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Quantitative determination of glycosylated and aglycon isoprenoid cytokinins at sub-picomolar levels by microcolumn liquid chromatography combined with electrospray tandem mass spectrometry

J.A. van Rhijn^{a,*}, H.H. Heskamp^a, E. Davelaar^b, W. Jordi^b, M.S. Leloux^{a,1},
U.A.Th. Brinkman^c

^aState Institute for Quality Control of Agricultural Products (RIKILT-DLO), Bornsesteeg 45, P.O. Box 230, 6700 AE Wageningen, The Netherlands

^bResearch Institute for Agrobiology and Soil Fertility (AB-DLO), Bornsesteeg 65, P.O. Box 14, 6700 AE Wageningen, The Netherlands

^cFree University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

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Abstract

Microcolumn liquid chromatography (μ LC) combined with electrospray tandem mass spectrometry is used for the determination of intact glycosylated cytokinins and the corresponding aglycons at picomole and sub-picomole levels in plant tissue. Routine analysis was done on C₈-bonded silica using a methanol–water gradient. Data acquisition was performed by multiple reaction monitoring. Quantification was carried out by using isotopically labelled analogues and applying linear regression to the response factor versus concentration data. For routine analysis a calibration range from 0.5 to 10 pmole injected on-column was used. The limits of detection ranged from 50 to 100 fmole injected on-column. The μ LC procedure was used to analyse plant tissue extracts from transgenic homozygote and hemizygote as well as wild-type *Nicotiana tabacum* species, and cauliflower samples. The data were compared with results obtained by conventional immunoassay and a satisfactory correlation was found. Validation data are presented. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Cytokinins; Zeatin; Dihydrozeatin; Isopentenyladenosine

1. Introduction

In recent years, the application of sophisticated techniques like LC–MS or LC–tandem-MS [1–4]

and LC–NMR [5–7] has increased rapidly in various areas of analytical chemistry. However, until now, relatively few papers are devoted to the use of such approaches in phytochemical analysis. Moritz [1] and Hostettmann and Wolfender [2] stated that although the benefits are very clear, hyphenated techniques are, in fact, currently being pioneered in this field of science.

In phytochemical analysis there is much interest in the qualitative and quantitative determination of

*Corresponding author. Tel.: +31-8-370-75400; fax: +31-8-370-17717.

E-mail address: j.a.vanrhijn@rikilt.wag-ur.nl (J.A. van Rhijn).

¹Present address: NIZO Food Research, Kernhemseweg 2, P.O. Box 20, 6710 BA Ede, The Netherlands.

aglycons and related glycosylated compounds [8]. To quote an example, the quantitative analysis of important plant growth regulators like cytokinins and gibberellines, and of other biologically active compounds such as saponins, auxins and flavonoids is urgently required [9–15]. Nevertheless, the application of conventional techniques like enzyme immuno assay (EIA), radio immuno assay (RIA) and LC–DAD/UV is still very common [16]. These techniques, although very useful, have some major drawbacks. Immunoassays cannot, in general, distinguish between closely related analytes such as aglycons and the corresponding glycosylated compounds common in plants, and extensive sample pre-treatment by, e.g. LC fractionation is required to obtain some more information on the identity of the analytes [8]. The same is true for LC–DAD/UV; it is well known that UV–Vis absorbance spectra of closely related compounds usually are very similar.

As regards more conclusive methods of analysis, GC–MS has been used successfully for several classes of analytes of phytochemical importance such as gibberellines [10]. However, most compounds of interest in phytochemistry, including the cytokinins, have to be derivatised to make them amenable to GC–MS analysis. Although several procedures have been reported, incomplete derivatisation, the formation of multiple derivatives and rapid hydrolysis of the derivatives are often encountered problems. Furthermore, GC–MS of intact glycosylated compounds is not possible, which is a serious drawback considering their biological importance. Again, LC fractionation yielding the aglycons and glycosylated compounds in separate fractions, followed by hydrolysis of the conjugated compounds to obtain the aglycons, may provide an answer, but more elegant and less laborious methods are certainly to be preferred.

Another development in phytochemical analysis is reduction of the sample size [10,17]. Using small samples will facilitate sample preparation, often the rate-limiting step of the procedure.

In addition specific small parts of plants with expected changes in cytokinin content, for instance expanding internodes, can then be analysed much more easily.

From the above it will be obvious that the quantification of cytokinins in plant material requires

highly selective and sensitive methods of analysis, preferably based on miniaturised LC with MS or MS/MS detection to provide the structural information required. In this paper, we present such a method for the compounds presented in Fig. 1, *trans*-zeatin, dihydrozeatin and isopentenyl adenine and their respective ribosides and N₉-glucosides, at low- and sub-picomole levels in plant tissue extracts. The method combines gradient LC on a 1 mm ID column and electrospray tandem mass spectrometry (ESP-MS/MS). Quantification relies on the use of isotopically labelled analogues of a number of the analytes. As an application, extracts of transgenic as well as wild-type *Nicotiana tabacum* are analysed, and the data are compared with results from EIA.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade unless stated otherwise. Demineralized water was prepared using Millipore Milli-Q system (Bedford, MA, USA).

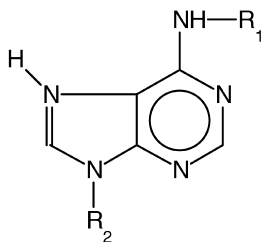
Deuterated cytokinin tracers (²H₅-Z, ²H₅-Z-N₉-G, ²H₃-DHZR, ²H₆-IPA, ²H₆-IPAR) and the unlabelled standards Z, ZR, Z-N₉-G, DHZ-N₉-G, DHZ, DHZR, IPA and IPAR were purchased from Apex Organics (Honiton, UK). For the full names of the cytokinins, the reader should consult Table 1. Acid phosphatase was purchased from Boehringer (Mannheim, Germany), DEAE-cellulose from Whatman (Springfield Mill, UK) and C₁₈-SPE columns from Varian (Harbor City, CA, USA). CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden).

2.2. Plant material

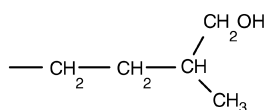
Tobacco seeds (*Nicotiana tabacum* L.) cv. Wisconsin 38, wildtype, P_{SAG12}-IPT hemizygote and P_{SAG12}-IPT homozygote were sown, germinated and, 2 weeks after sowing, transferred to 110-ml pots with Klasmann substrate No. 4 containing 210 mg/l nitrogen, 240 mg/l P₂O₅, 275 mg/l K₂O and 115 mg/l MgO, pH 5.5–6.0.

The plants were grown at a relative humidity of

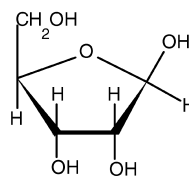
CYTOKININS



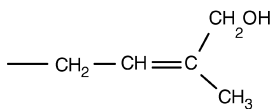
Compound	Abbreviation	R ₁	R ₂
Zeatin	Z	2-methyl-2-butenol	H
Zeatin-riboside	ZR	2-methyl-2-butenol	β-ribose
Zeatin-N ₉ -glucoside	Z-N ₉ -G	2-methyl-2-butenol	β-glucose
Dihydrozeatin	DHZ	2-methylbutanol	H
Dihydrozeatin-riboside	DHZR	2-methylbutanol	β-ribose
Dihydrozeatin-N ₉ -glucoside	DHZ-N ₉ -G	2-methylbutanol	β-glucose
Isopentenyladenine	IPA	2-methyl-2-butene	H
Isopentenyladenosine	IPAR	2-methyl-2-butene	β-ribose



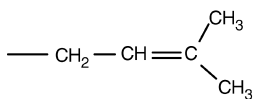
2-methylbutanol



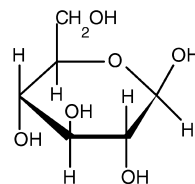
β-ribose



2-methyl-2-butenol



2-methyl-2-butene



β-glucose

Fig. 1. Molecular structures of the cytokinins studied.

Table 1
Diagnostic ions and CID conditions for the determination of cytokinins

Compound	Precursor mass	CID fragments (collision energy [eV])	Internal standard
Z	220	136 (–20)	$^2\text{H}_5\text{-Z}$
DHZ	222	136 (–20)	$^2\text{H}_5\text{-Z}$
Z–N ₉ –G	382	220 (–22)	$^2\text{H}_5\text{-Z-N}_9\text{-G}$
DHZ–N ₉ –G	384	222 (–22)	$^2\text{H}_5\text{-Z-N}_9\text{-G}$
ZR	352	220 (–20)	$^2\text{H}_3\text{-DHZR}$
DHZR	354	222 (–20)	$^2\text{H}_3\text{-DHZR}$
IPA	204	136 (–24)	$^2\text{H}_6\text{-IPA}$
IPAR	336	204 (–24)	$^2\text{H}_6\text{-IPAR}$
$^2\text{H}_5\text{-Z}$	225	136 (–20)	
$^2\text{H}_3\text{-DHZR}$	357	222 (–20)	
$^2\text{H}_6\text{-IPA}$	342	210 (–24)	
$^2\text{H}_6\text{-IPAR}$	210	136 (–24)	
$^2\text{H}_5\text{-Z-N}_9\text{-G}$	387	225 (–22)	

75% and the day/night temperatures in the greenhouse were 21/18.0°C. Supplementary light was provided for 16 h at an intensity of 140 $\mu\text{mol}/\text{m}^2/\text{s}$. Five weeks after sowing, the plants were transferred to 2500-ml containers with Klasmann substrate No. 4 which were arranged in the greenhouse according to a randomized block design. After 6 weeks each plant received 100 ml of a Steiner solution [18]. Subsequently, only water was supplied to the plants. Eleven weeks after sowing, the oldest leaves were collected and frozen in liquid nitrogen.

2.3. Sample preparation

Of freeze-dried samples, an amount corresponding to 5 g of fresh leaf material was powdered in liquid N₂ and extracted with boiling ethanol (10 min). After centrifugation, pellets were re-extracted twice with ethanol–water (80:20, v/v). Ethanol was removed by evaporation under reduced pressure and the aqueous residues were taken up in water. After freezing and thawing, the extracts were centrifuged. Total supernatants were treated with acid phosphatase to determine the amount of free plus bound cytokinin (like nucleotides), and partitioned against water-saturated butan-1-ol. A control experiment demonstrated that after the enzyme treatment no nucleotide and *O*-glucoside-forms are present.

The butanol layer, containing the cytokinins, was isolated and evaporated to dryness. The samples

were purified by passage through linked columns of DEAE–cellulose and C₁₈–SPE. The cytokinins were eluted from the C₁₈–SPE columns with ethanol–water (80:20 v/v). ^3H –Cytokinin standards were used for recovery studies which were found to yield a recovery percentage of at least 85% over the entire procedure. Since the overall recovery was high we did not correct for it. C₁₈–SPE column eluates were evaporated to dryness and dissolved in phosphate buffered saline–Tween solution (PBST) (pH 8.2) for the determination of the cytokinins as ZR- and IPAR-equivalents by EIA [19]. Alternatively, the column eluate was further purified by immunoaffinity column chromatography prior to the quantification of the individual cytokinins by LC–MS/MS.

2.4. Immunoaffinity chromatography

Samples were taken up in phosphate buffered saline (PBS) (pH 7.2). To allow comparison of ELISA and LC–MS/MS results, isotope-labelled analogues $^2\text{H}_5\text{-Z}$, $^2\text{H}_5\text{-Z-N}_9\text{-G}$, $^2\text{H}_3\text{-DHZR}$, $^2\text{H}_6\text{-IPA}$, and $^2\text{H}_6\text{-IPAR}$ (10 ng of each) were added as internal tracers in this stage instead of at the start of the sample preparation which would be the normal procedure. The samples were passed through a combination of a non-immuno column (1 ml) and an immunoaffinity column (0.5 ml). Non-immuno columns were prepared by coupling rabbit γ -globulins

to CNBr-activated Sepharose 4B. Immunoaffinity columns were prepared by coupling polyclonal antibodies against ZR, DHZR and IPAR [19] to CNBr-activated Sepharose 4B. These immunoaffinity columns can also bind the free bases and 9-glucosides of the cytokinins.

After washing with 5 ml of PBS buffer and, next, 10 ml of water, the immunoaffinity columns were eluted with 5 ml of ice-cold methanol. The resulting extract was evaporated to dryness and the dry residue redissolved in 100 μ l demineralised water and sonicated for 5 min to complete dissolution. The extract was centrifuged and transferred to the insert of a sample vial.

2.5. μ LC-ESP-MS/MS

Separation of the cytokinins was performed on a Phenomenex (Torrance, CA, USA) LUNA C₈, 5 μ m, 15 cm* 1.0 mm I.D. column at a flow-rate of 60 μ l/min using a gradient LC system comprised of GyncoTek (Germering, Germany) 300 and 480 pumps. The linear gradient was from 10 to 80 vol% methanol in 10 mM ammonium acetate in 20 min. Samples were injected each 45 min using a Gilson (Villiers-le-Bel, France) 231–401 autosampler. After each injection, the injection port and needle were thoroughly flushed with methanol–water (10:90, v/v) to prevent cross-contamination. A 20 μ l sample of the extracts were used for analysis.

The LC column was directly connected to a Finnigan MAT (San Jose, CA, USA) electrospray ion source mounted on a Finnigan MAT TSQ 700 mass spectrometer. The electrospray source was operated at standard settings (sheath gas pressure, 70 p.s.i.; spray voltage, 5 kV; heated capillary, 225°C). The mass spectrometer was tuned to obtain optimum performance with respect to sensitivity and spectral integrity. Tandem mass spectra were recorded in the product ion mode using argon at a pressure of 2 mTorr as the collision gas. The collision offset was adjusted to effect as complete a fragmentation of the precursor ion to the selected product ion as possible.

MS and tandem-MS spectra of all individual compounds were recorded by continuous infusion of a 1 ng/ μ l solution in methanol–10 mM ammonium acetate (50:50, v/v) at a flow-rate of 5 μ l/min. Data acquisition for quantitative analysis was performed

in the multiple reaction monitoring (MRM) mode recording the loss of the glycosyl moiety for the glycosylated compounds and the fragmentation of the N₆-substituent for the aglycons. For the isotopically labelled internal standards, analogous fragmentations were monitored (Table 1). Data processing was performed using LCQuan software.

3. Results and discussion

3.1. Mass spectra

Mass spectra and tandem mass spectra of all target analytes of the present study were recorded by using continuous infusion. Because of the limited availability of the isotopically labelled compounds, only one of these, ²H₅-*trans*-zeatin, was studied.

The cytokinins all form a protonated molecule. A minor ion at $[M+33]^+$ was recorded, that corresponds to the methanol adduct. A slight increase of the temperature of the heated capillary from 200 to 225°C causes this adduct ion to become completely dissociated. However, at higher capillary temperatures glycosylated compounds can be expected to dissociate to form the aglycons. This in-source fragmentation is highly undesirable since distinguishing the aglycons and their corresponding glycosylates on the basis of their mass spectra is a prerequisite for the quantitative determination of these substances in just one single analysis. Therefore the temperature of the capillary was optimised to avoid in-source fragmentation of the glycosides.

Collision-induced dissociation (CID) of the $[M+H]^+$ ions of glycosylated compounds in general involves the loss of the glycoside moiety [6]. This is also observed for the cytokinin glycosides. The resulting aglycons essentially yield four fragment ions upon further fragmentation, involving the loss of water followed by the loss of ammonia and the loss of C₄H₈O or C₅H₈O, the latter being the major fragment ion in all cytokinins that we studied. This fragmentation scheme, as presented in Fig. 2, is supported by the observations made in the tandem-MS spectrum of ²H₅-*trans*-zeatin where the proton rearrangements accompanying the proposed fragmentations can be readily followed due to the presence of deuterons. MSⁿ scanning on an ion-trap

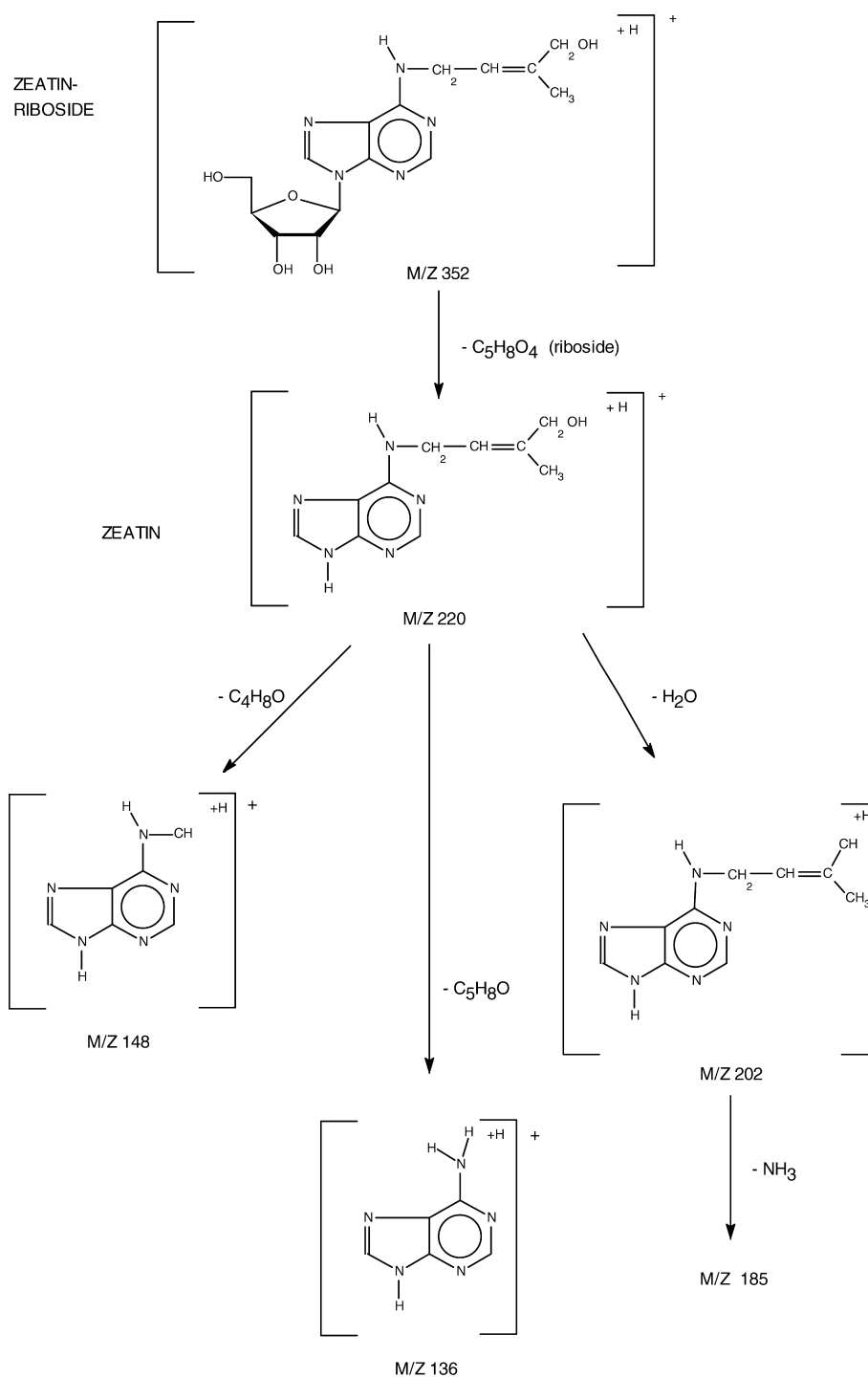


Fig. 2. Schematic representation of the major fragmentation reactions observed for cytokinin aglycons and glycosides during LC-ESP-MS/MS.

mass spectrometer indicated that the only fragmentation route yielding the ion at m/z 185 was via the ion at m/z 202. For the aglycons, CID conditions could be adjusted to obtain the protonated adenine almost exclusively. Since the ion current was preserved, it is obvious that a nearly quantitative fragmentation of the protonated molecule was achieved.

For the present set of cytokinins, which are all mono-substituted glycosides, such as ribosides and N_9 -glucosides, the corresponding aglycons result as the major fragment ion in all cases. A further increase of the collision energy beyond the point where the aglycon is quantitatively formed, yields further fragmentation similar to that of the aglycon. At the same time, a significant loss of ion current occurs. Hence, to achieve the sensitivity necessary for quantitative analysis at low-picomole levels, the glycosylated compounds are fragmented no further than to the aglycon, although this may be less informative than fragmentations of the aglycon arising from the glycoside.

The isotopically labelled compounds were found to exhibit fragmentations similar to the native analogues. The deuterium labelling is located on the aglycon part of the molecule and the labelled glycosides therefore yield the labelled aglycon. However, the labelled aglycons lose the N_6 -substituent containing the deuterons and, hence, yield the unlabelled adenine core. This fragmentation is accompanied by rearrangement of one hydrogen atom from the substituent to the adenine part of the molecule. Hence, an ion corresponding to the adenine core containing one deuteron is also observed and in much higher abundance than the mono-isotopic fragment ion. Although this fragment ion is not mentioned by Prinsen et al. [11], the ratio of m/z 136 to m/z 137 for 2H_5 -*trans*-zeatin is about 1:5, or in other words, the ion current is not concentrated in only one fragment ion in these cases. Nevertheless, since a sufficient amount of deuterated label was added to the samples and sensitivity was therefore not the limiting factor for the internal standard, the same reaction was monitored as for the native aglycons. To quote an example, for *trans*-zeatin, m/z 220 \rightarrow m/z 136 and for 2H_5 -*trans*-zeatin, m/z 225 \rightarrow m/z 136. The MRM transitions used for quantitative analysis are given in Table 1, together with the collision energy applied for each individual analyte.

3.2. μ LC-ESP-MS/MS

μ LC with gradient elution demanded rather long equilibration times between injections to obtain stable retention times. The retention time of the strongest retained compound, IPA, is about 20 min and it would seem to be possible to inject at 30-min intervals. In practice, however, we found that stable retention times could only be obtained when allowing at least 10 min extra for column equilibration. Of course, this reduces sample throughput but, actually, the sample preparation rather than the instrumental analysis is the rate-limiting step of the total procedure. It is gratifying that, if prolonged equilibration was used, the retention times of 2H_5 -Z recorded during a 45-h analytical study of biological samples, were essentially constant at 14.80 min with a coefficient of variation of 2% ($n=45$).

As a means to reduce sample preparation time, the immunoaffinity procedure was omitted and the samples were subjected to LC-MS/MS analysis immediately after the C_{18} clean-up. This approach was not successful. Especially for the compounds present at low concentration levels, considerable chemical background was recorded and it was often impossible to properly quantify the analyte of interest due to the interferences.

Using the proposed procedure, samples from *Nicotiana tabacum* were analysed, including both wild-type and genetically modified plant material. In the following, typically mass chromatograms are discussed to illustrate the practicability of the proposed method.

Fig. 3, panel I, presents mass chromatograms of a standard solution containing 100 nM of each test analyte; 20 μ l were injected on the 1 mm ID column which corresponds with 2 pmole of each compound. For clarity, only the mass chromatograms of Z, ZR, DHZ and DHZR are presented; the other compounds of interest yielded comparable S/N ratios.

It is obvious that detection poses no problems at this concentration level: the S/N ratios are at least 10 for all compounds of interest. Furthermore, although closely related compounds elute in a very short time window, separate determination is straightforward because of the use of MS/MS detection. *Trans*-zeatin and zeatinriboside (traces a and c, respectively), and dihydrozeatin and dihydrozeatinriboside (traces b and d, respectively), provide two interesting

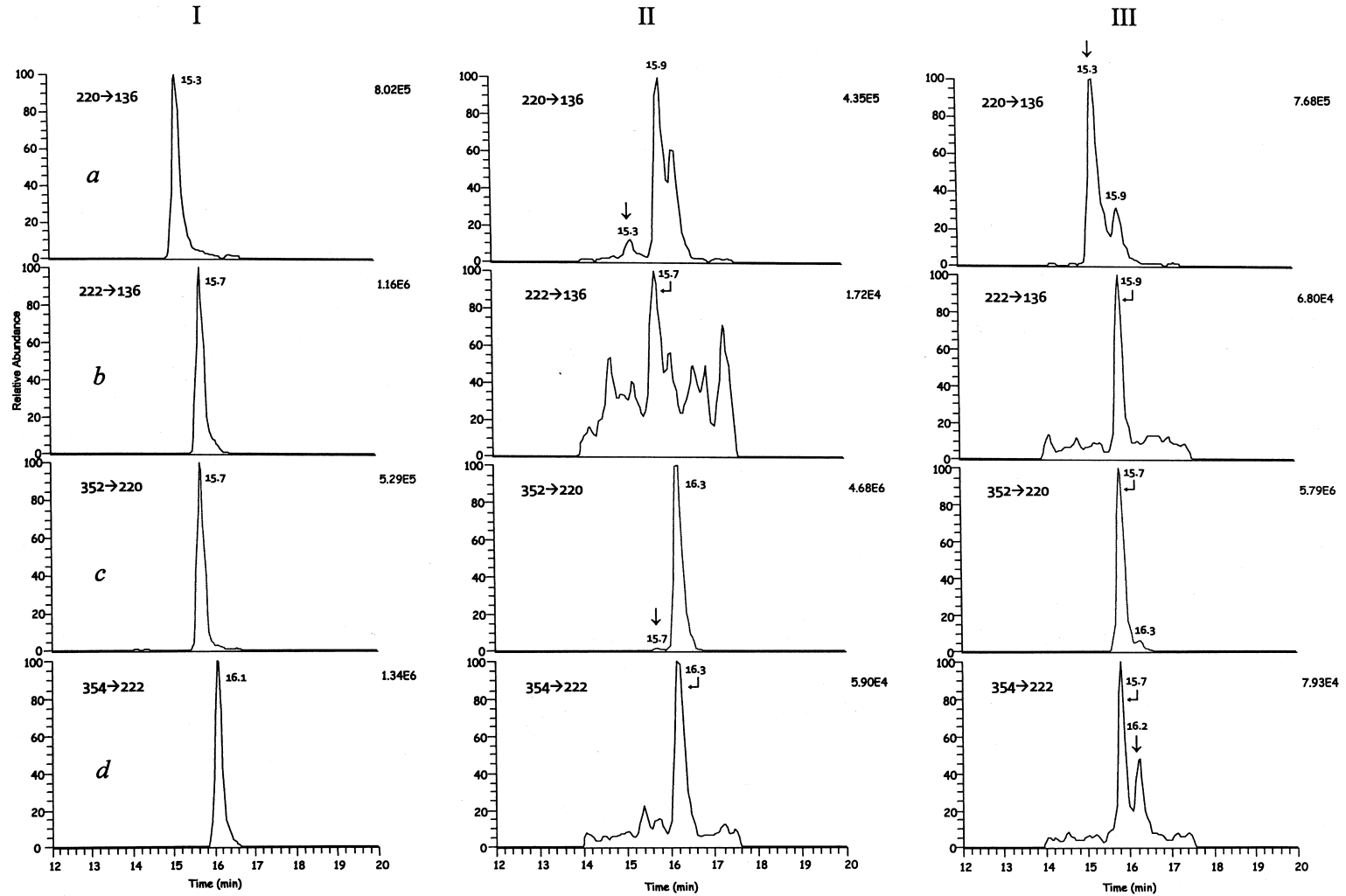


Fig. 3. μ LC-ESP-MS/MS mass chromatograms of (I) a standard solution, (II) a wild-type and (III) a hemizygote plant extract. Mass chromatograms of (a) zeatin, (b) dihydrozeatin, (c) zeatin-ribose and (d) dihydrozeatin-ribose are presented. The standards in panel I correspond to 2 pmole of each unlabelled compound injected on-column. For LC conditions, see text.

examples. Although both pairs of compounds have their peak maxima within 30 s of each other, the mass chromatograms convincingly demonstrate the complete absence of signals of the aglycons at the positions of the corresponding ribosides. Apparently there is no in-source fragmentation of the ribosides.

Fig. 3, panel II presents mass chromatograms recorded for the extract of a wild-type plant tissue sample. It is evident that this sample contains only few of the studied cytokinins and at very low levels of typically less than 0.2 pmol/g, or about 0.04–0.08 ng/g of fresh weight. Other compounds, notably ZR, DHZ, Z-N₉-G and DHZ-N₉-G are not present at or above the detection limit. For detailed quantitative results the reader is referred to Table 4 below. For comparison, Fig. 3, panel III presents mass chromatograms recorded for the hemizygote sample of Table 4. A marked increase in the *trans*-Z and ZR contents is apparent while DHZ and DHZR are present at the same or only slightly higher concentrations. These mass chromatograms clearly illustrate the ability of the present procedure to analyse cytokinins at biologically relevant levels in plant material since the sensitivity is sufficient to detect the cytokinins even at the naturally occurring background levels. The effect of genetic modification of plants on the cytokinin content in the leaves is therefore readily determined.

One remarkable aspect is that, despite the high selectivity of tandem-MS, quite a number of closely adjacent peaks are seen to be present in the zeatin and zeatinriboside traces (traces a and c, respectively in panel II and III). On the basis of its retention time, the compound eluting at t_{ret} 15.9 min could be identified as *cis*-zeatin. *Trans*-zeatin, at t_{ret} 15.3 min, is apparently present at much lower concentration than the *cis* isomer. Since *cis*- and *trans*-zeatin have identical mass spectra, co-chromatography of the sample in Fig. 3, panel II and a standard mixture was used to further confirm the identity of *trans*-zeatin.

The compound eluting at t_{ret} 16.3 min in the ZR trace has not been identified so far; however, co-chromatography of the sample in Fig. 3, panel II did show that ZR is indeed the minor compound eluting at t_{ret} 15.7 min as can also be concluded from Fig. 3, panel III where ZR is present in an appreciable concentration and a minor peak can be seen eluting just after ZR. Considering the selectivity of MS/MS

detection, the adjacent peaks have to originate from closely related compounds that have both the same molecular mass and a fragment ion of identical mass.

The peak at t_{ret} 15.7 min in the DHZR trace (trace d) in Fig. 3, panel III, is most likely due to the M+2 isotope peak of ZR. This isotope of ZR potentially undergoes the MRM transition 354→222, which is identical to the MRM transition monitored for DHZR. The same may be true for the peak at t_{ret} 16.3 min in Fig. 3, panel II, trace d, since this peak co-elutes with the unknown peak in the ZR trace. Comparison with the quantitative data of Table 4 supports this suggestion because the DHZR content was unexpectedly high in the wild-type sample when compared to the ZR content. However, definite proof is not yet available but these observations clearly demonstrate the necessity of optimum chromatographic separation even when such selective methods like MS/MS detection are used. It is therefore clear that isomer separation is of prime importance in order to obtain a correct estimate of the biologically active cytokinin content.

The presented mass chromatograms convincingly demonstrate the necessity of this type of hyphenated analysis, in which optimum LC separation is combined with MS/MS detection. The rapid separation procedures sometimes used by other authors [11,12] in order to increase sample throughput, would inevitably have caused the co-elution of *cis*- and *trans*-zeatin, and of zeatinriboside with the unknown, and consequently, a serious misinterpretation of the complexity of the sample and a consistent and considerable over-estimation of the concentrations of highly relevant cytokinins.

3.3. Analytical performance

Quantification was performed by means of isotope dilution. Response factors (RF) were calculated from the ratio of the peak area of an analyte and that of the appropriate isotope-labelled standard. The internal standard used for each analyte is indicated in Table 1.

Standard solutions containing 25, 50, 100, 200 and 500 nM of each of the analytes were prepared with each solution containing 10 ng of each isotope-labelled internal standard. Table 2 shows the regression data on the RF vs. concentration plots. It is

Table 2
Relevant calibration data for micro-LC–ESP-MS/MS of cytokinins^a

	Z	DHZ	ZR	DHZR	Z–N ₉ –G	DHZ–N ₉ –G	IPA	IPAR
Slope	0.020	0.023	0.038	0.063	0.103	0.186	0.034	0.049
Intercept	0.013	0.016	0.016	0.045	–0.030	–0.091	0.026	0.040
Correl. coeff.	0.9977	0.9987	0.9990	0.9992	0.9989	0.9981	0.9998	0.9997

^a Concentration range, 25–500 nM or 0.5–10 pmol injected on column ($n=3$).

obvious that good linear correlation was obtained for all analytes with correlation coefficients ranging from 0.997 to 0.999. Actually when absolute rather than relative peak areas were plotted versus analyte concentration, linear correlation was also acceptable with the largest discrepancy being found for *trans*-zeatin (0.991 vs. 0.998). These results clearly illustrate the stable operation of the μ LC–ESP-MS/MS system. This was confirmed during testing of the repeatability at concentration levels of 25, 50 and 100 nM. Preferably, repeatability should be tested in spiked samples but due to the laborious sample pre-treatment, this was not feasible and hence calibrants were used. The relative standard deviations (RSDs) typically were 3–6% ($n=5$) irrespective of the concentration level studied or analyte used. This was true even for compounds such as Z–N₉–G and DHZ–N₉–G which gave rather low S/N ratios. Especially considering the level at which the analyses were performed, repeatability is fully acceptable.

The accuracy of the LC–ESP-MS/MS procedure was tested by adding known amounts of individual compounds to cauliflower leave samples prior to sample clean-up. The same sample material was analysed with and without the addition of 4 or 8

pmol/g of fresh weight of Z, ZR, IPA and IPAR. The data of Table 3 show that, generally speaking, the results agree to within about 25%, which is most acceptable for the low spiking levels studied. As regards the exceptionally high result obtained for IPA at the 8 pmol/g spiking level, one main reason may be the limited solubility of this compound in aqueous solvents [11]. We can add that accuracies of 4–10% were found for all eight test analytes of Table 2 during the linearity and precision studies. This somewhat better result is not unexpected since, in that case, standard solutions were used, although these also have to be evaporated to dryness and redissolved in an aqueous solvent.

3.4. Applications

During our study, many extracts of tobacco leaves were subjected to μ LC–ESP-MS/MS. The results of three relevant examples, which are presented in Table 4, are all according to expectations from the point of view of the genetic modifications that were made, an aspect that is beyond the scope of the present study but has recently been discussed in detail by Jordi et al. [17]. The ion traces for the analytes in the wild-type *Nicotiana* sample are shown in Fig. 3, panel II; this sample was selected because here the lowest concentration levels were generally found.

Additionally, the present results were compared with EIA data. EIA analysis is by far the most widely used technique for the determination of cytokinins and, hence, a good correlation between LC–MS/MS and EIA data is important. Since the selectivity of EIA is governed by another mechanism than that of LC–MS/MS, a good match is a strong indication that the results of both types of analysis are correct. EIA was used to determine the concentrations of Z, ZR, IPA and IPAR in the samples of

Table 3
Analysis of extracts of cauliflower leaves spiked Z, ZR, IPA and IPAR

	Concentration (pmol/g fresh weight)			
	Z	ZR	IPA	IPAR
Non-spiked	0.2	0.3	0.1	0.3
Added (pmol/g)	3.6	4.0	3.6	3.8
Found	4.2	4.6	4.2	4.9
Added (pmol/g)	8.0	8.0	8.2	8.0
Found	10.2	7.2	12.3	9.3

Table 4
 μ LC–ESP–MS/MS of cytokinins in a wild type, a homozygote and a hemizygote sample of *Nicotiana tabacum* expressed as pmol/g of fresh weight

Sample	Z	DHZ	ZR	DHZR	Z–N ₉ –G	DHZ–N ₉ –G	IPA	IPAR
Homozygote	3.4	0.11	20	0.09	<0.1	<0.1	0.13	0.98
Hemizygote	3.2	0.18	40	0.16	<0.1	<0.1	0.11	0.90
Wild-type	0.06	<0.05	<0.05	0.14	<0.1	<0.1	0.07	3.5

Table 4. Although one usually expresses the EIA results for Z and ZR as summed ZR equivalents, for the comparison with LC–MS/MS the results of individual compounds were used. The correlation equation is $Y = 1.181X - 0.3525$ for a total number of data points of 9 with $r^2 = 0.96$ with X representing the EIA data. The essential confirmation of the EIA results by those of the MS-based procedure is an encouraging result. Although the μ LC–MS/MS data generally were somewhat higher than those of the EIA assay, the reverse was found for analyte levels near the limit of determination. For these very low amounts, found in old senescing leaves, the EIA results tend to be higher, probably as a result of interferences present in these leaves.

One more test was performed to assess the reproducibility of the procedure. The μ LC–MS/MS analysis of a number of samples was repeated at a later stage. This would normally have been done if the first analysis yields an unexpected result such as low recovery or distinctly outlying quantitative data. These repeated analyses yielded 39 duplicate results of individual compounds determined in eight individual sample extracts. Fig. 4 presents the correlation between the first and second sets of results, which features a correlation coefficient of 0.987. Actually, the differences between the duplicate results were below 25% in 35 out of 39 cases. This is quite acceptable when it is compared with the situation in the analysis of residues of microcontaminants and veterinary drugs. In that field it is generally accepted that within-lab variability at the level of 0.1 μ g/kg, which is the level at which the present analyses are performed, may be as high as 20%, expressed as the coefficient of variation of repeated analysis [20]. With such a C.V., differences between individual results may well exceed 30% without being unexpectedly high. Even though it is only the instrumental analysis which is considered in

our study, the performance of the μ LC–MS/MS procedure can be said to be quite acceptable.

4. Conclusions

μ LC–ESP–MS/MS can be recommended for the routine determination of low- and sub-picomole quantities of individual glycosylated and aglycon cytokinins. The results obtained for tobacco leave samples show that, despite the selectivity of MS/MS detection, efficient LC separation of the analytes from interfering compounds is necessary to avoid problems of co-elution and erroneous quantification. The technique is user-friendly and the experimental data correlate well with those of commonly used immunoassays.

The present approach offers the phytochemical analyst a new tool to detect, quantify and characterise biologically active compounds in plant tissue extracts at biologically relevant levels. Future work will be directed at further improving cytokinin

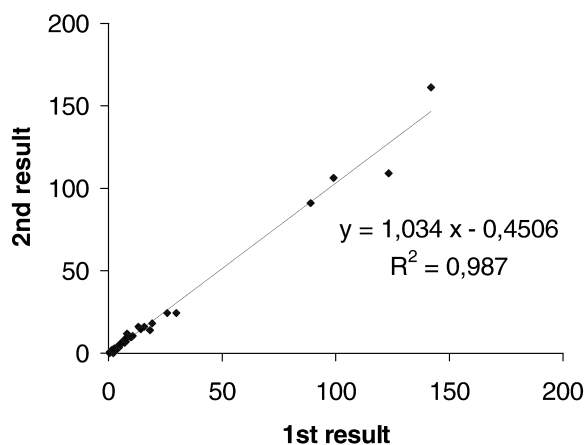


Fig. 4. Correlation of duplicate LC–ESP–MS/MS analysis of tobacco leaf extracts. For details, see text.

detectability, one main aim of our work being miniaturisation of the sample clean-up procedure. Initial experiments with capillary LC instead of μ LC show that considerably improved LODs can be obtained. Future work will address this issue in more detail.

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