$^2H_5$]ZRMP and [$^2H_6$]iPRMP were added separately to the frozen powder of plant material at the beginning of the extraction, and the deuterated products were determined using HPLC/MS/MS after extraction and purification by mixed-mode-SPE. Both riboside monophosphates were hydrolyzed almost exclusively to the corresponding ribosides, and further hydrolysis of the ribosides to the corresponding bases was very low ($\leqslant$1.5%, results not shown). Among all of the tested solvents, iPRMP appeared to be much more sensitive to hydrolysis than ZRMP undergoing nearly 60% conversion during extraction with 80% (v/v) MeOH, as compared to 12% and 18% following extraction with Bieleski's and modified Bieleski's solvents, respectively. Hydrolysis of ZRMP was also the highest in 80% (v/v) MeOH (13%), as compared to Bieleski's and modified Bieleski's solvents (both 3%) (Fig. 3). Correspondingly, Horgan and Scott (1987) reported that AMP as a model riboside monophosphate was also hydrolyzed to adenosine

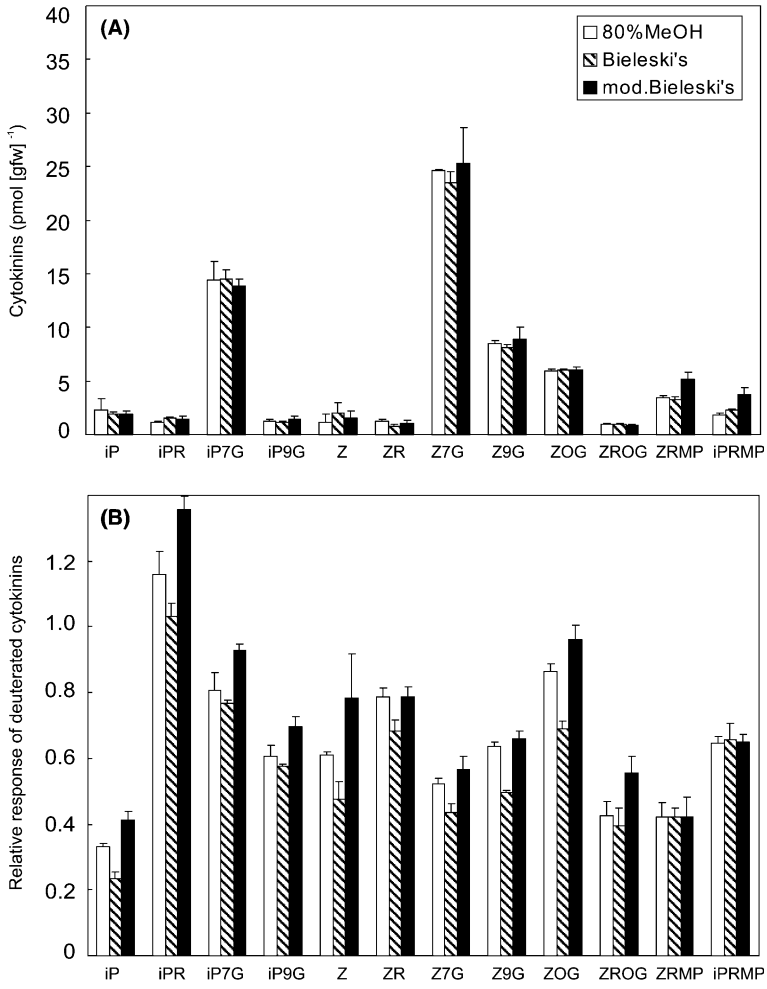


Fig. 2. The effect of three different extraction solvents on cytokinin levels (A) and RISRs, representing the ratio of HPLC/MS/MS responses of deuterated cytokinins from plant extracts to the responses of pure cytokinin standards (B). The plant material was extracted as described in Section 4.2, and the cytokinins were purified as specified in Section 4.4. Results are averages of the analyses of three independent samples. Vertical bars indicate SD.

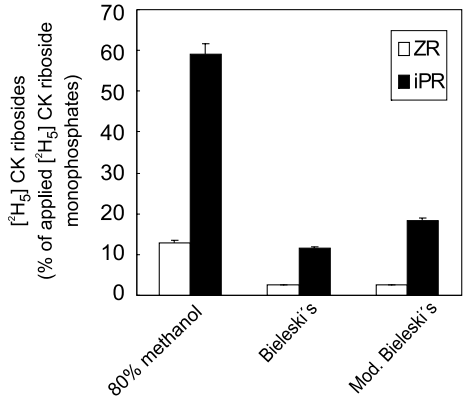


Fig. 3. Conversion of cytokinin (CK) riboside monophosphates ([$^2H_5$]ZRMP and [$^2H_6$]iPRMP) to corresponding CK ribosides following extraction of *Arabidopsis* plants with different solvents. The plant material was supplied with 200 pmol of [$^2H_5$]ZRMP and [$^2H_6$]iPRMP and extracted as described in Section 4.2. Cytokinins were purified as specified in Section 4.2. Results are expressed as a percentage of the cytokinin riboside monophosphates converted to the corresponding ribosides, and represent averages of the analyses of three independent plant samples. Vertical bars indicate SD.

more readily during extraction of soybean callus tissue with 80% MeOH than with Bieleski's solvent.

Evidently, the activity of phosphatases is sufficiently suppressed when plant tissue is powdered in liquid nitrogen and extracted at −20 °C with diluted MeOH acidified with HCO_2H (modified Bieleski's solvent). Moreover, according to Bieleski (1964), extraction in acid solvents at −25 °C speeded-up the deactivation of phosphatases. Unfortunately, he did not test the extraction efficiency of acid solvents lacking $CHCl_3$. As shown in Fig. 3, the omission of $CHCl_3$ from the Bieleski's MCF-7 solvent in the modified Bieleski's solvent did not affect hydrolysis of ZRMP, and only slightly increased hydrolysis of iPRMP.

2.2. Comparison of purification methods

Two different purification procedures, (a) DEAE Sephadex RP-C_{18} and (b) mixed-mode-SPE were compared, using extracts of plant material with modified Bieleski's solvent. Both methods provided similar yields of analyzed cytokinins but iPR and ZRMP where the latter method

provided significantly higher yields (Fig. 4A). The two methods differed significantly in RISR of cytokinins that were almost twice as high in the case of mixed-mode-SPE, as compared to the DEAE Sephadex RP-C_{18} (Fig. 4B). The ratios of RISRs, recorded by the mixed-mode-SPE and DEAE Sephadex RP-C_{18} varied between 1.25 (iP) and 2.3 (Z9G). Values of RISRs for some cytokinins exceeded 1.0, with a maximum of 1.1 (iP7G), most probably due to the increase of the signal by a matrix effect. These results indicate that the DEAE Sephadex RP-C_{18} method was much less efficient in the removal of those contaminants suppressing RISRs. This view is supported by the comparison of total ion current (TIC) of the MS analysis. TIC values were approximately twice as high during the whole interval of elution of cytokinins when plant samples were purified by DEAE Sephadex RP-C_{18}, as compared to mixed-mode-SPE. Moreover, extracts purified by the DEAE Sephadex RP-C_{18} exhibited a much greater variation of TIC profiles of the samples from parallel extractions, than those purified by mixed-mode-SPE (Fig. 5).

It is possible that the differences in RISRs of the tested cytokinins, following their purification using the DEAE Sephadex RP-C_{18} and dual-mode-SPE, are predominantly due to the different recoveries of the deuterated standards. Moreover, the recoveries may be concentration-dependent. To determine the recoveries of cytokinins achieved by the two purification procedures, independent of mass spectrometric analysis, plant extracts corresponding to 1 g of plant material were supplied with [2-^{3}H]Z and [2-^{3}H]ZR, representing cytokinin base and riboside, just before their application onto DEAE Sephadex and dual-mode SPE columns. So as to know if recovery is affected by cytokinin concentration, a separate set of plant extracts was supplied with 40 pmol of the corresponding cold cytokinins, in addition to those labeled [2-^{3}H]. This concentration exceeds the endogenous concentrations of Z and ZR in non-transformed plants (Corbesier et al., 2003; Werner et al., 2003; Ananieva et al., 2004). The recoveries of both [^{3}H]Z and [^{3}H]ZR were very high ($\geqslant$90%) and statistically significant differences were found neither between the two purification procedures nor between samples with and without the addition of cold cytokinins. However, the differences (1.4–2.9%) were not statistically significant, showing that within the tested concentration range recoveries are not dependent on cytokinin concentration (results not shown). This strongly indicates that differences in the levels of tested deuterated cytokinins in plant extracts, purified by

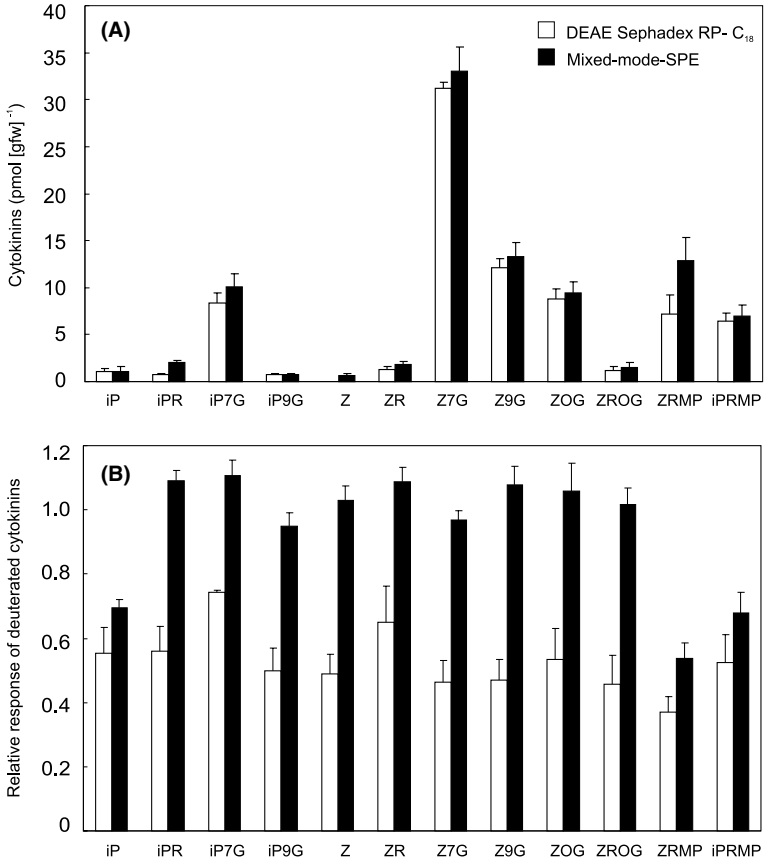


Fig. 4. The effect of purification *of Arabidopsis* plant extracts with DEAE Sephadex RP-C_{18} and mixed-mode-SPE on cytokinin levels (A) and RISRs, representing the ratio of HPLC/MS/MS responses of deuterated cytokinins from plant extract to the responses of pure cytokinin standards (B). The plant material was extracted with modified Bieleski's solvent, as described in Section 4.2, and the cytokinins were purified, as specified in Sections 4.3 and 4.4. Results are averages of the analysis of three independent plant samples. Vertical bars indicate SD.

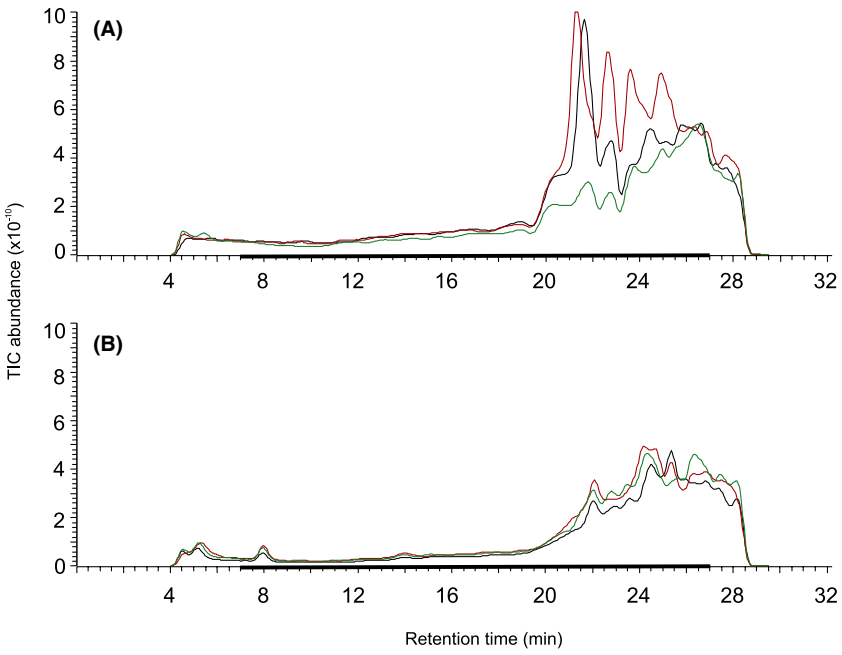


Fig. 5. MS total ion current profiles (TIC) of *Arabidopsis* plant extracts purified with DEAE Sephadex RP-C_{18} (A) and mixed-mode-SPE (B), expressed in arbitrary units. The plant material was extracted with modified Bieleski's solvent, as described in Section 4.2, and the cytokinins were purified as specified in Sections 4.3 and 4.4. Each curve corresponds to one separate analysis. Cytokinins were eluted at retention time from 7 min (Z7G) to 27 min (iPR) as marked by bold lines on the time scales.

the two methods, are caused by the different efficiency of removal of those contaminants interfering with MS analysis, rather than by their differential recoveries.

Purification of cytokinins using dual-mode-SPE is apparently extremely efficient in the removal of UV absorbing contaminants, decreasing the content of UV absorbing material by 90%, as compared to DEAE Sephadex RP-C_{18} (Fig. 6). This demonstrates its potential suitability for the purification of plant extracts prior to the determination of cytokinins by methods other than HPLC/MS/MS, such as ELISA and RIA.

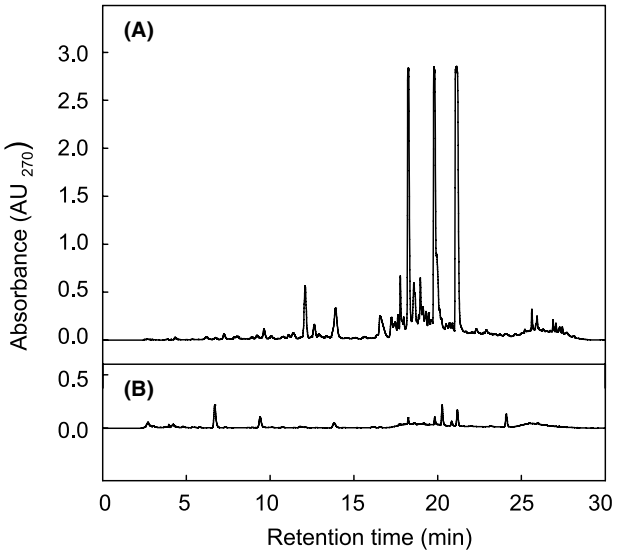


Fig. 6. HPLC UV traces of *Arabidopsis* plant extracts, purified with DEAE Sephadex RP-C_{18} (A) and mixed-mode-SPE (B). The plant material was extracted with modified Bieleski's solvent, as described in Section 4.2, and the cytokinins were purified, as specified in Sections 4.3 and 4.4. No internal standards were added.

3. Conclusion

The modified Bieleski's, as compared to two other tested extraction solvents, sufficiently suppressed dephosphorylation of cytokinin riboside monophosphates and reduced the extraction of compounds decreasing the RISRs of tested deuterated cytokinins. This solvent, lacking $CHCl_3$, is easier and safer to handle and appeared to be the most suitable for extraction of cytokinins. Purification of cytokinins using mixed-mode-SPE, as compared to DEAE Sephadex RP-C_{18} method, was powerful in the removal UV absorbing contaminants providing preparations exhibiting high RISRs of deuterated counterparts of natural cytokinins. This method was found simpler, faster and more operational. It also allows more complex plant hormone analysis by providing a partially purified fraction containing auxin and abscisic acid.

4. Experimental

4.1. Plant material and chemicals

Arabidopsis thaliana (ecotype Columbia) plants were grown in a greenhouse under 16 h light/8 h dark at 23 °C/20 °C daylight cycle. Leaf rosettes of 55-day old plants were frozen in liquid nitrogen and stored at −80 °C.

Deuterium-labeled cytokinins were purchased from Apex (Honiton, Devon, UK). [2-^{3}H]Z and [2-^{3}H]ZR (both 0.59 TBq mmol^{-1}) were obtained from the Isotope Laboratory of the Institute of Experimental Botany, Prague, Czech Republic. EtOH of spectrophotometric grade, MeOH, HCO_2H, NH_4OH, NH_4HCO_3, all of analytical grade, were supplied by Lachema a.s. (Neratovice, Czech Republic). Calf intestine alkaline phosphatase (4U/mg) was a product of Sigma–Aldrich (St. Louis, USA). Sep-Pak Plus †C_{18} cartridges (containing 400 mg sorbent of C_{18} phase bonded on silica gel matrix), and Oasis MCX columns (containing 150 mg sorbent of a sulfonated copolymer, capacity 1 mequiv g^{-1}, particle diameter 30 μm) were obtained from Waters (Milford, MA, USA). DEAE Sephadex A-25, capacity 3.5 mequiv g^{-1} and particle size 40–120 μm was product of Pharmacia (Uppsala, Sweden). Syringes (20 ml) with frits (porosity 20 μm) were purchased from Isolute SPE Accesories (Alltech, UK). The SPE procedures were performed using a vacuum manifold equipped with vacuum control valve that allowed the concurrent processing of 12 samples (Supelco's Visiprep, Sigma, St. Louis, USA).

4.2. Extraction of cytokinins

Three different extraction solvents: (a) 80% (v/v) MeOH, (b) Bieleski's MCF-7 solvent (MeOH–$CHCl_3$–HCO_2H–H_2O 12:5:1:2, v/v/v) and (c) modified Bieleski's solvent (MeOH–HCO_2H–H_2O; 15/1/4, v/v/v) were compared. *Arabidopsis* leaf rosettes were frozen by dipping in liquid nitrogen and then homogenized to a powder with a pestle in a ceramic mortar in liquid nitrogen. One gram plant powder aliquots were distributed to 50 ml polypropylene centrifuge tubes. Ten millilitre aliquots of cold (−20 °C) tested solvents were immediately added together with a mixture of the following deuterium-labeled cytokinin standards (50 pmol each in total volume of 50 μl of 50% MeOH, v/v): [^{2}H$_6$]iP), [^{2}H$_5$]Z, [^{2}H$_3$]DHZ), [^{2}H$_6$]iPR, [^{2}H$_5$]ZR, [^{2}H$_3$]DHZR, [^{2}H$_6$]iP7G, [^{2}H$_6$]iP9G, [^{2}H$_5$]Z7G, [^{2}H$_5$]Z9G, [^{2}H$_5$]ZOG, [^{2}H$_5$]ZROG, [^{2}H$_6$]iPRMP, [^{2}H$_5$]ZRMP and [^{2}H$_3$]DHZRMP. After overnight extraction at −20 °C solids were removed by centrifugation (13,000*g*, 20 min, 4 °C) and re-extracted with 5 ml of corresponding extraction solvent by vortexing for 30 s, followed with standing at −20 °C for 1 h. Pooled extracts were passed through Sep-Pak Plus †C_{18} to remove pigments and lipids and evaporated under vacuum at 40 °C near to dryness prior purification.

4.3. Purification of cytokinins using DEAE Sephadex RP-C_{18}

The method was adapted according to MacDonald and Morris (1985) and Redig et al. (1996). Plant extracts containing deuterated cytokinin standards were diluted with 10 ml water and alkalized with ammonium to pH 7.2–7.5. The solution was applied to a column of DEAE Sephadex (10 ml, HCO_3^- form) connected in tandem with a Sep-Pak Plus †C_{18}. The column was washed with 20 ml of distilled water. At this point, the Sep-Pak Plus †C_{18} column (containing cytokinin bases, ribosides and glucosides) and the DEAE Sephadex column (with entrapped cytokinin riboside phosphates) were disconnected and each column was separately washed with 10 ml water. Cytokinins from Sep-Pak Plus †C_{18} were eluted with 5 ml of 80% (v/v) MeOH and solvent was evaporated under vacuum at 40 °C. The DEAE Sephadex column was connected with a new Sep-Pak Plus †C_{18} column, and the CK riboside phosphates were eluted from the former column into Sep-Pak Plus †C_{18} with 10 ml 1 M NH_4HCO_3. Finally, cytokinin riboside phosphates were eluted from the Sep-Pak Plus †C_{18} with 5 ml of 80% (v/v) MeOH. The solution was evaporated under vacuum at 40 °C to water phase, and the riboside phosphates were hydrolyzed to the corresponding ribosides, as described in Section 4.6.

4.4. Purification of cytokinins mixed-mode-SPE

The method of Dobrev and Kamínek (2002) was used. Plant extracts were diluted with 5 ml of 1 M formic acid and applied on an Oasis MCX column. The column was washed with 5 ml 1 M formic acid, and plant hormones auxin (IAA) and abscisic acid (ABA) were eluted with 5 ml MeOH. Subsequently, the cytokinin riboside phosphates were eluted with 5 ml 0.35 M ammonia in water, and hydrolyzed by alkaline phosphatase, as described in Section 4.6. Cytokinin bases, ribosides and glucosides were eluted in the next step with 5 ml 0.35 M ammonia in 60% (v/v) MeOH. Solvents were evaporated at 40 °C under vacuum.

4.5. Preservation of cytokinin riboside phosphates

Preservation of cytokinin riboside monophosphates during extraction was tested using three different solvents: (a) 80% (v/v) MeOH, (b) Bieleski's MCF-7 and (c) modified Bieleski's solvent. *Arabidopsis* leaf rosettes were frozen and extracted as described in Section 4.2. Two hundred pmol of [^{2}H$_5$]ZRMP and [^{2}H$_6$]iPRMP were added to 1 g aliquots of frozen plant powder at the beginning of extraction, instead of the mixture of deuterated cytokinin standards. Extracted [^{2}H$_5$]ZRMP and [^{2}H$_6$]iPRMP, as well as their ribosides and bases released during extraction, were purified using mixed-mode-SPE (as described in Section 4.4), and determined by HPLC/MS/MS using the standard addition method. The corresponding deuterated ribosides were the only clearly quantified products. The results were expressed as the percentage of deuterated riboside phosphates converted to the corresponding deuterated ribosides.

4.6. Hydrolysis of cytokinin riboside monophosphates

Cytokinin riboside monophosphates were determined as cytokinin ribosides after their dephosphorylation, prior to

HPLC/MS/MS analysis. The MCX column fractions containing cytokinin riboside monophosphates were evaporated to water phase and supplied with 2 M $CH_3CO_2NH_4$ to final 100 mM concentration. After addition of alkaline phosphatase (0.3 U), the solution was incubated at 37 °C for 1.5 h, and then neutralized with formic acid to pH 5–6. The solution was passed through a Sep-Pak Plus †C_{18} column and entrapped cytokinin ribosides were eluted with 5 ml 80% (v/v) MeOH.

4.7. Recoveries of [^{3}H] cytokinin standards and content of contaminants

To determine how the purification procedures affect cytokinin recovery, independent of the cytokinin extraction and MS analysis, *Arabidopsis* leaf rosettes were powdered in liquid nitrogen and extracted with modified Bieleski's solvent under conditions specified in Section 4.2 without the addition of deuterated cytokinin standards. Prior to purification of the cytokinins using DEAE Sephadex RP-C_{18} and mixed-mode-SPE (as described in Sections 4.3 and 4.4, respectively), 4 kBq of [2-^{3}H]Z and [2-^{3}H]ZR were separately added to extract aliquots corresponding to 1 g of plant material. To determine the potential effect of cytokinin concentration on recovery, a separate set of extract aliquots was supplied with 40 pmol of cold cytokinins (Z and ZR), in addition to [^{3}H] cytokinins. The radioactive compounds were separated by HPLC using a series 200 autosampler and quaternary pump (both Perkin–Elmer, Wellesley, MA, USA), a Luna C_{18} (2) column (150 × 4.6 mm, 3 μm, Phenomenex, Torrance, CA, USA) maintained at 35 °C, and two detectors coupled in series: a 235 C diode array detector (Perkin–Elmer, Wellesley, MA, USA), and a Ramona 2000 flow-through radioactivity detector (Raytest, Straubenhardt, Germany). Two solvents (A: 40 mM CH_3CO_2H adjusted with NH_4OH to pH 4 and B: CH_3CN/CH_3OH, 1/1, v/v) were used at a flow rate of 0.6 ml min^{-1} with linear gradients of 10–15% B in 2 min, 15–20% B in 9 min, 20–34% B in 0.1 min, 34–45% B in 7.9 min, 45–100% B in 2 min, 100% B for 2 min, and 100–10% B in 2 min. The column eluate was monitored at 270 nm on the diode array detector and, after on-line mixing with three volumes of liquid scintillation cocktail Flo-Scint III (Packard BioScience, Meriden, CT, USA), on the Ramona 2000 radioactivity detector. The radioactive analytes were identified on the basis of coincidence of their retention times with authentic standards.

4.8. HPLC/MS/MS analysis

The analyses were carried out on a HPLC/MS system, consisting of a PAL HTS autosampler (CTC Analytics, Zwingen, Switzerland) and quaternary HPLC pump Rheos 2000 (Flux Instruments, Basel, Switzerland) connected to an Ion-Trap mass spectrometer LCQ (Finnigan, San Jose, CA, USA) equipped with an electrospray interface (ESI).

Table 1
MS/MS transitions for cytokinin quantitation

Cytokinin	Parent ion	Product ion
ZxG-d5 ($x = 7,9,O$)	387	225
ZROG-d5	519	387
trans-Z-d5	225	207
ZR-d5	357	225
IPxG-d6 ($x = 7,9$)	372	210
IPR-d6	342	210
IP-d6	210	137 + 148
ZxG ($x = 7,9,O$)	382	220
ZROG	514	382
trans-Z	220	202
ZR	352	220
IPxG ($x = 7,9$)	366	204
IPR	336	204
IP	204	136 + 148

Dry samples of purified cytokinins were dissolved in 20 μl 50% (v/v) acetonitrile. Solutions were diluted by the addition of 80 μl H_2O and passed through Micro-Spin centrifuge nylon filters (0.2 μm, Alltech, Deerfield, IL, USA). The filtrate aliquots of 5 μl were injected onto a C_{18} HPLC column (AQUA 250 mm × 2.0 mm, 5 μm, Phenomenex, Torrance, CA, USA) and eluted at a flow rate of 0.2 ml min^{-1} using a linear gradient of acetonitrile (B) in 0.0005% (v/v) acetic acid in water (A): 10% B for 5 min, to 17% B in 10 min; then to 50% B in 11 min; and finally increased to 90% B and maintained for 5 min. The column was equilibrated by the starting composition of the mobile phase for 20 min before each analytical run. MS data were collected in positive MS/MS product ion mode for quantification and the TIC was recorded in a MS full scan (50–2000 amu) mode.

Endogenous natural cytokinins were determined by the same HPLC/MS/MS method using the internal standard calibration and the corresponding deuterated cytokinins as internal standards. The measured masses (m/z) are shown in Table 1. The relative responses of internal standards (RISR) were used to characterize the effectiveness of extraction and purification procedures (recovery) as well as the effectiveness in removal of residual contaminants affecting the MS signal (matrix effect). RISRs are expressed as the ratio of MS/MS responses of deuterated standards (used as test compounds) added to plant samples at the beginning of extraction and responses of corresponding pure deuterated standards.

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