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Cytokinin profiling in plant tissues using ultra-performance liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

We have developed a simple, high-throughput batch immunoextraction (IAE) micropurification procedure for extracting a wide range of naturally occurring cytokinins (bases, ribosides, *O*- and *N*-glucosides, and nucleotides) from plant tissues in solutions that are compatible with ultra-performance liquid chromatography (UPLC), thereby facilitating sensitive subsequent analysis. The UPLC system was coupled to a tandem quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface (ESI). Small (mg) amounts of tissues were purified by solid-phase extraction (SPE) followed by an immunoaffinity clean-up step and two fast chromatographic separations of most cytokinin metabolites (bases, ribosides, and 9-glucosides in the first, *O*-glucosides and nucleotides in the second). Using UPLC, the runs were up to 4-fold faster than in standard cytokinin analyses, and both retention times and injection volumes were less variable (RSDs, 0.15–0.3% and 1.0–5.5%, respectively). In multiple reaction monitoring (MRM) mode, the detection limit for most of the cytokinins analyzed was close to 1 fmol (5–25 fmol for *O*-glucosides and nucleotides) and the linear range spanned at least five orders of magnitude. The extraction and purification method was optimized using poplar (*Populus* × *canadensis* Moench, cv *Robusta*) leaf samples, and the analytical accuracy was further validated using IAE-purified 10-day-old *Arabidopsis thaliana* plants spiked with 1 and 10 pmol of cytokinin derivatives. This approach can be used for rapid, sensitive qualitative and/or quantitative analysis of more than 50 natural cytokinins in minute amounts of plant tissues with high performance, robustness, and accuracy.

1. Introduction

Cytokinins (CKs), a group of phytohormones, play crucial roles in the control of plant growth and development. Since their discovery in the 1950s (Miller et al., 1955), it has been established that cytokinins play important regulatory roles in various physiological processes in plants, including the promotion of cell division, the counteraction of senescence, the regulation of apical dominance and the transmission of nutritional signals. Naturally occurring cytokinins are adenine derivatives (Mok and Mok, 2001; Table 1), substituted at the N^6 position by either an isoprenoid side chain or an aromatic ring (designated isoprenoid and aromatic cytokinins, respectively). In each group, there are small variations in the side-chain structure between individual representatives, e.g. in the presence/absence of double bonds, additional hydroxyl or methyl groups, and their stereoisomeric positions. In most cases, nucleosides, nucleotides and other sugar and amino acid conjugates have also been found. The physiological significance of these variations has not yet been fully elucidated.

In recent years, the development of user-friendly bench-top mass spectrometers has revolutionized many aspects of analytical chemistry. Mass spectrometry has become an increasingly important technique in plant hormone analysis (Moritz, 1996; Ljung et al., 2004), and many workers in this field have switched from using relatively non-specific bioassays, thin layer and high-performance liquid chromatography (HPLC) to combined chromatographic-detection techniques that are both very sensitive and accurate. The rapid technological developments in the design and construction of mass spectrometers have provided new tools that enable plant hormones to be detected and quantified in milligram amounts of plant tissue (Ljung et al., 2004), greatly facilitating attempts to characterize their distribution and to elucidate their metabolism and mode of action. However, in order to determine amounts of cytokinins present at low levels in relatively impure (complex) extracts, efficient separation and purification techniques as well sensitive mass spectrometry are required.

Analysis of cytokinins is associated with a number of problems related to the chemical nature of the compounds, the small quantities present in samples and the complexity of plant matrices. Firstly, an appropriate solvent (e.g. 80% methanol, 70% ethanol or Bieleski buffer) must be selected to extract them efficiently from the targeted tissues (Bieleski, 1964; Faiss et al., 1997; Hoyerová

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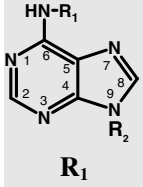
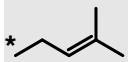
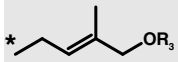
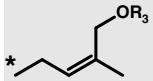
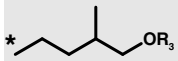
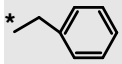
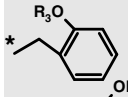
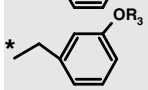
Table 1
Structures, generic names and abbreviations of naturally occurring cytokinins

	Base
R_1	R_3: H
	N^6-(Δ^2-isopentenyl) adenine (iP)
	*trans*-Zeatin (tZ)
	*cis*-Zeatin (cZ)
	Dihydrozeatin (DHZ)
	N^6-Benzyladenine (BA)
	*ortho*-Topolin (oT)
	*meta*-Topolin (mT)
R_2: β-D-Ribose	Riboside (R)
R_2: β-D-Glucose	9-Glucoside (9G)
R_2: β-D-Ribose	Riboside
5′-Monophosphate	5′-Monophosphate (RMP)
R_3: β-D-Glucose	*O*-Glucoside (OG)

et al., 2006). It is also essential to add at least one appropriate internal standard to monitor possible losses during cytokinin extraction and purification (Ljung et al., 2004). The extracts must then be cleaned-up, generally using a suitable combination of materials such as octadecylsilica (C18), strong cation exchanger (SCX; Åstot et al., 1998), MCX (combined C18 and SCX material; Dobrev and Kamínek, 2002), DEAE-cellulose, DEAE-Sephadex or anion-exchangers like phosphocellulose (Prinsen et al., 1995a,b). They can also be fractionated if desired by these procedures, notably into nucleotides and other cytokinins. However, immunoaffinity chromatography (IAC) offers the most powerful method for purifying specific classes of cytokinin metabolites (MacDonald and Morris, 1985; Faiss et al., 1997). Furthermore, cytokinin-*O*-glucosides and nucleotides washed out of IAC columns can be subsequently purified, using the same columns, following hydrolysis by β-glucosidase and alkaline phosphatase, respectively (Novák et al., 2003).

The most widely used techniques to analyze phytohormones (after cleaning-up samples) are gas or liquid chromatography combined with mass spectrometry (GC–MS and LC–MS, respectively) and radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs) of LC-fractionated samples (MacDonald et al., 1981; Strnad et al., 1992). For a long time, GC–MS was the most commonly used technique for analyzing cytokinins (Moritz, 1996), but LC–MS has become increasingly popular in recent years (Prinsen et al., 1995a,b; van Rhijn et al., 2001; Novák et al., 2003). The potential utility of coupling a capillary liquid chromatograph with a double-focusing magnetic sector mass spectrometer via a frit–fast-atom bombardment ion source (interface) has been investigated (Imbault et al., 1993; Åstot et al., 1998), as has derivatization of cytokinin-containing samples before LC–MS/MS analysis (Nordström et al., 2004), and combined HPLC (high-performance liquid chromatography)–MS/MS (tandem mass spectrometry) has become a standard method in recent years (i.e. Zhang et al., 2001; Chiwocha et al., 2003; Bredmose et al., 2005; Ge et al., 2005).

In addition, new advances in separation technology have led to the introduction of the so-called ultra-performance liquid chromatography (UPLC), in which a column packed with 1.7 μm bridged ethylsiloxane/silica hybrid particles is used, providing higher peak capacity, greater resolution, and greater sensitivity than HPLC (Zhao et al., 2006). The analytical parameters of mass spectrometry measurements are significantly enhanced by using UPLC (Leandro et al., 2006), and several studies published in the last 2 years have demonstrated that UPLC–MS has high potential in pharmaceutical, environmental, cosmetic, explosive, proteomic, and metabolomic analyses (Nguyen et al., 2006). However, few publications to date have described the use of UPLC–MS/MS for qualitative or quantitative analysis of plant extracts, although Li et al. (2006) and Fekete et al. (2007) have described its use for analyzing *T. ledibouri* Reichb. flower extracts and bacterial *N*-acylhomoserine lactones, respectively. Very recently, UPLC–MS/MS methods have been developed also for some plant hormone groups (Naito et al., 2007; Arite et al., 2007; Zentella et al., 2007; Hirano et al., 2007). However, no previous applications of UPLC–MS/MS for cytokinin analysis have been reported up-to-date.

Since plant hormones are present in minute quantities in the small amounts that are generally available of many plant tissues (e.g. organelles, protoplasts, cells, buds, apical roots, stem regions, seedlings, and seeds) of interest in modern phytohormone research, rapid but sensitive techniques are essential for analyzing them. Moreover, fairly large numbers of biological samples containing broad ranges of metabolites of multiple groups of phytohormones must often be analyzed to investigate physiological processes thoroughly. Our recent effort comprises development of a protocol for extracting and analyzing cytokinins involving immunoextraction using generic monoclonal anticytokinin antibodies followed by UPLC–ESI(+)–MS/MS separation and quantification. For the first time, developed method was lately applied on determination of CK levels in moss (*Physcomitrella patens*) (von Schwartzenberg et al., 2007). In the present study, detailed description and evaluation of developed method, including off-line batch immunoextraction, is presented and whole method is carefully compared with the classical HPLC–MS method. The method provides substantial improvements in terms of robustness, sensitivity, selectivity, convenience, throughput and cost-effectiveness over previous methods.

2. Results and discussion

The main aim of the work described in this article was to develop a new, fast, sensitive procedure for analyzing naturally occurring isoprenoid and aromatic cytokinins in plant tissues, combining convenient, high-throughput purification steps, rapid but reliable separations and highly sensitive detection and quantitative analysis by electrospray tandem mass spectrometry.

2.1. Development of the UPLC–ESI(+)–MS/MS method

Since certain cytokinins (zeatins and topolins) yield identical parent $[M+H]^+$ ions and basic fragment peaks under electrospray mass spectrometry conditions, it is essential to clearly separate the isomers to obtain accurate estimates of levels of the natural

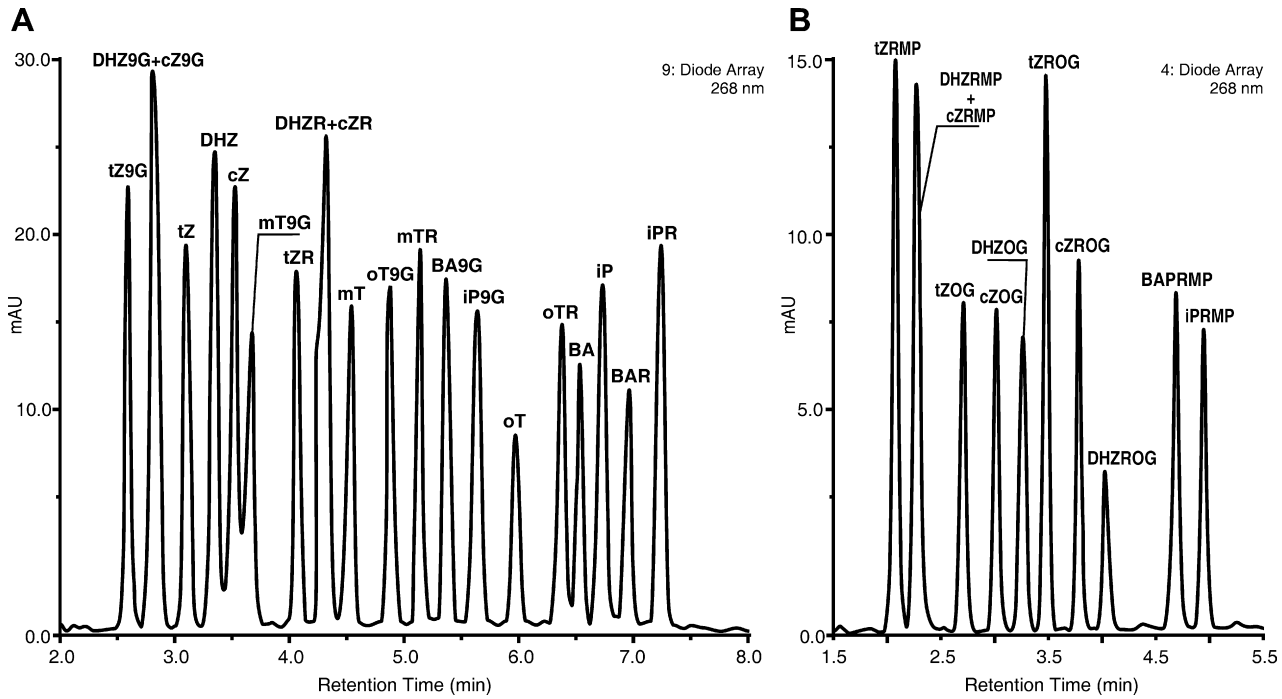


Fig. 1. Separation of cytokinin standards by ultra-performance liquid chromatography (UPLC). UPLC-UV chromatogram (268 nm) of 21 cytokinin bases, ribosides, and 9-glucosides (A) and 11 cytokinin *O*-glucosides and nucleotides (B) containing 10 pmol of each derivative per injection.

cytokinins. The analyses without and/or with imperfect separation are used sometimes in order to increase sample throughput (Prinsen et al., 1995a,b, 1998), but this inevitably raises high risks of overestimating concentrations of some endogenous cytokinins. Thus, there is a clear need for highly hyphenated analyses, in which baseline HPLC separation is combined with mass spectrometric detection. A recently described HPLC–MS method for cytokinin analysis (Novák et al., 2003) is capable of separating 20 naturally occurring cytokinins in 30 min. Using UPLC, similar baseline separation of isoprenoid (*trans*/*cis*-zeatin, dihydrozeatin, isopentenyladenine groups) and aromatic (benzyladenine, *meta*/*ortho*-topolin groups) cytokinins was obtained and (as shown in Fig. 1A) a mixture containing 21 unlabeled and 15 labeled internal CK standards could be separated using a short reversed-phase column (2.1 × 50 mm) over 7.5 min, reducing the run time of the analysis 4-fold. The total run time of the UPLC method, including equilibration, was 12 min while that of the HPLC method was at least 50 min. It also provides much more robust separation, in terms of the consistency of retention times, than the HPLC method. The chromatographic stability and injection accuracy of the UPLC system was tested in detail by 10 consecutive measurements of cytokinins in biological matrices (data not shown), and coefficients of variation for the retention times were found to range between 0.15% and 0.30% RSD, compared to 0.27–0.54% RSD for the HPLC method, and 1.0–5.5% RSD for injection volumes. The statistical evaluation of the method also showed that the system retained high levels of consistency during the chromatographic separations, allowing the chromatographic run to be reliably split into eight retention windows (2.0–3.0 min, 3.0–3.8 min, 3.8–4.2 min, 4.2–4.7 min, 4.7–5.6 min, 5.6–6.2 min, 6.2–7.1 min, 7.1–8.0 min) to increase the sensitivity of subsequent ESI(+)–MS/MS measurements.

Previously, direct estimation of *O*-glucosides and nucleotides has been almost impossible. These groups of cytokinins are usually determined following β-glucosidase or acid/alkaline phosphatase treatment of partially purified extracts, respectively (Novák et al., 2003; Takei et al., 2003; Hoyerová et al., 2006). However, using identical chromatographic conditions, baseline separation of 11 cytokinin *O*-glucosides and nucleotides (obtained in the PBS fraction following the IAE step, see below) was also achieved using UPLC with the bridged ethylsiloxane/silica hybrid column (Fig. 1B). Furthermore, the retention times were sufficiently consistent to split the chromatographic run of this fraction into three reliable retention windows (1.5–2.5 min, 2.5–4.3 min, 4.3–5.5 min) to increase the sensitivity of subsequent determinations.

To select characteristic diagnostic MRM transitions, 10 pmol of each labeled and unlabeled standard was injected separately onto the column, and for each compound, precursor and product ions were automatically defined by QuantOptimize™. Strong consistency was found between the MRM transitions obtained here using the ESI with QuantOptimize, and those obtained using single spray systems (for basic isoprenoid CKs) by Prinsen et al. (1995a,b), van Rhijn et al. (2001) and Chiwocha et al. (2003). Electrospray capillary and cone voltages were tuned to generate the required precursor ions in positive ion mode. Subsequently, the collision energy was optimized for dissociation of molecular ions into product ions for each cytokinin. Table 2 shows all product and precursor ions of most isoprenoid and aromatic cytokinins determined by UPLC–ESI(+)–MS/MS with sustained cone voltages of 17–38 V and collision energies of 15–24 eV. Summarizing the results, collision-induced dissociation of the $[M+H]^+$ ions of cytokinins generates: (i) identical characteristic product ions for cytokinin isomers (*cis*- and *trans*-zeatin: 220.1 > 136.1; *ortho*- and *meta*-topolin: 242.1 > 136.1); (ii) a 136.1 amu product ion (adenine) for all free bases except BA (91) and mT (107); (iii) an aglycone fragment from monoglycosylated compounds and a nucleotide; and (iiii) an appropriate *O*-glucoside ion from cytokinin riboside-*O*-glucoside conjugates.

2.2. UPLC–ESI(+)–MS/MS parameters for quantification

The cytokinin analytes were quantified by standard isotope dilution analysis, which provides quantitative results with high

Table 2
Optimized MS conditions

	Transition	CV (V)	CE (eV)	DT (s)		Transition	CV (V)	CE (eV)	DT (sec)
tZ9G	382.2 > 220.1	35	22	0.13					
[$^2H_5$]tZ9G	387.2 > 225.1	34	23	0.13	oT9G	404.2 > 242.1	32	20	0.09
DHZ9G	384.2 > 222.1	32	22	0.13	mTR	374.2 > 242.1	31	21	0.09
[$^2H_3$]DHZ9G	387.2 > 225.1	34	23	0.13	BA9G	388.2 > 226.1	35	24	0.09
cZ9G	382.2 > 220.1	35	22	0.13	[$^2H_7$]BA9G	395.2 > 233.1	35	24	0.09
tZ	220.1 > 136.1	24	17	0.07	iP9G	366.2 > 204.1	33	21	0.09
[$^{13}C_5$]tZ	225.1 > 141.1	26	18	0.07	[$^2H_6$]iP9G	372.2 > 210.1	33	21	0.09
DHZ	222.1 > 136.1	32	21	0.07	oT	242.1 > 136.1	23	16	0.09
[$^2H_3$]DHZ	225.1 > 136.1	32	21	0.07	[$^{15}N_4$]oT	247.1 > 141.1	23	16	0.09
cZ	220.1 > 136.1	24	17	0.07	oTR	374.2 > 242.1	28	18	0.04
[$^{13}C_5$]cZ	225.1 > 141.1	26	18	0.07	BA	226.1 > 91.0	33	22	0.04
mT9G	404.2 > 242.1	35	24	0.07	[$^2H_7$]BA	233.1 > 91.0	33	22	0.04
tZR	352.2 > 220.1	30	19	0.18	iP	204.1 > 136.1	22	15	0.04
[$^2H_5$]tZR	357.2 > 225.1	30	19	0.18	[$^2H_6$]iP	210.1 > 137.1	22	15	0.04
DHZR	354.2 > 222.1	23	21	0.07	BAR	358.2 > 226.1	33	21	0.04
[$^2H_3$]DHZR	357.2 > 225.1	24	22	0.07	[$^2H_7$]BAR	365.2 > 233.1	35	22	0.04
cZR	352.2 > 220.1	22	17	0.07	iPR	336.2 > 204.1	28	19	0.18
mT	242.1 > 107.0	33	21	0.07	[$^2H_6$]iPR	342.2 > 210.1	28	19	0.18
[$^{15}N_4$]mT	246.1 > 107.0	33	21	0.07					
tZRMP	432.2 > 220.1	32	20	0.13	tZOG	382.2 > 220.1	31	18	0.04
[$^2H_5$]tZRMP	437.2 > 225.1	32	21	0.13	[$^2H_5$]tZOG	387.2 > 225.1	30	17	0.04
DHZRMP	434.2 > 222.1	34	23	0.13	cZOG	382.2 > 220.1	31	18	0.04
[$^2H_3$]DHZRMP	437.2 > 225.1	32	20	0.13	DHZOG	384.2 > 222.1	32	21	0.04
cZRMP	432.2 > 220.1	32	20	0.13	[$^2H_7$]DHZOG	391.2 > 229.1	33	22	0.04
BARMP	438.2 > 226.1	33	21	0.09	tZROG	514.2 > 382.2	37	20	0.04
[$^2H_7$]BAMP	445.2 > 233.1	32	21	0.09	[$^2H_5$]tZR	519.2 > 387.2	35	22	0.04
iPRMP	416.2 > 204.1	33	19	0.09	cZROG	514.2 > 382.2	37	20	0.04
[$^2H_6$]iPRMP	422.2 > 210.1	32	20	0.09	DHZROG	516.2 > 384.2	38	21	0.04

Optimized product ion scanning, diagnostic transition, cone voltage (CV), collision energy (CE) and dwell time (DT) parameters of the triple quadrupole mass spectrometer (electrospray interface in positive ion mode) for each of the analyzed cytokinins.

accuracy and precision (Ljung et al., 2004). A known amount (1 pmol) of an internal standard was added to each sample during microextraction, and the final concentration was calculated from the peak area in MRM chromatograms. The ratio of unlabeled (endogenous) to labeled internal cytokinin standard was used to estimate the concentration of endogenous compound in the sample. For most of the measured naturally occurring cytokinins, appropriate labeled standards were added (Table 2) to determine the endogenous cytokinin levels, to check recoveries during purification and to validate the determination. For quantification of cZR, cZ9G, cZROG, cZRMP and DHZROG, the corresponding *trans*-zeatin tracers were used. All topolins were analyzed using internal [$^{15}N_4$]mT and [$^{15}N_4$]oT standards since no other labeled aromatic cytokinins were available. Therefore, the values of other cZ and topolin metabolites may have been subject to errors due to imperfect internal standardization.

Calibration solutions containing varying concentrations of each unlabeled cytokinin and a defined, fixed concentration of the corresponding stable isotope-labeled internal standard (IS) were used to create calibration curves. The linear range was at least five orders of magnitude when all data were plotted after logarithmic transformation. The resulting calibration curves were mostly linear across concentration ranges from 1–50 fmol to 100 pmol, with correlation coefficients (R^2) between 0.9996 and 0.9986 (illustrative data for isopentenyladenine-type cytokinins are shown in Fig. 2).

Limits of detection (LOD) and quantification (LOQ), defined as signal-to-noise ratios of 3:1 and 10:1, respectively, were determined for each cytokinin. In MRM mode, the LODs for most cytokinins were lower than 5.0 fmol (Table 4), while the LOQs ranged from 0.75 fmol to 10 fmol for DHZ(R) and mT (oT9G), respectively. For all the cytokinins measured, the developed procedure is the most sensitive method described to date for non-derivatized compounds. For classical cytokinins (Z and iP type), the method provided 5–10-fold greater sensitivity than previously published HPLC–MS techniques (Witters et al., 1999; Novák et al., 2003). We introduced not only a new approach for separating intact cytokinin *O*-glucosides and nucleotides, but also direct mass spectrometry measurements of these metabolites in positive [M+H]$^+$ mode. For cytokinin *O*-glucosides and nucleotides, LODs in the range 5–25 fmol were achieved (Table 4). Although these detection limits are somewhat higher than those obtained for free CK bases and ribosides, this is the first published method allowing the analysis of intact CK nucleotides with no need for derivatization.

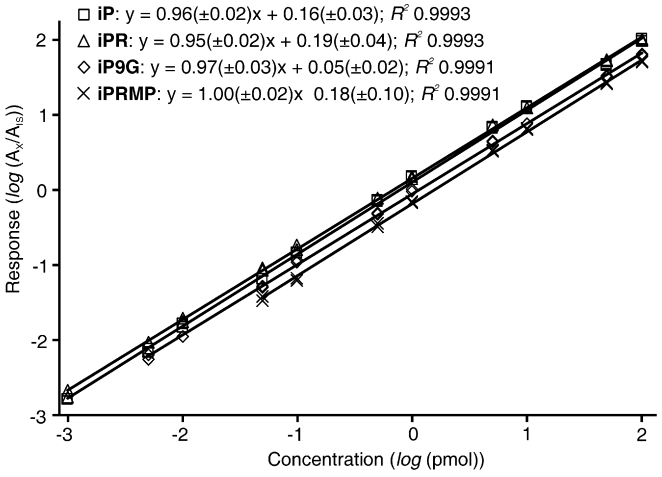


Fig. 2. Linearity ranges for isopentenyladenine cytokinins (iP, iPR, iP9G, iPRMP). Confidence limits and correlation coefficients, with dynamic ranges, for four cytokinin derivatives analyzed by UPLC–ESI(+)–MS/MS are shown (based on values obtained from five consecutive measurements).

2.3. Purification and IAE method development

Immunoaffinity chromatography can be used as a valuable final purification step for samples of various biological matrices (Hage, 1999), and its applicability for purifying cytokinins in plant material has been demonstrated by several authors (i.e. Faiss et al., 1997; Novák et al., 2003). IAC has higher selectivity than conventional solid-phase extraction, but the throughputs offered by previous IAC procedures for cytokinins have generally been low. However, Hauserová et al. (2005) recently published an off-line batch immunoextraction method (IAE) that could be used to isolate unknown aromatic cytokinins with high recovery levels relatively cheaply from plant tissues. In the study reported here we extended this approach by applying it to the high throughput purification of all classes of endogenous cytokinins from various plant tissues, and combining it with UPLC–ESI(+)–MS/MS. The resulting procedures provide potent purification and analytical tools for cytokinin research.

2.3.1. Optimization of batch immunoextraction

An important parameter of immunoaffinity gel (IAG) is its capacity, which is defined as the maximum amount of the target analyte(s) that can be bound by a given volume of the immunosorbent (Hennion and Pichon, 2003; Delaunay-Bertoncini and Hennion, 2004). Clearly, when using an IAG for purifying plant extracts, in which multiple cross-reacting metabolites are often present, the immunosorbent capacity must be sufficient for each metabolite. Therefore, we determined the dynamic capacities, for 21 cytokinin standards, of different volumes of an IAG (containing immobilized generic anticytokinin monoclonal antibodies), which has proved to be useful for cytokinin immunoaffinity chromatography, calculating the binding capacity and recovery parameters for 10 µl, 25 µl, and 50 µl of the gel and each CK in mixtures with amounts ranging from 0.05 to 100 pmol. Recoveries higher than 50% were obtained for most cytokinins in the range 0.05–5 pmol, but beyond 10 pmol the immunoextraction recovery declined rapidly. Fig. 3 shows the recoveries obtained with different volumes of IAG for two cytokinin species, tZ and mTR, as representatives of isoprenoid and aromatic cytokinins, respectively. The recoveries of tZ were substantially lower than those of mTR, and the recoveries of all the cytokinins tested were maximal when 50 µl of the gel was used (Fig. 3A and B). Recoveries were high (>90%) for topolin derivatives across the entire studied range, and their recovery curves were similar to that of mTR (Fig. 3B). The recoveries were the lowest, 30–50% for isopentenyladenine cytokinins (and very low for amounts of these compounds higher than 10 pmol) and intermediate for other cytokinins, including benzyladenine, *cis*- and *trans*-zeatin, dihydrozeatin, their ribosides and 9-glucosides. Cytokinin *O*-glucosides and nucleotides were not retained by the IAG at all. In summary, the IAG is capable of binding free bases, ribosides and 9-glucosides of isoprenoid and aromatic cytokinins, and it has sufficient capacity and utility for the co-purification of many cytokinins from milligram amounts of plant material, since the total amount of cytokinins in non-transgenic plant tissues is very rarely greater than 30 pmol g^{-1} FW.

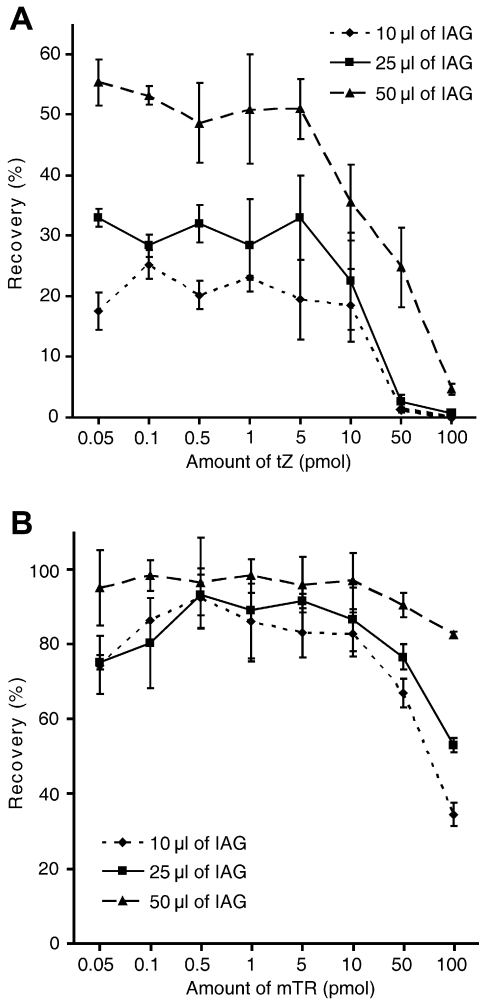


Fig. 3. Recovery curves for *trans*-zeatin (tZ, A) and *meta*-topolin riboside (mTR, B) obtained using an immunoaffinity gel with bound generic anticytokinin monoclonal antibody. Curves obtained using mixtures of 21 naturally occurring cytokinin derivatives with concentrations ranging from 0.5 to 100 pmol and 10 µl, 25 µl or 50 µl portions (in triplicate) of the immunoaffinity gel.

2.3.2. Development of the rapid extraction and purification method

Initially, the rate-limiting step in preparing extracts was tissue homogenization. To accelerate the extraction, we modified the procedure by crushing the plant tissues with tungsten carbide beads in a vibration mill. As well as being much faster, this method is very efficient for extracting small plant samples and it reduces sample losses and cross-contamination. This approach is frequently used for isolating RNA and DNA from plant tissues and can be readily adapted for plant hormones (Zhou et al., 2003).

We then compared the precision and efficiency (using internal standards and spiking the samples) of two extraction and purification protocols: (a) microextraction with ice-cold 70% ethanol followed by C18 column purification and (b) microextraction in Bieleski buffer followed by purification using a SCX column. Samples containing 20–200 mg of *Populus* × *Robusta* leaf tissues were used in this study. In addition, we tested two variants of immunopurification (using extracts obtained by both procedures): immunoaffinity chromatography essentially as described by Faiss et al. (1997) and the batch modification described by Hauserová et al. (2005). Similar conditions and solutions (PBS buffer for application, methanol for elution) were used in both immunopurification procedures.

The cytokinin profiles (rich in both isoprenoid and aromatic forms) obtained from the poplar leaf samples were consistent with published profiles (Novák et al., 2003), and the measured amounts of CKs were proportional to the amount of plant material used, across the range of tested amounts. The main variable affecting the behavior of the cytokinins during the purification procedure was found to be whether they were aromatic or isoprenoid (Table 3). Higher contents of isoprenoid CKs were always obtained when Bieleski buffer extraction followed by SPE/SCX column purification was used for the sample preparations, while there were non-significant differences in the levels of endogenous aromatic cytokinins

Table 3
Extraction and purification efficiency

$n = 3$	Weight (mg)	Total cytokinin content (pmol g^{-1} FW) and recovery (%)			
		70% Ethanol + C18		Bieleski buffer + SCX	
		IAC	IAE	IAC	IAE
Isoprenoid	20	19.73 ± 1.22 (75%)	17.26 ± 2.12 (45%)	26.22 ± 1.38 (80%)	27.62 ± 1.50 (51%)
	50	19.63 ± 1.24 (65%)	17.97 ± 1.02 (37%)	28.42 ± 1.09 (89%)	27.35 ± 2.50 (46%)
	200	20.96 ± 0.76 (42%)	18.88 ± 1.70 (30%)	27.07 ± 1.21 (60%)	26.01 ± 1.88 (49%)
Aromatic	20	81.48 ± 5.03 (74%)	76.49 ± 8.79 (41%)	88.05 ± 2.94 (83%)	83.65 ± 2.63 (61%)
	50	92.27 ± 7.20 (64%)	84.83 ± 7.63 (33%)	68.25 ± 3.70 (85%)	77.70 ± 4.72 (48%)
	200	87.32 ± 7.70 (50%)	77.49 ± 8.14 (29%)	70.44 ± 3.04 (75%)	71.20 ± 5.64 (49%)

Comparison of cytokinin recoveries and amounts found in *Populus* × *canadensis* Moench, cv *Robusta* leaves using two extraction procedures in combination with two immuno clean-up procedures: immunoaffinity chromatography (IAC) and batch immunoextraction (IAE).

detected following application of the six tested combinations of protocols.

As shown in Table 3, the tested extraction solvents gave quite similar yields of analyzed cytokinin metabolites. The small differences in levels of endogenous cytokinins detected confirm the efficiency of both all of the extraction procedures, and the internal calibration method. However, the Bieleski buffer provided the highest recoveries for all cytokinins tested, recovering 60–95% of internal standards from the poplar leaf extracts, while the proportions recovered using 70% ethanol were usually 20–30% lower. In addition, batch IAE usually gave substantially lower (ca. 2-fold) recoveries than immunoaffinity chromatography, due to the limited volume of the IAG used (50 µl). However, use of internal standards allows such differences to be accounted for, thus the calculated concentrations obtained using the different extraction and purification procedures were almost identical (as illustrated by the data in Table 3).

We also compared apparent concentrations in samples based on recoveries subjected to one extraction and both two and three pooled extractions (data not shown). For the results of such comparisons to be consistent, both the internal standards and endogenous analytes must reach equilibrium between the solid and liquid phases during the extractions. The data confirmed that at least two extractions are required to approach close to 100% analyte extraction. The protocol (see Fig. 4) involving double microextraction in Bieleski buffer then purification on an SCX column followed by a batch immunoextraction (50 µl IAG) was subsequently used for validating application of the developed UPLC–ESI(+)–MS/MS method to 100 mg *A. thaliana* samples (Table 5).

2.4. Validation of the method

Large numbers of UV-absorbing substances are present in plant samples that have not been subjected to effective immunoextraction (Hauserová et al., 2005; Hradecká et al., 2007), even extracts from small quantities of *A. thaliana* tissues that have been cleaned by reversed phase (Sep-Pak C18) chromatography. Therefore, immunoaffinity techniques are being used increasingly frequently for purifying phytohormones (Prinsen et al., 1995a,b, 1998; van Rhijn et al., 2001; Novák et al., 2003). The effectiveness of the IAC step for purifying cytokinins from plant extracts applied in this study was evaluated using the UPLC equipped with either a diode array detector or mass spectrometer. Comparison of UPLC chromatograms obtained before (0.2–2.5 AU) and after (0.001–0.006 AU) IAE (Fig. 5) shows that considerable purification was achieved by this step, and the absence of interfering peaks in the vicinity of the cytokinin peaks even allowed the amounts of the corresponding cytokinins to be quantified from UV peak area measurements. The advantages of using IAC prior to LC–ESI(+)–MS for cytokinin analyses have not previously been shown, but the LC–ESI(+)–MS

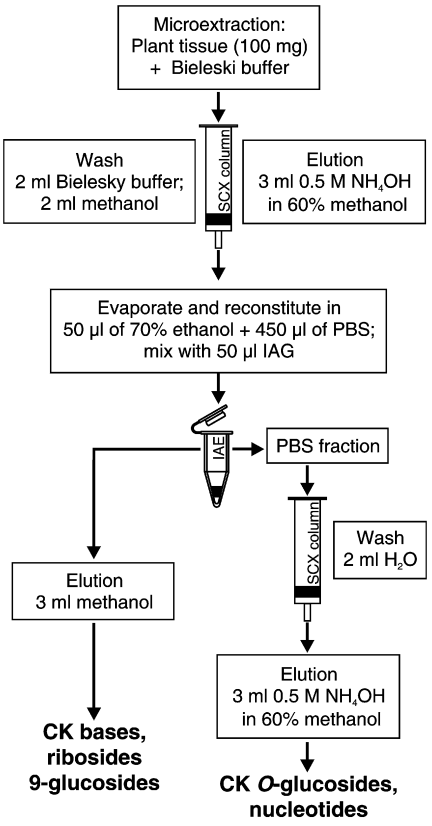


Fig. 4. New cytokinin extraction and purification protocol. Plant material (<100 mg) is homogenized and extracted in Bieleski buffer (1 ml) containing labeled internal standards. Pooled supernatant is applied to a pre-conditioned SCX column (100 mg), which is washed and eluted with the indicated solutions. The eluate is evaporated to dryness, then mixed with 50 µl of IAG and incubated for 2 h at room temperature. After incubation, the PBS fraction is collected and desalted using a SCX column (100 mg). The compounds retained on IAG are eluted by 3 ml methanol and the fraction is evaporated to dryness and stored at −20 °C.

chromatograms of partially purified (C18) and immunopurified plant extracts showed that the IAE can also increase the selectivity and sensitivity of UPLC–ESI(+)–MS/MS analysis. In contrast, the partially purified (C18) extracts of the same sample were contaminated by many impurities and the signals were, consequently, weaker and more difficult to resolve (signal/noise ratios of 30/38 for iPR and 78/89 for tZR). Moreover, it is very important for high-throughput analysis to minimize content of sample impurities in order to reduce risk of frequent system clean-up necessity.

In order to verify the effects of the sample matrix on the whole method (isolation and quantification procedure) and to assess its

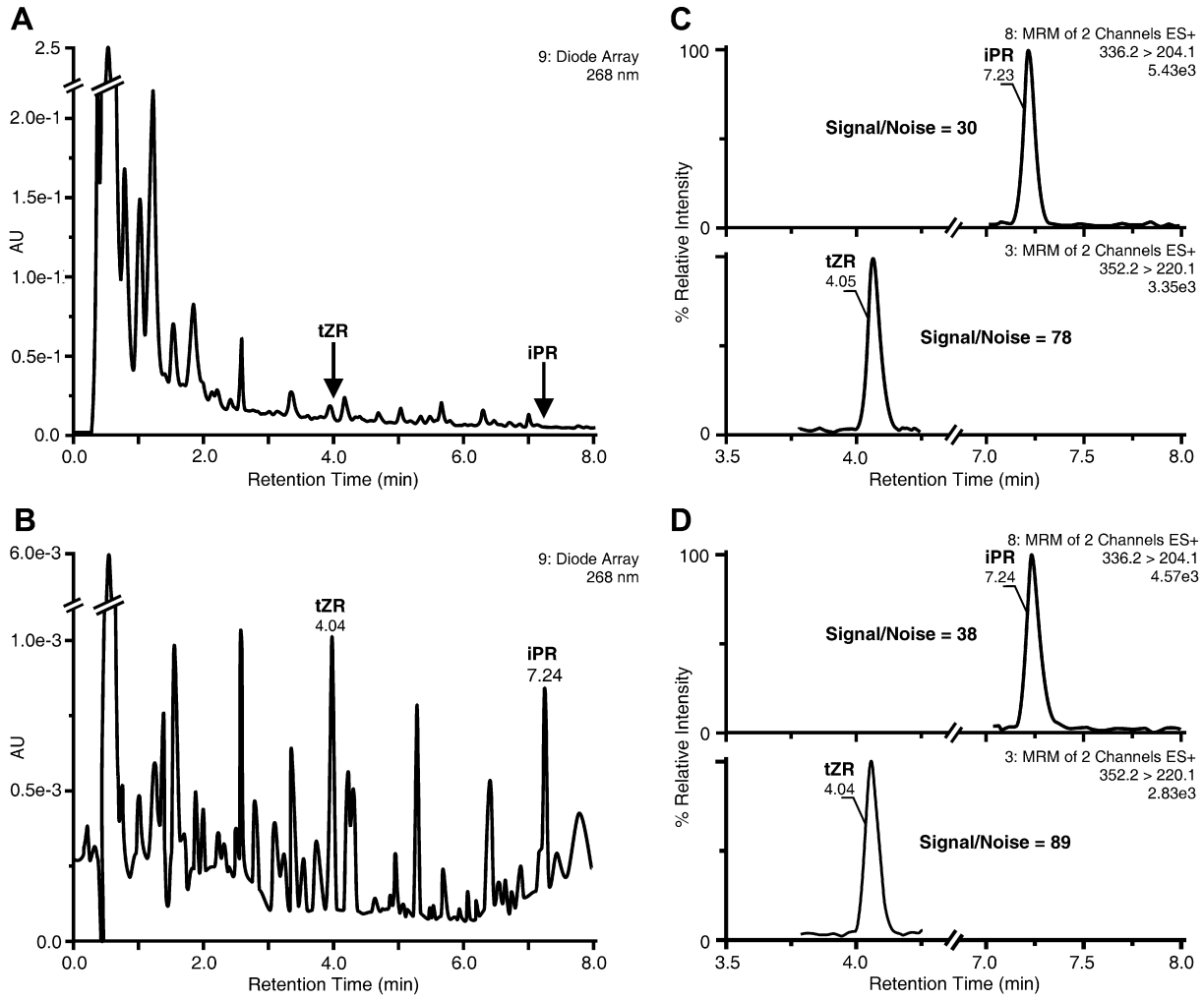


Fig. 5. Effects of immunoaffinity purification on selectivity and sensitivity of analysis. Comparison of UV (268 nm) and MRM chromatograms of authentic tZR and iPR present in 100 mg *Arabidopsis thaliana* extracts before (A,C) and after using batch IAE (B,D).

efficiency and analytical accuracy, spiked samples were analyzed (Table 4). Three types of samples were used in this experiment: a mixture of 21 cytokinin standards (1 pmol of each), and 100 mg extracts of 10-day-old *A. thaliana* seedlings with and without additions of the standard cytokinin mixture (1 and 10 pmol each). All the samples contained 1 pmol of internal standards.

After purification on an SCX column followed by batch immunoextraction, recoveries of samples without plant matrices (with cold standards only) reached on average 65% and 85% for isoprenoid and aromatic cytokinins, respectively (Table 4). These data are consistent with results obtained from the IAG capacity testing. Matrix effects strongly influenced recovery levels during purification of *A. thaliana* extracts, reducing them by ca. a third in comparison to levels recovered from non-plant samples, for which average values of 40% and 60% were obtained for isoprenoid and aromatic cytokinins, respectively. Finally, the accuracy of the developed analytical procedure was determined using spiked *A. thaliana* samples. The acquired data showed that the variations in recoveries could be accounted for by standard isotope dilution analysis, and the amounts of the cytokinins estimated by the fast UPLC–ESI(+)–MS/MS method were generally within ±20% of the true amounts, which is satisfactory for such determinations of trace components in complex plant matrices (van Rhijn et al., 2001), although the determined levels of other cZ derivatives – except for cZ (IS: [$^{13}C_5$]cZ) – were usually lower, probably due to differences in the losses of *cis*- and *trans*-zeatin cytokinins during the IAE, which were not accounted for because of the imperfect internal standardization of *cis*-zeatins. Thus, the rapid SPE-IAE purification and UPLC–ESI(+)–MS/MS quantitation protocol provided adequately accurate estimates of levels of endogenous cytokinins in the sampled tissues, as validated by the internal standardization procedure. The addition of labeled derivatives to the extracts further facilitated the detection of natural cytokinins, providing better resolution of products that eluted close together, as well as a measure of the percentage recovery of different cytokinins throughout the purification procedure. Ideally, recovery markers should be included for every hormone metabolite that is being measured (Chiwocha et al., 2003) as shown here in the case of *cis*-zeatins. However, in many studies only a few internal standards have been used, often added at late stages during the extraction process, or just before quantitative analyses (Witters et al., 1999; van Rhijn et al., 2001). Clearly, our methodology presented here could also be further improved by internal standardization of some of the missing labeled cytokinins.

The analyses of the model samples showed that the immunoaffinity extraction increased both the selectivity and sensitivity of the UPLC–ESI(+)–MS/MS analysis. Using batch IAE, some of the analytes in the plant samples are inevitably lost, but it still has a strongly positive effect on the signal-to-noise ratios of the MS measurement. These results also show that the purification step

Table 4
Detection limits and sample matrix effects on purification and analytical accuracy

	LOD (fmol) [a]	Recovery (%)[b]		Analytical accuracy (%)[c]	
		STD[b]	Plant matrix	1 pmol	10 pmol
tZ	2.5	76 ± 6	41 ± 7	106 ± 6	96 ± 7
tZR	1.0	82 ± 11	64 ± 10	121 ± 6	103 ± 5
tZ9G	2.5	82 ± 9	45 ± 6	112 ± 5	100 ± 4
tZOG	5.0	70 ± 5	29 ± 4	105 ± 6	99 ± 3
tZROG	10.0	65 ± 9	38 ± 9	107 ± 5	101 ± 4
tZRMP	25.0	62 ± 11	49 ± 10	104 ± 8	96 ± 5
cZ	2.5	45 ± 4	24 ± 5	110 ± 8	110 ± 3
cZR	1.0	64 ± 10	31 ± 6	72 ± 9	69 ± 1
cZ9G	1.0	52 ± 8	24 ± 6	71 ± 8	67 ± 2
cZOG	5.0	71 ± 8	28 ± 7	61 ± 3	59 ± 3
cZROG	5.0	53 ± 9	33 ± 9	55 ± 8	54 ± 6
cZRMP	25.0	66 ± 9	35 ± 7	58 ± 3	57 ± 5
dHZ	0.75	66 ± 6	40 ± 6	112 ± 5	111 ± 3
dHZR	0.75	84 ± 10	51 ± 9	105 ± 4	96 ± 4
dHZ9G	1.0	71 ± 11	45 ± 8	108 ± 3	103 ± 1
DHZOG	1.0	70 ± 9	42 ± 7	109 ± 4	109 ± 5
DHZROG	5.0	71 ± 8	45 ± 7	89 ± 3	87 ± 5
DHZRMP	10.0	64 ± 10	36 ± 8	94 ± 3	103 ± 3
iP	0.75	60 ± 9	36 ± 6	107 ± 3	104 ± 2
iPR	0.75	64 ± 5	38 ± 7	104 ± 7	97 ± 6
iP9G	1.0	55 ± 5	30 ± 5	102 ± 6	94 ± 2
iPRMP	10.0	80 ± 10	53 ± 10	102 ± 8	104 ± 7
BAP	2.5	73 ± 4	50 ± 8	111 ± 3	106 ± 3
BAPR	2.5	73 ± 8	46 ± 7	96 ± 3	87 ± 2
BAP9G	2.5	59 ± 6	43 ± 7	101 ± 1	96 ± 4
BARMP	25.0	86 ± 9	54 ± 11	97 ± 3	107 ± 2
mT	5.0	96 ± 8	70 ± 10	103 ± 2	92 ± 5
mTR	5.0	91 ± 9	65 ± 10	111 ± 8	108 ± 8
mT9G	5.0	95 ± 6	70 ± 10	116 ± 5	115 ± 5
oT	2.5	95 ± 5	75 ± 11	110 ± 2	111 ± 7
oTR	5.0	93 ± 8	72 ± 10	112 ± 5	117 ± 6
oT9G	5.0	92 ± 7	69 ± 6	108 ± 5	112 ± 6

Recoveries of cytokinins from standard solutions with and without plant matrices following the purification procedure and analytical accuracy with 1 pmol and 10 pmol spiking concentrations of the standard mixture.
[a] Limit of detection, defined as signal-to-noise ratio of 3:1.
[b] STD – standards.
[c] Mean value of three independent measurements ± standard deviation.

Table 5
Cytokinin levels in *Arabidopsis thaliana* extracts determined by HPLC–ESI(+)–MS and UPLC–ESI(+)–MS/MS

CKs	Cytokinin content (pmol g^{-1} FW)	
	HPLC–ESI(+)–MS	UPLC–ESI(+)–MS/MS
tZ	0.66 ± 0.20	0.61 ± 0.16
tZR	2.76 ± 0.85	2.84 ± 0.81
tZ9G	3.66 ± 0.17	3.72 ± 0.25
tZOG	–	4.77 ± 0.49
tZROG	–	0.52 ± 0.22
tZRMP	–	1.62 ± 0.34
cZ	n.d.[a]	0.32 ± 0.08
cZR	1.12 ± 0.62	1.13 ± 0.54
cZ9G	n.d.	0.15 ± 0.02
cZOG	–	1.62 ± 0.22
cZROG	–	0.48 ± 0.03
dHZ	n.d.	0.02 ± 0.01
dHZR	n.d.	0.09 ± 0.03
dHZ9G	n.d.	0.03 ± 0.01
iP	0.55 ± 0.05	0.42 ± 0.07
iPR	2.33 ± 0.71	2.38 ± 0.44
iP9G	1.97 ± 0.10	1.99 ± 0.12
iPRMP	–	5.69 ± 1.78

10-Day-old *Arabidopsis thaliana* seedlings (100 mg) were extracted, purified by SCX column chromatography followed by batch immunoextraction then measured by single or tandem mass spectrometry. Mean values (±SDs) from triplicate measurements of real samples exceeding detection limit are shown.
[a] n.d. – Not detected.

effectively removes interfering substances, thus reducing analyte ion-suppressing effects of the matrix.

2.5. Analysis of cytokinins in milligram amounts of plant material

The purification protocol developed here (see Fig. 4) was tested for UPLC–ESI(+)–MS/MS analysis of cytokinins from the model plant *A. thaliana*. To assess the sensitivity of the analytical approach we applied it to small samples of plant tissues (100 mg fresh weight). Cytokinin bases, ribosides, and 9-glucosides were simultaneously analyzed in three replicates by both the combined IAE-UPLC–ESI(+)–MS/MS and IAC-HPLC–ESI(+)–MS methods. Direct analysis of *O*-glucosides and nucleotides was possible only by using UPLC–ESI(+)–MS/MS. The results are presented in Table 5 (solely for isoprenoid cytokinins, data for aromatic cytokinins not shown). The responses were generally weaker in HPLC–ESI(+)–MS analyses than in corresponding UPLC–ESI(+)–MS/MS analyses, and only seven isoprenoid cytokinins were detected in them, compared to 12 in the latter. Use of UPLC–ESI(+)–MS/MS led to a general increase in sensitivity, and allowed more endogenous cytokinins to be measured in the 100 mg fresh weight samples. Notably, dihydrozeatin and N^6-benzyladenine cytokinins (data for BA cytokinins not shown) were not measurable or detectable by HPLC–ESI(+)–MS (sensitivity limits > 50 fmol), but appeared to be present in the low femtomolar range (<20 fmol) according to the UPLC–ESI(+)–MS/MS determinations. These data clearly show that measurement of endogenous plant hormones strongly depends on the sensitivity limits of the method used for analyses, and thus previously undetected compounds can be identified and quantified by using more advanced analytical approaches. The sensitivity of the method could be further improved by using more sophisticated mass spectrometry techniques. It is also worth mentioning that the generally low number of scans per MRM transition, due to the small peak-eluting volumes in UPLC, may be an important factor to consider in attempts to improve limits of detection (LODs). When MRM transition channels are monitored simultaneously, the inter-channel delay and dwell time must be not more than 0.1 s and 0.06 s per MRM transition, respectively, to obtain at least 16 scan points for each transition. The amount of scans could be further increased by using fast scanning mass detectors exploiting T-Wave technology with an acquisition rate of 100 data points per second with 0.005 ms interchannel delays and dwell times. Use of such technology could allow the quantification of plant hormones in very limited amounts of material, for example meristems, embryos and possibly even individual plant cells.

3. Conclusions

The need to quantify plant hormones spanning large ranges of hydrophobicity in attempts to elucidate various physiological processes calls for new analytical strategies. HPLC–MS is widely applied because of its ability to separate and detect phytohormones without derivatization, but as shown here the sensitivity and separation efficiency of HPLC–MS can be increased by new approaches. The immunopurification procedure presented here provides possibilities to automate cytokinin analysis since the whole extraction and purification procedure can be performed in an Eppendorf tube. The immunoaffinity extraction step also simplifies and increases the sensitivity of the final mass spectrometric cytokinin determinations. The modified method described here, combining microextraction with solid-phase extraction and batch immunoextraction, is also much easier and faster than previous techniques. More than 50 samples per day can be extracted and purified by this approach. In summary, the procedure is less time-consuming and much more effective than previous methods.

The liquid chromatographic separation of 32 cytokinin derivatives by UPLC combined with tandem mass spectrometric detection provides accurate, reproducible quantification of most cytokinin metabolites. The approach represents an improvement over previous methods, enabling direct, detailed analyses of diverse cytokinin forms in limited, specific samples of plant tissues, which were not previously possible. The newly developed UPLC–ESI(+)–MS/MS method may thus be very useful for studying cytokinins physiological roles, metabolism and modes of action in plants.

4. Experimental procedures

4.1. Reagents and materials

Naturally occurring and deuterium-labeled CK standards were obtained from Olchemim Ltd., Czech Republic. Methanol (HPLC grade), acetic acid and chloroform were from Sigma–Aldrich (St. Louis, MO, USA); ammonium hydroxide and formic acid from MERCK (Darmstadt, Germany); sodium dihydrogenphosphate and sodium chloride from Lach-Ner (Brno, Czech Republic); Murashige & Skoog medium from Duchefa Biochemie B.V. (Haarlem, Nederland); and both C18 and SCX cartridges (100 mg) from Agilent Technologies (Palo Alto, CA, USA). The PBS buffer contained 7.8 g $NaH_2PO_4 \cdot 2H_2O$, 0.877 g NaCl l^{-1}, pH 7.2. Deionized (Milli-Q) water was obtained from a Simplicity 185 system (Millipore, Bedford, MA, USA). Monoclonal antibody based immunoaffinity gel was prepared, used and stored as previously described (Novák et al., 2003).

4.2. Instrumentation

An Acquity UPLC™ System (Waters, Milford, MA, USA), including a Binary solvent manager, Sample manager, and 2996 PDA detector, combined with a Micromass Quattro *micro*™ API (Waters MS Technologies, Manchester, UK) were used for cytokinin analysis. All data were processed by MassLynx™ software with QuanLynx™ and QuantOptimize™ programs (version 4.0, Waters, Milford, MA, USA).

4.3. Biological material

Arabidopsis thaliana, ecotype Colombia, seedlings were grown *in vitro* in Petri dishes containing Murashige & Skoog medium including vitamins (4.4 g MS medium, 10 g of sucrose, 10 g of plant agar l^{-1}, pH 5.7) at 23 °C under 16 h photoperiods. Ten-day-old seedlings were harvested, weighed and immediately plunged into liquid nitrogen.

Leaves of *Populus* × *canadensis* Moench, cv. *Robusta* were harvested at 7 a.m. (time of sunrise 4:11 a.m.), immediately frozen in liquid nitrogen, and stored until extraction at −80 °C.

4.4. Liquid microextraction and solid-phase extractions

Two variants of extraction and purification protocols were tested. One set of samples (“A” samples) of poplar leaves (20–200 mg, FW) was placed individually in 750–1000 μl of Bieleski buffer (60% methanol, 25% $CHCl_3$, 10% HCOOH and 5% H_2O) and re-extracted in 300 μl of 50% methanol with 2% formic acid in 1.5 ml Eppendorf tubes. A second set (“B” samples) was extracted twice in 70% ethanol containing diethyldithiocarbamic acid (DDC; 400 μg g^{-1} fresh weight). Stable isotope-labeled CK internal standards were added to each of the samples (1 pmol of each compound per sample) to check the recovery during purification and to validate the quantification. The following internal standards (IS) were used: [$^{13}C_5$]tZ, [$^{2}H_5$]tZR, [$^{2}H_5$]tZ9G, [$^{2}H_5$]tZOG, [$^{2}H_5$]tZROG, [$^{2}H_5$]tZRMP, [$^{13}C_5$]cZ, [$^{2}H_3$]DHZ, [$^{2}H_3$]DHZR, [$^{2}H_3$]DHZ9G, [$^{2}H_7$]DHZOG, [$^{2}H_3$]DHZRMP, [$^{2}H_6$]iP, [$^{2}H_6$]iPR, [$^{2}H_6$]iP9G, [$^{2}H_6$]iPRMP, [$^{2}H_7$]BA, [$^{2}H_7$]BAR, [$^{2}H_7$]BA9G, [$^{2}H_7$]BARMP, [$^{15}N_4$]mT, and [$^{15}N_4$]oT. Plant material was extracted using an MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) at a frequency of 30 Hz for 3 min after adding 3-mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany) to each tube to increase the extraction efficiency. The tube contents were ultrasonicated for 3 min and then stirred for 30 min at 4 °C. After centrifugation (3 min, 15,000 rpm, 4 °C) the supernatants were transferred to glass tubes and stored at 4 °C. The pellets were re-extracted in the same way for 30 min at 4 °C and after centrifugation (3 min, 15,000 rpm, 4 °C) both supernatants were combined and immediately purified.

The second purification step, for set A, involved passing the samples through 100 mg SCX columns, activated with 2 ml of methanol and equilibrated with 2 ml of 50% methanol containing 2% HCOOH. In each case, after applying the extract, the column was washed with 2 ml of Bieleski buffer followed by 2 ml of methanol. Target compounds were subsequently eluted with 3 ml of 0.5 M NH_4OH in 60% methanol (v/v). Samples of set B were purified using 100 mg C18 cartridges, conditioned with 2 ml of methanol and 2 ml of water. In each case, after applying an extract the column was washed with 2 ml of water and eluted with 3 ml of 80% acidified methanol (containing 2% CH_3COOH). Partially purified samples (sets A and B) were evaporated to dryness, dissolved in 50 μl of 70% ethanol and 450 μl of PBS (25 mM NaH_2PO_4, 15 mM NaCl, pH 7.2), then purified by passage through a pre-immune column and then by chromatographic or batch immunoextraction, as described below.

4.5. Immunoaffinity purification

Immunoaffinity gel (IAG) based on a generic monoclonal antibody raised against *ortho*-topolin riboside, as described by Novák et al. (2003), was used for the sample purification and cytokinin isolation in all our experiments. Samples were prepared in Eppendorf tubes. Firstly, the capacity of the IAG was tested using standard solutions containing 0.01–100 pmol of each of 21 tested cytokinin standards in ethanol:PBS buffer (5:95, v/v). A portion (10–50 μl) of IAG was added to each of the standard solutions, mixed, incubated (2 h), and eluted by 3 ml of ice-cold methanol (−20 °C) using a 1 ml plastic column with a frit. Each concentration was prepared in triplicate, and the optimized volume of IAG (50 μl) was used for the subsequent purification and isolation of cytokinins from different plant samples.

In all cases the plant samples were pre-purified, using pre-immune gel in the following procedure, prepared by coupling rabbit IgG to Affi-Gel 10 (25 mg ml^{-1}), as a pre-cleaning step before batch IAE or IAC to eliminate compounds that non-specifically bound to IAG. A portion of 400 μl of pre-immune gel was added to each sample, dissolved in 50 μl of 70% ethanol and 450 μl of PBS, following purification by passage through SCX or C18 columns. Filtrates and eluates obtained by adding the mixtures to plastic column cartridges (1 ml SPE tubes, 20 μm frits, Supelco) and washing with 250 μl of PBS were then mixed with 50 μl of IAG in Eppendorf tubes and agitated for 2 h by a rotary shaker at laboratory temperature. The samples were again placed in plastic column cartridges (1 ml SPE tube, 20 μm frit, Supelco), and the filtrates, containing CK *O*-glucosides and nucleotides, were collected and desalted using 100 mg SCX cartridges (pre-activated with 2 ml methanol and 2 ml H_2O containing 2% HCOOH). After applying the fraction, each column was washed with 2 ml H_2O and CK *O*-glucosides and nucleotides were eluted with 3 ml of 0.5 M NH_4OH in 60% methanol (v/v). The IAG was washed with water and compounds were eluted by 3 ml ice-cold methanol. In the methanol fraction, cytokinin bases,

ribosides, 9-glucosides were obtained. The used IAG was collected and regenerated with a cycle of PBS–H_2O–MeOH–H_2O–PBS (1 ml of each). Purified samples were evaporated to dryness in a Speed-Vac concentrator (UniEquip) and analyzed by UPLC–ESI(+)–MS/MS.

4.6. UPLC–ESI(+)–MS/MS conditions

Samples were dissolved in 20 μl of mobile phase (initial conditions), and 10 μl of each sample was injected onto a reversed-phase column (BEH C18, 2.1 × 50 mm, 1.7 μm; Waters). The samples were eluted with an 8-min linear gradient of 90:10 A:B to 50:50 A:B (v/v) where A was 15 mM ammonium formate (pH 4.0) and B was methanol at a flow rate of 0.25 ml min^{-1} and column temperature of 40 °C. At the end of the gradient the column was washed with 100% B (1 min) and equilibrated to initial conditions for 3 min. Under these conditions, retention times for the monitored compounds ranged from 2.5 to 7.5 min. The effluent was passed through an ultraviolet-diode array detector (scanning range 210–400 nm, resolution 1.2 nm, sampling rate 10 spectra s^{-1}) and the tandem mass spectrometer without post-column splitting. Analytes were quantified by multiple reaction monitoring of $[M+H]^+$ and the appropriate product ion. For selective MRM experiments, optimized conditions were as follows: capillary voltage, 0.6 kV; source/desolvation gas temperature, 100/350 °C; cone/desolvation gas flow rates, 2.0/550 l h^{-1}; LM/HM resolution, 12.5; ion energy 1, 0.3 V; ion energy 2, 1.5 V; entrance, exit and multiplier voltages, 2.0 V, 2.0 V and 650 eV, respectively. Argon was used as collision gas with an optimized pressure of 5×10^{-3} mbar. The dwell times, cone voltages, and collision energies for particular diagnostic transitions were optimized (Table 2) to maximize sensitivity. The dwell time of each MRM channel has been calculated to provide 16 scan points per peak, during which time the inter channel delay was 0.1 s and the cycle times were in the range 0.55–0.65 s.

4.7. Calibration curves

Calibration curves were created from triplicate analyses of cytokinin free bases, ribosides, nucleotides, *O*- and 9-glucosides by plotting the known concentration of each unlabeled analyte against the calculated response area of the analyte/IS ratio 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 pmol/0.5 pmol, where IS concentration is the known amount of the internal standard added. The levels of cytokinins in the plant samples were determined from the area ratios of endogenous to corresponding stable isotope-labeled cytokinins. Equations were fitted to the calibration curves and corresponding correlation coefficients were determined following logarithmic transformation for each measured cytokinin by QuanLynx™ software (Waters, Manchester, UK). This software calculated limits of detection (LOD) and quantification (LOQ) for individual cytokinins during every UPLC–ESI(+)–MS/MS run, from which average LOD and LOQ values (n = 10) were determined. As mentioned above, the LODs and LOQs were determined from signal-to-noise ratios of 3:1 and 10:1, respectively.

4.8. Validation of the analytical procedure

Three sets of validation experiments were performed, in each of which extraction buffer A contained an internal standard (IS) mixture of the 22 isotopically-labeled cytokinins (1 pmol each). Firstly, the reproducibility, sensitivity and accuracy of the method for plant samples were determined from dose–response curves obtained using aliquots of extracts, each corresponding to 100 mg fresh weight of 10-day-old *A. thaliana* seedlings extracted in 1 ml of Bieleski buffer, re-extracted, and purified according to the protocol for sample set A. The endogenous cytokinin levels had been predetermined in these samples. Secondly, aliquots of the extracts were then spiked with different amounts (1 and 10 pmol) of authentic cytokinin standards, extracts were processed using procedure A and IAE, then analyzed by UPLC–ESI(+)–MS/MS. The amounts of the added standards were then calculated from each series of extracts, based on the known, predetermined amounts of endogenous compounds, which served as reference levels. Thirdly, the extraction buffer was supplemented with a mixture of cold cytokinin standards only (1 pmol of each) and was processed as for the two sets of samples mentioned above. The content and RSD for the retention time of each cytokinin were determined from the first series. The analytical accuracy at each spiking level was determined from the results obtained with the second set of samples, by dividing the determined concentration by the concentration derived from the first set of samples plus the spiked amount.

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