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Quantitative analysis of cytokinins in plants by liquid chromatography–single-quadrupole mass spectrometry

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Abstract

A sensitive method for the quantitative analysis of all natural isoprenoid cytokinins in plant material by electrospray single-quadrupole mass spectrometry is presented. A baseline chromatographic separation of 20 non-derivatised naturally occurring cytokinins has been developed. Precise analyses of *O*-glucoside and ribonucleotide fractions were also performed by the high-performance liquid chromatography–mass spectrometry (HPLC–MS) but run separately from the basic cytokinin metabolites. Using post-column splitting, the flux from narrow-bore (2.1 mm i.d.) reversed-phase liquid chromatography column was simultaneously introduced into the diode array and mass detector. Optimal conditions, including final flow rate, desolvation temperature, desolvation gas flow, capillary and cone voltage for effective ionisation in the electrospray ion source were found. When low cone voltage (20 V) was applied, all studied cytokinins were determined in aqueous methanol as dominant quasi-molecular ions of $[M + H]^+$ with limits of detection ranging between 10 and 50 fmol. For routine analysis a linearity range between 25 (75) fmol and 100 pmol was obtained. Developed liquid chromatography–mass spectrometry (LC–MS) method in selective ion monitoring mode was employed to quantify cytokinin species in tobacco BY-2 suspension culture and poplar leaves (*Populus* × *canadensis* Moench, cv *Robusta*).

Purified plant cell (BY-2) and plant tissue (poplar leaves) extracts were obtained by using two different ion-exchange chromatography steps, in combination with immunoaffinity purification using a broad-spectrum monoclonal anti-cytokinin antibody. The antibody strongly recognises the presence of N^6-substituent on purine skeleton and thus does not bind adenine and related compounds. The presence of authentic cytokinins in the extracts quantified by LC–MS was further verified by enzyme-linked immunosorbent assays (ELISAs) with prior LC preparation. The combination of liquid chromatography–single-quadrupole mass spectrometry with immunoaffinity chromatography offers an efficient and elegant method for detection and quantification of cytokinin metabolites.

Keywords: Liquid chromatography–mass spectrometry; Cytokinins; ELISA; Tobacco; Poplar

1. Introduction

Cytokinins represent one group of phytohormones, defined as substances that promote cell division in the presence of auxins. All native cytokinins are derivatives of adenine with at least one substituent at

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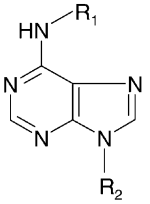
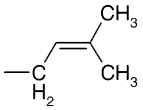
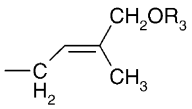
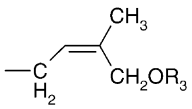
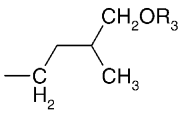
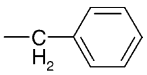
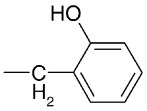
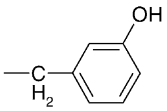
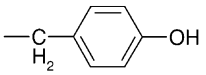
Table 1
Structures, names and abbreviations of studied cytokinins



R_1	R_2	R_3	Compound	Abbreviation
	H	–	N^6-Isopentenyladenine	iP
	R	–	N^6-Isopentenyladenosine	iPR
	G	–	N^6-Isopentenyladenine-9-glucoside	iP9G
	RP	–	N^6-Isopentenyladenosine-5′-monophosphate	iPMP
	H	H	*trans*-Zeatin	tZ
	R	H	*trans*-Zeatin riboside	tZR
	H	G	*trans*-Zeatin *O*-glucoside	tZOG
	R	G	*trans*-Zeatin riboside *O*-glucoside	tZROG
	RP	H	*trans*-Zeatin riboside-5′-monophosphate	tZMP
	H	H	*cis*-Zeatin	cZ
	R	H	*cis*-Zeatin riboside	cZR
	H	G	*cis*-Zeatin *O*-glucoside	cZOG
	R	G	*cis*-Zeatin riboside *O*-glucoside	cZROG
	RP	H	*cis*-Zeatin riboside-5′-monophosphate	cZMP
	H	H	Dihydrozeatin	DHZ
	R	H	Dihydrozeatin riboside	DHZR
	RP	H	Dihydrozeatin riboside-5′-monophosphate	DHZMP
	H	G	Dihydrozeatin *O*-glucoside	DHZOG
	R	G	Dihydrozeatin riboside *O*-glucoside	DHZROG
	H	–	6-Benzylaminopurine	BAP
	R	–	6-Benzylaminopurine riboside	BAPR
	G	–	6-Benzylaminopurine-9-glucoside	BAP9G
	H	–	*ortho*-Topolin	oT
	R	–	*ortho*-Topolin riboside	oTR
	G	–	*ortho*-Topolin-9-glucoside	oT9G
	H	–	*meta*-Topolin	mT
	R	–	*meta*-Topolin riboside	mTR
	G	–	*meta*-Topolin-9-glucoside	mT9G
	H	–	*para*-Topolin	pT

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

the N^6 position (see Table 1). Depending upon this N^6-substituent, these compounds may be classed into isoprenoid (zeatin-type), isoprenoid-derived (dihydrozeatin-type) and aromatic cytokinins (benzyladenine-type) [1], which all can occur as free bases, as well as conjugates with sugars and aminoacids [2]. There are also other substances with cytokinin activity, such as certain phenylurea derivatives.

Since plant tissue extracts represent complex multicomponent mixtures and, moreover, cytokinins

occur in minute quantities (less than 50 pmol/g fresh weight), sensitive and sufficiently selective analytical tools are required for determination of their endogenous levels. The most common techniques utilised recently in field of the phytohormone analysis are gas and liquid chromatography combined with mass spectrometry (GC–MS, LC–MS) as well as enzyme- or radioimunoassays (ELISA, RIA) of LC fractionated samples. Regardless of the analytical tool used, highly purified samples are preferred.

Immunoassay techniques can be used as sensitive and relatively cheap alternative for estimation of cytokinin levels. When individual HPLC fractions of plant extracts are analysed by radioimmunoassay or enzyme-linked immunosorbent assay, the main disadvantage—cross-reactivity of the antibodies—could be overcome [3,4]. However, such experimental arrangement is labour-intensive and moreover, usage of radiolabelled standards is indispensable.

Naturally occurring cytokinins are not volatile compounds. The necessity to derivatise studied substances before analysis is the main drawback of using gas chromatography for their analysis. Many complications associated with derivatisation have been reported. Trimethylsilyl [5,6], trifluoroacetyl [7] and *t*-butyldimethylsilyl [8] derivatives are subjected to hydrolysis [9]. Further, permethylation [10,11] and trimethylsilylation results in formation of multiple derivatives. Acetylation provides stable and easy to prepare derivatives, but their volatility is not satisfactory [12]. Pentafluorobenzylation of cytokinin free bases for negative ion mass spectrometry has also been reported by Hocart et al. [13].

A few different ionisation techniques were used for mass spectrometric analysis of cytokinins in combination with reversed-phase high-performance liquid chromatography (RP-HPLC), including electrospray (ES), thermospray (TS), atmospheric pressure chemical ionisation (APCI) and fast-atom bombardment (FAB). Prinsen et al. [14] reported the first use of LC–ESI–MS/MS with multiple reaction monitoring (MRM) for cytokinin determination in the isopentenyltranserase-transformed *Nicotiana tabacum* calli. A fast method allowed quantification of 16 different compounds with a detection limit of 1 pmol injected. Later publication of this group [15] presents the main advantages of on-column and column switch focusing for the trace enrichment, when large volumes of analyte are injected onto micro or capillary chromatographic column. A slightly improved gradient elution together with capillary column usage gives a detection limit at the low femtomolar level, despite almost no chromatographic separation was used and signal suppression effect could be expected. It is clear, that without proper chromatographic separation, certain physiologically important isomers and metabolites cannot be distinguished and thus quantified by this method. A similar approach was recently reported by another group [16] for quantitative analysis of zeatin, dihydrozeatin and isopentenyladenine-type cytokinins at the sub-picomolar level. Using thermospray interface LC–MS method for zeatin, dihydrozeatin and isopentenyladenine-type cytokinins determination has been developed [17]. Bartók et al. [18] examined the potential of ESI (in-source) collision induced dissociation (CID) single-quadrupole mass spectrometry for the mapping of cytokinins structures. ESI–CID–MS spectra of appropriate cytokinins provides valuable structural information about both the side-chain attached to N^6 and the substitution of the purine ring by sugars, however no precursor ion selection was involved. Combination of LC–APCI–MS with enzyme-linked immunosorbent assay for qualitative and semi-quantitative analysis of tZR was also reported [19]. In the APCI–MS approach by single ion monitoring mode, sensitivity for zeatin riboside at 35 nmol level was found. The use of capillary liquid chromatography coupled with double-focusing magnetic sector mass spectrometer via frit–fast-atom bombardment ion source (interface) was also investigated. Imbault et al. [20] presented identification and quantification of underivatised isopentenyladenosine. Later, Åstot et al. [21] suggested precolumn derivatisation for LC–frit–FAB–MS cytokinin analysis in order to increase sensitivity. New types of propionyl ester derivatives of cytokinin nucleosides and glucosides and *N*-benzyl derivatives of cytokinin bases gave stronger quasi-molecular ion currents and more spectral information, in comparison with underivatised compounds.

In this paper, we report a sensitive LC–ESI–MS method using a benchtop, single quadrupole instrument, for determination of endogenous cytokinin levels. The presented method is based on baseline chromatographic separation of non-derivatised, immunopurified biological samples. Different parameters

such as retention time stability, linear ranges and limits of detection are also discussed in this communication. The baseline separation of isoprenoid and aromatic cytokinin isomers was of prime importance for method as we would like to obtain a correct estimate of the active cytokinin content.

2. Experimental

2.1. Chemicals

Cytokinin standards, authentic and deuterium-labelled, were purchased from Apex Organics (Haniton, UK). *Ortho*- and *meta*-topolins were synthesised as previously described [22,23]. HPLC-gradient grade methanol, acetic acid, chloroform, 6-chloropurine and 6-chloropurine-9-β-D-ribofuranoside were from Sigma–Aldrich (St. Louis, MO, USA). Ammonium hydroxide and formic acid were obtained from MERCK (Darmstadt, Germany). Sodium hydroxide, sodium dihydrogenphosphate, butanol and sodium chloride were purchased from Lachema (Brno, Czech Republic). All chemicals were at least analytical-reagent grade. Deionised (Milli-Q) water was obtained from Simplicity 185 (Millipore, Bedford, MA, USA). Monoclonal antibody based immunoaffinity columns were prepared as will be described in more details by Lenobel et al. [24]. Used monoclonal antibody raised against oTR via hybridoma technology was purified by affinity chromatography on protein G. The purified antibody was used for preparation of immunoaffinity gel based on coupling on *N*-hydroxysuccinimide-ester activated agarose (Affi-Gel 10, Bio-Rad, Hercules, USA). Each immunoaffinity (IA) column contained 200 μl of immunoaffinity gel packed in 3 ml-polypropylene cartridge. The IAC columns were stored in phosphate-buffered saline (PBS; 50 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) with 0.1% NaN_3 at 4 °C.

2.2. Plant material and sample preparation

The cytokinin-autonomous tobacco BY-2 cell suspension culture was grown in Murashige and Skoog medium (Duchefa, The Netherlands), which was enriched with sucrose (30 g/l), KH_2PO_4 (200 mg/l), thiamine (1 mg/l) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2 mg/l). Four days after last subcultivation the cells were harvested on a sintered glass filter (mesh size 40–100 μm), carefully rinsed with distilled water and immediately frozen in liquid nitrogen.

Fully expanded leaves of field grown *Populus* × *canadensis* Moench., cv *Robusta* were harvested 3 h after the daybreak. The leaves were harvested, immediately frozen in liquid nitrogen, and until extraction and purification were stored at −80 °C.

The extraction and purification method was essentially the same as described by Åstot et al. [21]. Frozen material was ground in liquid nitrogen with a mortar and pestle. Fresh mass (1 g) was extracted overnight in Bieleski solvent [25]. At the beginning of extraction, [$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_5$]tZOG, [$^2H_5$]tZROG, [$^2H_5$]tZRMP, [$^2H_3$]DHZRMP, [$^2H_6$]iPMP were added as internal tracers for recovery studies and quantification. After centrifugation and two ion-exchange chromatography (IEC) steps (SCX, DEAE-Sephadex combined with SPE C18-cartridges) the samples were split into two fractions. The first fraction included cytokinin free bases, ribosides, 9-glucosides and *O*-glucosides, the second fraction contained ribonucleotides. The first fraction was purified by immunoaffinity chromatography (IAC). The samples were dissolved in 50 μl 70% ethanol and 450 μl PBS (50 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) and subsequently applied onto immunoaffinity columns. The immunoaffinity column was equilibrated with 10 ml PBS before sample loading. The sample was repeatedly (fives times) applied onto immunoaffinity column. Then the column was rinsed with 10 ml PBS and 10 ml water. The bound cytokinins were eluted by 3 ml 100% methanol.

O-glucoside derivatives not retained on the IA-columns were treated with β-glucosidase and immuno-purified again, giving a second so-called OG-fraction. Cytokinin ribonucleotides were treated with alkaline phosphatase and subsequently purified using the same IAC, as described above. According to this purification protocol, ribonucleotides and *O*-glucosides are analysed after enzyme treatment as appropriate ribosides and bases, respectively. All samples were evaporated in vacuo and stored in a freezer (−20 °C) until further analysis.

2.3. Equipment

HPLC–MS experiments were carried out on Alliance 2690 Separations Module (Waters, Milford, MA, USA) linked simultaneously to a PDA 996 (Waters) and a ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK). Data were processed by Masslynx software (Data Handling System for Windows, version 3.3, Micromass, Altrincham, UK).

The HPLC system consisted of SP 8800 pump (Spectra-Physics, Mountain View, CA), Rheodyne 7125 injection valve (200 μl loop) (Rheodyne, Cotati, CA, USA), SPD-M6A diode array detector (Shimadzu, Tokyo, Japan) and Frac-100 fraction collector (Pharmacia, Uppsala, Sweden) was utilised for preparative purpose prior to ELISA.

2.4. LC–MS conditions

Samples were dissolved in mobile phase (initial conditions), filtered through the micro-filter (PTFE, 4 mm, 0.45 μm, Waters) and 25 μl (50% of total sample volume before filtration) was injected on RP-column (150 mm × 2.1 mm, 5 μm) (Symmetry C18, Waters). The column thermostat was set at 30 °C. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide. Solvent (B) consisted of methanol. At flow-rate of 250 μl/min, the following binary gradient was used: 0 min, 10% B; 0–25 min, a linear gradient to 50% B; followed by 5 min isocratic elution of 50% B. At the end of the gradient the column was washed by 100% B (5 min) and equilibrated to initial conditions for 15 min. Using the post-column splitting (1:1), effluent was simultaneously introduced into the DAD (scanning range 210–300 nm; with 1.2 nm resolution) and an electrospray source (source temperature 100 °C, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 250 °C). Nitrogen was used both as desolvation gas (400 l/h) and as cone gas (50 l/h). The detector parameters were: span size ± 0.50 *m/z*, interchannel delay 0.02 s. Quantitation was done by SIM of quasi-molecular ions of $[M + H]^+$. Dwell time of

Table 2
Basic set-up of ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK) for detection of specific diagnostic ions and deuterium labelled internal tracers

Compound	$[M + H]^+$	Internal tracer	$[M + H]^+$	Dwell time (s)	Retention window (min)	
Z9G	382.10	$^2H_5$-Z9G	387.18	0.75	I	11.0–13.8
DHZ9G	384.08	$^2H_3$-DHZ9G	387.18	0.75		
tZ	220.07	$^2H_5$-tZ	225.03	0.56	II	13.8–17.3
DHZ	222.18	$^2H_3$-DHZ	225.03	0.56		
cZ	220.07	$^2H_5$-tZ	225.03	0.56		
mT9G[a]	404.05			0.56		
tZR	352.09	$^2H_5$-tZR	357.17	0.45	III	17.3–21.1
DHZR	354.16	$^2H_3$-DHZR	357.17	0.45		
cZR	352.09	$^2H_5$-tZR	357.17	0.45		
mT[a]	242.10			0.45		
oT9G[a]	404.05			0.45		
mTR[a]	374.10			0.45	IV	21.1–25.2
BAP9G[a]	388.18			0.45		
iP9G	366.10	$^2H_6$-iP9G	372.18	0.45		
oT[a]	242.10			0.45		
oTR[a]	374.10			0.32	V	25.2–30.0
BAP	226.14			0.32		
iP	204.10	$^2H_6$-iP	210.16	0.32		
BAPR	358.16			0.32		
iPR	336.21	$^2H_6$-iPR	342.16	0.32		

[a] These compounds were not quantified.

each SIM channel has been calculated to obtain 16 scan points per peak and individual mass. Particular specific diagnostic ions, appropriate SIM windows, dwell time and internal tracers for each cytokinin derivative are listed in Table 2.

2.5. LC–ELISA conditions

Samples were dissolved in mobile phase (initial conditions) and separated on analytical RP-column (150 mm × 4.6 mm, 5 μm) (Microsorb C18; Varian, Walnut Creek, CA, USA) in sequence with a pre-column (150 mm × 4.6 mm) of the same material. The elution was performed with a methanolic gradient in (A) 10% methanol, 40 mM acetic acid adjusted to pH 3.35 with triethylamine, and (B) 80% methanol, 50 mM acetic acid. The following protocol was used for gradients: 0 min, 90% A; 0–10 min, a linear gradient to 60% A; 10–14 min, a linear gradient to 65% A; 14–18 min, a linear gradient to 50% A, 18–24 min, isocratic elution of 50% A, 24–26 min, a linear gradient to 0% A (100% B), followed by 10 min isocratic elution of 100% B (wash step) and equilibrated to initial conditions for 20 min.

The flow-rate was 0.6 ml/min. The UV absorbance was scanned between 240 and 300 nm. HPLC was combined with specific ELISAs for each cytokinin group [26]. For the ELISA analysis, the fractions of 0.5 min were collected and evaporated to dryness in vacuo. To examine immunoreactivity of individual cytokinin peaks, aliquot volumes of fractions of each HPLC run were analysed with antibodies against the studied cytokinin nucleosides [26]. The resulting immunohistograms were further quantified on the basis of cross reactivity. This means that the same antibody was utilised for the quantifying of cytokinin ribosides and free bases as well as 9-glucosides. The antibody, originally raised against appropriate riboside (antigen), also recognises free base and 9-glucoside. Response equivalent depends on level of structural similarity with the antigen. For instance, antibody raised against tZR exhibits 90% cross-reactivity with tZ9G, which means that certain amount of tZ9G gives 90% of the response when compared with the same amount of tZR. The preparation of immunogens and enzyme tracers, as well as the production of antibodies for the immunoassays have been described in detail by Strnad and co-workers [4,26–28].

3. Results and discussion

Different methanolic mobile phases as well as reversed-phase chromatography columns were tested. In respect of baseline separation necessity, mobile phase stability, ionisation efficiency and run time, 15 mM HCOOH (pH 4.0, adjusted by NH_4OH) has been chosen. Three tested chromatographic columns: (150 mm × 2.0 mm, 5 μm) (Microsorb C18; Varian, Walnut Creek, CA, USA); (150 mm × 2.1 mm, 5 μm) (Symmetry C18, Waters); (150 mm × 2.1 mm, 5 μm) (Symmetry C8, Waters) were noted to have acceptable selectivity for mixture of 20 cytokinin standards. The Symmetry C18 has been chosen for further utilisation, because of its ability to separate all isomers (*cis*/*trans*-zeatin, *ortho*/*meta*-topolin) in the shortest time (see Fig. 1). However, a signal of DHZR ($m/z = 354$) is still at least partially obscured by the natural isotope of cZR (MH + 2; $m/z = 354$). This fact has little impact on our result quality, since both derivatives are present at comparable levels (see Table 5). However, this situation should be taken in the account when different types of plant material will be analysed by our method.

Since *cis*- and *trans*-zeatin, their ribosides, nucleotides as well as *O*- and *N*-glycosides (valid also for *ortho*- and *meta*-topolins) have identical molecular mass and basic fragment peaks under the ES–MS conditions, but in many cases different or unknown physiological properties, the isomer separation is of prime importance in order to obtain a correct estimate of the natural cytokinins. Using the present method we were able to obtain baseline separation of all zeatin and topolin isomers, respectively (see Fig. 1). Furthermore, another remarkable observation supporting development of such technology is, that, despite the high selectivity of IAC purification and LC–MS technology used, quite a number of unidentified peaks are seen to be present in the individual mass chromatograms (as an example see Fig. 2B, retention window IV for iP9G). It was also noted by Rhijn et al. [16] that quite a number of closely adjacent peaks of the same molecular masses and fragments are seen to be present in the Z and ZR traces even if highly selective MS/MS detection have been used. The simple separation procedures sometimes used by other labs [15] in order to increase sample throughput, would inevitably have caused co-elution not only of zeatin

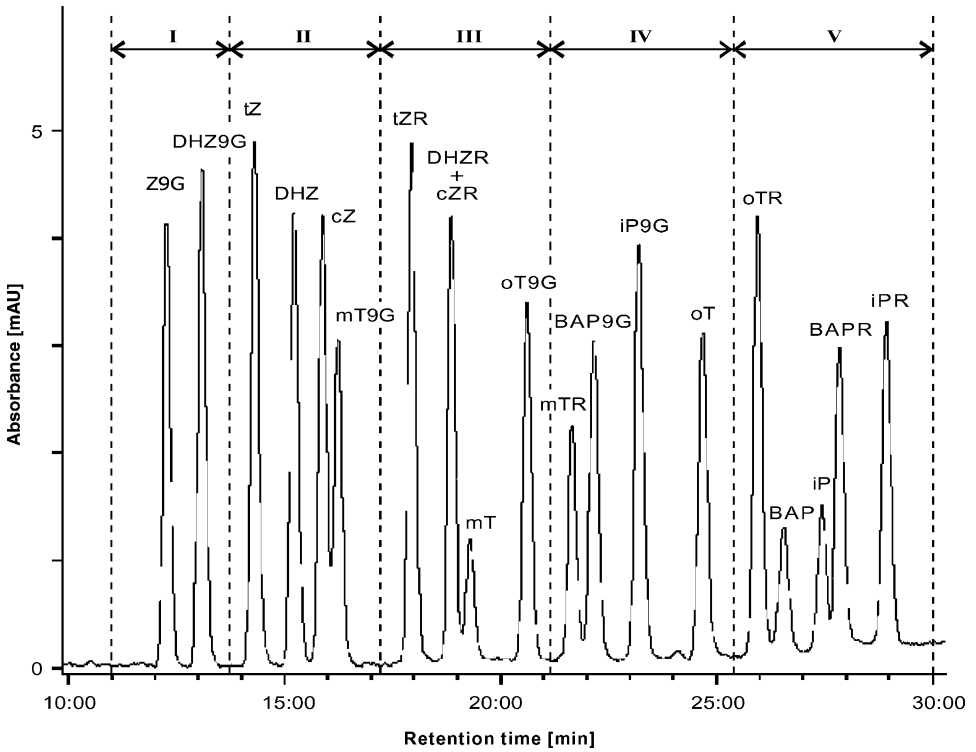


Fig. 1. UV-profile (268 nm) of cytokinin standards mixture separation. The separation of standard mixture, containing 12.5 pmol of each derivative, diluted in mobile phase (initial conditions) and injected on RP-column. Chromatography was performed as described in Section 2.

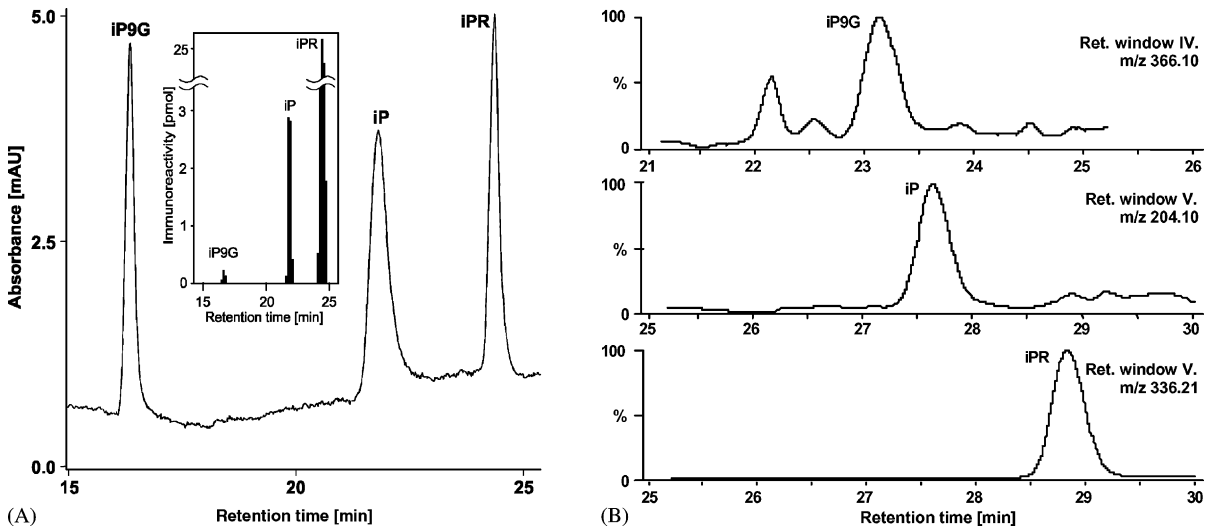


Fig. 2. Immunohistogram (A) and SIM ion chromatograms (B) of authentic (0.23 pmol) iP9G, (3.2 pmol) iP, (23 pmol) iPR present in 1 g of poplar leaves. Immunoreactive regions (black bars) correspond with UV-profile (268 nm) of the standards.

or topolin isomers, but also of unknown compounds with the classical cytokinins. Thus, a consistent and considerable over-estimation of endogenous levels could be obtained. The developed IAC–HPLC–MS technology thus convincingly demonstrates the necessity of highly hyphenated analyses, in which optimum IAC chromatography and LC separation is combined with MS detection. It has been already mentioned [16], that proper sample purification is required prior a cytokinin quantification. Any simplification of the purification protocol results directly in chemical background increase, which consequently makes analysis difficult, even impracticable. Moreover, omission of an immunoaffinity step results in selectivity and sensitivity decrease of LC–MS, especially when simple SIM mode is used (data not shown). The considerable fall of analytical parameters was also observed in LC–ELISAs of natural samples [26,28]. This fact is in contrast to the latest approach used for other plant hormone groups, for instance auxins or gibberellins [34,35], apparently due to lower cytokinin levels. On the other hand, it must be emphasised, that these methods were based on MRM methodology. Since purification is still the most time consuming part of the entire analysis, chromatographic run duration is not crucial.

In spite of our effort to develop HPLC gradient separation as short as possible, total run time verges on 50 min. At the end of gradient elution, a washing step (100% methanol for 5 min) is followed by only 15 min column equilibration, but the stability of chromatographic profiles of all subsequent runs were found to be highly satisfactory. A basic disadvantage of the prolonged equilibration and chromatographic separation is that it reduced sample throughput, but, actually, the sample preparation rather than the instrumental analysis is the rate-limiting step of the total methodology of cytokinin determination. Employing the developed method, it is possible to analyse 26 samples in 24 h.

Stability of retention times was tested with three cytokinins eluted from LC column at the beginning (Z9G), in the middle (ZR) and at the end (iP) of chromatographic run in both, mixture of standards and real samples (see Table 3). Response for 12 consecutive injections of nine different cytokinin concentrations was measured. The retention times of Z9G, ZR and iP were essentially constant at 12.23, 17.86, and 27.31 min with coefficient of variation between 0.27 and 0.54. The chosen compounds represent different cytokinin groups (free base, riboside, and glucoside), hence number of replicates for real samples varied according individual occurrence of these metabolites. For example, ZR is present, after appropriate enzymatic treatment, in all three fractions (bases, nucleotides, *O*-glucosides), while iP and Z9G occur in the first basic fraction only.

Table 3
Retention time stability for selected compounds

Compound	R.S.D. (%)		
	Standard	BY-2	Poplar leaves
ZR	0.27[a]	0.22[c]	0.19[c]
Z9G	0.54[a]	0.21b[b]	0.35[b]
iP	0.27[a]	0.12b[b]	0.12[b]

R.S.D. values represent mean values of 12 consecutive measurements of cytokinin standards followed by two sets of six consecutive measurements of real samples (BY-2, poplar leaves).
[a] Twelve replicates.
[b] Six replicates.
[c] Eighteen replicates.

On the basis of retention time stability, the chromatographic run has been split into five retention windows. Each window is marked in Fig. 1 and time borders are described in Table 2. Usually 2–4 cytokinins were selected for each window on the basis of their retention times. Such approach allowed us to increase sensitivity of the analysis by dwell time prolongation. Cytokinins are weak bases, which exhibit two dissociation constants ($pK_{a1} \sim 4.2$ and $pK_{a2} \sim 9.8$) [29,30]. Each member of a cytokinin class becomes protonated if pH drops to approximately 4 and deprotonated in the alkaline region [31]. Hence, all studied cytokinins showed dominant quasi-molecular ions of $[M + H]^+$ in positive electrospray (ESI+), when low cone voltage (around 20 V) was applied. We investigated effects of various parameters (mobile phase splitting ratio, desolvation temperature, capillary and cone voltage) on the sensitivity of mass spectrometric detection. Optimal values have been found for “average” mobile phase content (1:3 = solvent (A):solvent (B)) and consequently used for LC–MS method (see Section 2.5). Since the electrospray mass spectrometer is a concentration-selective detector [32,33], the flux from the chromatography column can be split without consequent loss of sensitivity.

Table 4
Dynamic range, detection limit and expression of linearity (linear regression and correlation coefficients) for different compounds quantified using ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK)

Compound	Detection limit[a] (fmol)	Dynamic range (pmol)	Equation of linear regression	ρ^2	n
tZ	25	0.050–100.00	$y = 0.99 \pm (0.02)x + 0.19 \pm (0.43)$	0.9996	6
cZ	25	0.050–100.00	$y = 0.98 \pm (0.03)x + 0.30 \pm (0.44)$	0.9996	7
tZR	25	0.050–100.00	$y = 0.99 \pm (0.02)x + 0.11 \pm (0.02)$	0.9994	7
cZR	25	0.050–100.00	$y = 0.95 \pm (0.04)x + 0.41 \pm (0.07)$	0.9994	5
Z9G	25	0.050–100.00	$y = 0.99 \pm (0.01)x - 0.09 \pm (0.01)$	0.9996	5
DHZ	25	0.050–100.00	$y = 0.96 \pm (0.01)x + 0.01 \pm (0.08)$	0.9994	7
DHZR	50	0.075–100.00	$y = 0.93 \pm (0.04)x + 0.16 \pm (0.31)$	0.9990	7
DHZ9G	50	0.075–100.00	$y = 0.92 \pm (0.02)x + 0.56 \pm (0.47)$	0.9990	6
iP	10	0.025–100.00	$y = 1.10 \pm (0.13)x - 0.36 \pm (0.29)$	0.9995	5
iPR	10	0.025–100.00	$y = 0.94 \pm (0.03)x - 0.28 \pm (0.46)$	0.9993	8
iP9G	50	0.075–100.00	$y = 0.92 \pm (0.02)x + 0.10 \pm (0.00)$	0.9994	6
BAP	10	0.10–100.00			
BAPR	25	0.10–100.00			
BAP9G	25	0.50–100.00			
mT	25	0.10–100.00			
mTR	25	0.10–100.00			
mT9G	50	0.50–100.00			
oT	25	0.25–100.00			
oTR	50	0.25–100.00			
oT9G	50	0.50–100.00			

All measurements were made using 25 μl injections.
[a] Signal to noise ratio was set to 3:1.

In the present method 50% of flow was introduced into DAD, in order to obtain additional spectral information. Such approach allows us to detect other cytokinin UV-absorbing peaks (λ_{max} = 265–270 nm) in the immunopurified extracts, whose retention times does not correspond with those known for classical cytokinins. At LC separation conditions the linear ranges, limits of detection (LOD) and equations of linear regression found for all analysed standard compounds are given in Table 4. LODs ranges from 10 towards 50 fmol. The best results have been obtained for strongly retained derivatives: BAP, iP, iPR. Prinsen et al. [15] also obtained the best sensitivity for iP. The simple explanation for this observation is, that higher methanol content results in higher chromatographic (narrower peaks) and ionisation efficiency [33]. The linearity of SIM using the diagnostic ions listed in Table 2 was measured for different cytokinins. The data shown were obtained by measuring of each analyte solution at nine different concentrations. After log-transformation, a linear regression function adequately described the ratios between concentration and integrated area units of the corresponding peak signals within the concentration range. It is obvious that good linear correlation has been obtained for all cytokinin metabolites with correlation coefficients ranging from 0.9990 to 0.9996. The linear ranges found are in good agreement with earlier published data [15]. Although Prinsen et al. [15] did not investigate the 2 mm i.d. column linear range, our sensitivity results correspond with a position between conventional (4.6 mm i.d.) and micro (1 mm i.d.) column.

To evaluate the developed method, the cytokinin content in BY-2 cells and poplar leaves was determined. Quantification was performed by using standard isotope dilution method. Final concentrations were calculated from area of the SIM chromatograms. Ratio of the endogenous cytokinin to appropriate labelled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to the knowledge of quantity of added internal standard. Results are summarised in Table 5.

The accuracy of developed LC–MS method was tested. Poplar leaf extract was spiked with known amount of tZ, tZR, iP, and iPR. An identical plant

Table 5
Cytokinin content quantified in BY-2 cells and poplar leaves

Compound	BY-2 cells		Poplar leaves	
	Content[a]	*n*	Content[a]	*n*
tZ	3.50 ± 0.82	5	1.29 ± 0.12	5
cZ	0.17 ± 0.02	4	0.26 ± 0.03	6
tZR	2.84 ± 0.34	5	3.31 ± 0.16	5
cZR	0.26 ± 0.01	5	1.44 ± 0.02	5
Z9G	3.02 ± 0.70	5	0.20 ± 0.02	4
DHZ	0.60 ± 0.01	5	0.04 ± 0.01[b]	5
DHZR	0.49 ± 0.04	5	0.34 ± 0.02	6
DHZ9G	Not detected	5	Not detected	6
iP	2.12 ± 0.17	5	3.20 ± 0.60	6
iPR	1.34 ± 0.37	5	25.66 ± 1.71	6
iP9G	Not detected	5	0.23 ± 0.02	6
tZOG	5.38 ± 0.92	5	18.49 ± 1.03	6
cZOG	0.56 ± 0.10	5	8.78 ± 0.48	6
tZROG	3.14 ± 0.37	5	10.45 ± 0.23	5
cZROG	1.64 ± 0.20	5	10.92 ± 0.68	6
DHZOG	3.70 ± 0.74	5	4.01 ± 0.12	5
DHZROG	14.95 ± 1.57	5	7.76 ± 0.22	3
tZRMP	40.68 ± 3.23	3	51.58 ± 3.19	6
cZRMP	2.99 ± 0.51	5	2.38 ± 0.23	5
DHZRMP	0.45 ± 0.09	5	0.88 ± 0.25	6
iPRMP	89.17 ± 1.44	3	66.63 ± 6.40	4

Alliance 2690 Separations Module (Waters, Milford, MA, USA) connected to ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK) was used for quantifications. Samples were dissolved in mobile phase (initial conditions), filtered, injected on RP-column (150 mm × 2.1 mm, 5 μm) (Symmetry C18, Waters) and separated in methanol-15 mM formic acid (pH 4.0) linear gradient. A biological variation is indicated by the standard deviation.

[a] Fresh weight (pmol g^{-1}) (mean ± S.D.).

[b] Out of calibration range.

material was analysed with and without the addition of 1 or 10 pmol of each standard compound per gram of the fresh weight. The data we obtained are summarised in Table 6. In case of 1 pmol addition, results for all tested compounds are fully satisfactory for trace analysis [16]. Concerning results for iP and iPR spiked with 10 pmol, determined levels are about 17 and 9% lower than expected. It is probably caused by due a sorption of these compounds on cell debris during overnight extraction. Also lower solubility in aqueous solvents may result in decreased recovery of added standards, especially when post-SCX extract (after the first purification step) is dissolved in 10 mM ammonium formate.

Table 6
Cytokinin levels in poplar leaves spiked with tZ, tZR, iP, and iPR

	Cytokinin levels (pmol/g fresh weight)			
	tZ	tZR	iP	iPR
Non-spiked	1.29	3.31	3.20	25.66
Spiked with 1 pmol	2.38	4.50	4.02	26.83
Spiked with 10 pmol	12.00	12.24	10.94	32.58

Because measuring of specific diagnostic ions (SIM) for the appropriate derivatives is not a sufficiently selective mode, we confirmed the presence of authentic cytokinins by LC–ELISA. Using specific anti-cytokinin antibodies, HPLC fractions of real samples exhibit immunoreactivity at retention times equivalent to standards. As a result of the application of different principles of enzyme immunoassay and mass spectrometry together with the use of two different chromatographic systems, the identity of quantified derivatives is well verified. As an example, immunohistrogram of iP-type cytokinins determined in poplar leaves is shown (Fig. 2).

Our methods achieving selectivity in the presence of other cytokinin signals are based on co-chromatography of UV, mass and immunoactivity traces with signals of authentic heavy labelled standards. All applied ^{2}H-cytokinins co-eluted with authentic natural compounds, according to their retention times. The addition of deuterated derivatives to the extracts facilitated detection of natural cytokinins further, giving better resolution of mass traces that were eluted close together, as well as giving a measure of the percentage of recovery of different cytokinins throughout purification. The recoveries averaged approximately from 20 to 40%, which is acceptable for a multistep purification procedure including such critical steps as enzymatic treatment. Recovery for the compounds purified without enzymatic treatment (free bases, etc.) reached 60%. However, cytokinin concentrations in plant samples are usually much higher than detection limits obtained using our method, which means that very high recovery was not critical parameter in the development of our method. On the other hand, accuracy of our method is quite high. Differences obtained for poplar samples spiked with 1 and 10 pmol of four different cytokinin standards were below 17% (Table 6). The results to within about 25% are most acceptable for the low spiking levels studied [16]. Ideally, recovery markers are required for each cytokinin metabolite that one is attempting to measure. Hence, in many

cases only few internal standards have been used, usually added later during the extraction process or just before LC–MS quantitative analyses [14–16]. Thus, the accurate determination of endogenous cytokinin metabolites presented above enabled purification by DEAE-C18-IAC chromatography and quantitation by LC–MS to be validated by very sophisticated internal standardisation procedures, respectively.

Topolins are, as naturally occurring and highly biologically active cytokinins, the object of our long-term interest. That is why we included aromatic cytokinin standards into chromatographic separation and mass spectrometric method development. However, lack of heavy labelled analogues was the reason why we did not quantify levels of aromatic cytokinins in the biological materials. The use of *para*-topolin as internal standard for determination of endogenous topolin derivatives was reported by Horgan and co-workers [36]. Since different isomers exhibit different recoveries on immunoaffinity columns, this procedure was not utilised in this LC–MS method.

4. Conclusion

According to our knowledge, this is the first liquid chromatography–single-quadrupole mass spectrometry method, enabling routine determination of endogenous levels of isoprenoid, as well as isoprenoid-derived cytokinins in a single analysis. Single-quadrupole instruments do not allow the use of selective MS/MS detection. Therefore, the selectivity of the newly developed method has been verified by co-chromatography of UV and immunoactivity traces. The results obtained for tobacco BY-2 cells and poplar leaves show, that the utilised LC–MS method permit us to quantify physiological cytokinin levels. Future work will be mainly focused on aromatic cytokinin determination, including 6-benzylaminopurine and topolin-type derivatives.

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