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Qualitative and Semi-Quantitative Analyses of Cytokinins Using LC/APCI-MS in Combination with ELISA

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Abstract. Application of liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC/APCI-MS) for the analysis of cytokinins was examined. The fragmentation of cytokinins was studied using authentic *trans*-zeatin (*t*-Z), *trans*-zeatin riboside (*t*-ZR), isopentenyl adenine (i^6Ade), isopentenyl adenosine (i^6Ado), benzyl adenine (BAde), benzyl adenosine (BAdo), and kinetin. These cytokinins were effectively ionized by APCI in aqueous acetonitrile. *t*-Z, i^6Ade, BAde, and kinetin showed prominent quasi-molecular ions of $[M+H]^+$, and ribosylcytokinins clearly showed both $[M+H]^+$ and a characteristic fragment ion ($[M+H-\text{ribose}]^+$), giving some information about their structures. The qualitative and semi-quantitative analyses of cytokinins by LC/APCI-MS were validated in combination with enzyme-linked immunosorbent assay (ELISA) through the analysis of *t*-ZR in the teratoma of *Nicotiana tobacum*. *t*-ZR was conclusively identified and a semi-quantitative estimate of its endogenous levels were provided by the combination of LC/APCI-MS and ELISA. The quantified values obtained by LC/APCI-MS (single ion detection) and ELISA are in close agreement.

Combined gas chromatography–mass spectrometry (GC/MS) has been used as a highly sensitive and reliable method for the identification and quantification of plant hormones. However, there are some problems associated with the analysis of cytokinins by GC/MS. For instance, in trimethylsilylation, which is necessary for GC/MS analysis, zeatin riboside (ZR) gives a tetratrimethylsilylated or a pentatrimethylsilylated derivative, depending on the conditions (Palni et al. 1983, Watanabe et al. 1982).

Recently, it has been shown that combined high-performance liquid chromatography–mass spectrometry (LC/MS) provides a convenient method for the analysis of plant hormones, in which derivatization is not necessary. We applied this methodology to the analysis of gibberellin-conjugates using an LC/MS apparatus equipped with an atmospheric pressure chemical ionization (APCI) interface. The mechanism of LC/APCI-MS and its application for gibberellins were briefly reported previously (Murofushi et al. 1992).

On the other hand, an immunoassay using highly specific antibodies was also reported to be a sensitive and convenient method for the analysis of plant hormones (Weiler and Wieczorek 1981). However, using this methodology, it is difficult to identify hormone species conclusively, because immunoassay results can be confounded by unexpected cross-reactivities.

With this background, we examined the effectiveness of combining HPLC and immunoassay with LC/APCI-MS. Since the same HPLC conditions can be applied to both HPLC-immunoassay and LC/APCI-MS, a direct comparison of histograms from HPLC-immunoassay and mass chromatograms from LC/APCI-MS is possible. This ap-

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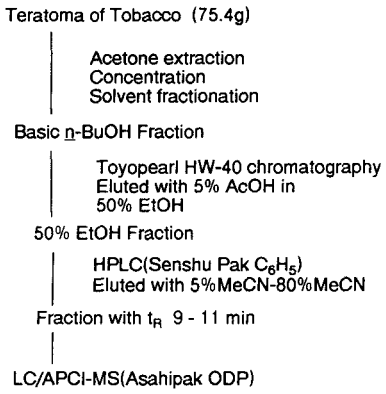

Fig. 1. Procedure for purification of *t*-ZR from teratoma for LC/APCI-MS analysis.

proach appears to provide additional evidence for the identification of the cytokinins.

We describe herein the characteristics of LC/APCI-MS of cytokinins and consider the application of this methodology, as a case study, to the analysis of endogenous *trans*-zeatin riboside (*t*-ZR) in plant materials.

Materials and Methods

The authentic cytokinins used to obtain standard mass spectra included *trans*- and *cis*-zeatins (*t*-Z, *c*-Z), *trans*- and *cis*-zeatin riboside (*t*-ZR, *c*-ZR), isopentenyl adenine (i^6Ade), isopentenyl adenosine (i^6Ado), benzyl adenine (BAde), benzyl adenosine (BAdo), and kinetin. Those not commercially available were prepared in our laboratory. Our LC/APCI-MS consisted of a Hitachi M-2000 mass spectrometer equipped with an atmospheric pressure ionization interface and a Hitachi L-6200 HPLC system.

Plant Materials and Processing

The purification procedure for the analysis is shown in Fig. 1. The teratoma (75.4 g fresh wt) of *Nicotiana tobacum* induced by the Ti plasmid (Ishikawa et al. 1988), which was grown in hormone-free MS medium containing 4% sucrose and 0.9% agar for 3 weeks under continuous light (4000 lux) at 25°C, was extracted with 80% acetone in water. The extract was concentrated in vacuo. The aqueous residue was adjusted to pH 10 by adding concentrated NH_4OH, and partitioned against *n*-butanol three times. The *n*-butanol fractions were combined and concentrated in vacuo. The concentrate was dissolved in 5% acetic acid in water and charged into a column of Toyopearl HW-40 swelled in water (100 × 12 mm), which was eluted with 50 ml of 5% acetic acid in water and subsequently with 50 ml of 50% ethanol in water. Aliquots of the eluates were subjected to enzyme-linked immunosorbent assay (ELISA) using an anti-*t*-ZR-antibody (Eberle et al. 1986). Those which were obviously immunoreactive were concentrated and fractionated by HPLC on a Senshu Pak Phenyl column (100 × 6 mm, Senshu Scientific Co., Tokyo, Japan), eluted with a linear gradient of acetonitrile (CH_3CN) in H_2O at a flow rate of 1 ml/min (0–1 min 15% CH_3CN, 1–30 min 15–80% CH_3CN linear gradient). An aliquot of each 1-ml fraction which showed clear immunoreactivity was further purified by HPLC using an Asahipak ODP column (100 × 6 mm, ODS on polymer, Asahi Kasei Co., Tokyo, Japan), eluted with a linear gradient of CH_3CN in H_2O at a flow rate of 1 ml/min (0–5 min 20% CH_3CN, 5–30 min 20–50% CH_3CN). An aliquot of these 1-ml fractions was subjected to LC/APCI-MS under the same HPLC conditions.

Conditions for LC/APCI-MS

The outlet of the column was directly connected to a nebulizer through a teflon tube (600 × 0.25 mm) and a stainless steel capillary (150 × 0.1 mm). The effects of the nebulizer and the vaporizer temperatures, the drift voltage (Sakairi and Kambara 1988), and the needle current for corona discharge on the fragmentation of cytokinins were examined using 1 μg of *t*-ZR and i^6Ado. On the basis of the results the temperature of the nebulizer was fixed at 380°C, and that of the vaporizer at 400°C in the analysis of *t*-ZR from plant material. The needle current for corona discharge was set at 12 μA and the drift voltage at 180 V.

Reliability of the Quantitative Estimates and Sensitivity

The quantitative estimates obtained from LC/APCI-MS were examined in a single ion detection (SID) mode, monitoring the $[M+H]^+$ (m/z 352) of *t*-ZR. For this experiment an Asahipak HPLC column (100 × 6 mm) was eluted isocratically with 50% CH_3CN at 1 ml/min.

Immunoassay

ELISA was carried out using the monoclonal antibodies and protocol reported by Eberle et al. (1986).

Results

LC/APCI Condition

The nebulizer and vaporizer temperatures were examined to give the lowest possible background and to maximize the intensity of the quasi-molecular ion, $[M+H]^+$. The effects of the drift voltage on the intensities of the $[M+H]^+$ ion and a fragment ion $[M+H\text{–ribose}]^+$ of *t*-ZR are shown in Fig. 2. While the highest intensity of $[M+H]^+$ was observed in the range of 156–180 V of the drift voltage, the intensity of $[M+H\text{–ribose}]^+$ was highest in the range of 204–216 V. The drift voltage for the analysis of cytokinins was determined to be 180 V, which gave sufficient intensities for both the quasi-molecular ion and the fragment ion.

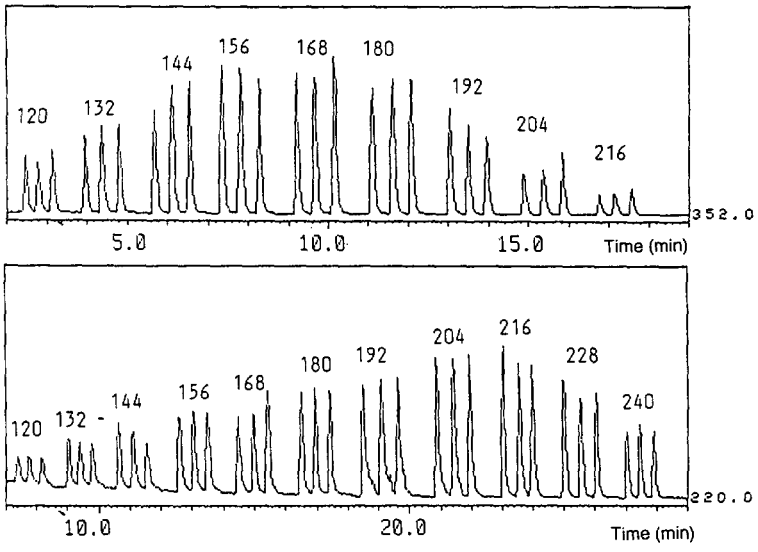

Fig. 2. Fluctuation of intensity of the molecular ion (m/z 352) and the fragment ion (m/z 220) of *t*-RZ with increasing drift voltage.

Spectra of Authentic Cytokinins

The LC/APCI mass spectra obtained using authentic standards are presented in Fig. 3. In all spectra, quasi-molecular ions $[M+H]^+$ are observed at high intensity. In the spectra of ribosylcytokinins, the fragment ions of $[M+H\text{–ribose}]^+$ were also clearly observed.

Sensitivity and Quantitativity

The calibration curve is shown in Fig. 4. The detection limit by SID was approximately 200 pg, and a good linearity was observed in the range of 500 pg to 10 ng on the calibration curve.

Identification and Semi-Quantification of t-ZR from Plant Material

Separation of *t*-ZR in each step is shown in Table 1. Figure 5 a and b shows the histogram of *t*-ZR (t_R 11–12 min) detected by ELISA after the final HPLC purification (Asahipak ODP) (Fig. 5a), and the profiles of UV (269 nm) absorption and mass chromatogram corresponding to that region (Fig. 5b). The full-scan mass spectrum at the t_R of *t*-ZR (11.7 min) in the LC/APCI-MS analysis of the plant extract is shown in Fig. 5c. The ions, m/z 352 $[M+H]^+$ and 220 $[M+H\text{–}C_5H_8O_4]^+$, are characteristic of ZR.

The t_R of the peak in the mass chromatogram monitoring m/z 352, a quasi-molecular ion $[M+H]^+$ of ZR, is coincident to that of the peak in the histogram of immunoreactivity which is measured using anti-*t*-ZR-antibody specific to *t*-Z and *t*-ZR but

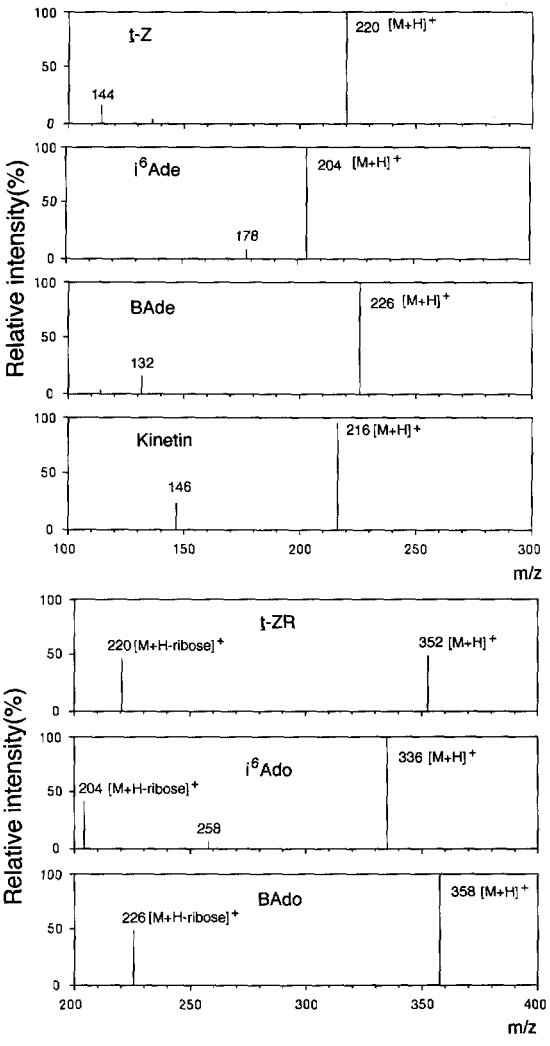

Fig. 3. LC/APCI mass spectra of several cytokinins.

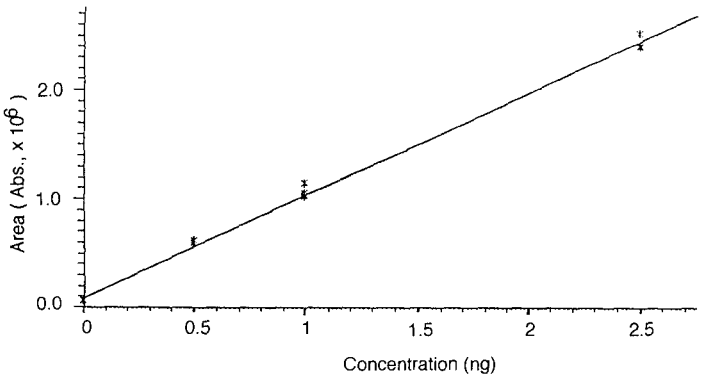

Fig. 4. Quantitativity of *t*-ZR. Calibration curve obtained by 0.5, 1, and 2.5 ng injection. APCI-MS conditions: temperature of nebulizer, 380°C; temperature of vaporizer, 400°C; needle current, 12 μA; drift voltage, 180 V.

Table 1. Quantified values (ng/g fw) of *t*-ZR after each purification step.

Purification steps (column)	Active fraction[a]	Devices	
		ELISA[b]	LC/APCI-MS[c]
Toyopearl HW-40	50% EtOH eluate	58 ± 4.6	
Senshu Pak Phenyl	t_R 9–11 min	51 ± 5.4	
Asahipak ODP	t_R 11–12 min	51 ± 3.9	54 ± 1.2

[a] For chromatographic conditions, see the text.
[b] Values are averages of triplicated analyses for each fraction.
[c] Value is the average of peak areas obtained from three injections of the immunoreactive fraction after separation with a Senshu Pak Phenyl column.

not to their *cis* isomers. Table 1 shows the quantified values by ELISA at each purification step and that by LC/APCI-MS at the final HPLC. The quantified values by both methods varies within an acceptable range to show that they selectively detected *t*-ZR.

Discussion

In the LC/APCI-MS of cytokinins, aqueous acetonitrile was used as a developing solvent. Since an adenyl group in cytokinins is an effective proton acceptor, addition of an ionization promoter such as ammonium, which is usually required for the ionization in LC/APCI-MS, was unnecessary.

As clearly shown in the spectra in Fig. 3, all ribosylcytokinins showed a quasi-molecular ion $[M+H]^+$ and a fragment ion $[aglycon+H]^+$ with prominent intensity; free cytokinins showed only a quasi-molecular ion $[M+H]^+$ without any fragment ions. The relative intensity of the $[M+H]^+$ and $[aglycon+H]^+$ in the APCI-MS of ribosylcytokinins could be influenced by the temperatures of the nebulizer and the vaporizer, and by the drift voltage. Judging from the pattern of the intensities of $[M+H]^+$ and $[aglycon+H]^+$ affected by the drift voltage, the drift voltage seems to be a major parameter to determine the relative intensity of $[M+H]^+$ and $[aglycon+H]^+$. Less fragmentation in APCI-MS is disadvantageous for structural elucidation of unknown compounds. It is also impossible to differentiate *trans* isomers of Z and ZR from their *cis* isomers by their APCI mass spectra.

It is partly an advantage, however, with respect to sensitivity, because less fragmentation gives rise to a quasi-molecular ion with higher intensity, which is favorable for detecting small amounts of cytokinin. Thus, the combination of LC/APCI-MS with immunoassay using an antibody with a high specificity as to the substituent on the 6-amino group of an adenine moiety is quite effective.

The sensitivity of LC/APCI-MS in the analysis of ribosylcytokinins by SID mode was fairly high (a few 100 pg), which is comparable to that of GC/MS, using the selected ion monitoring mode. For full-scan LC/APCI-MS, however, approximately 30 ng of *t*-ZR was required. In the quantitative analysis of *t*-ZR by SID, the calibration curve was linear within the range of 500 pg to 10 ng.

As demonstrated in the analysis of *t*-ZR in the teratoma of tobacco, combination of immunoassay and LC/APCI-MS was quite effective. LC/APCI-MS gave good information for the molecular weight of the compound, but not for its stereochemistry. The uncertainty in LC/APCI-MS for the stereochemistry was complemented by immunoassay using specific antibody for the isomer in question. The good agreement of the quantified values by both methods also supports the identification of *t*-ZR. Combination of these two methods may be a promising technique in the analysis of cytokinins.

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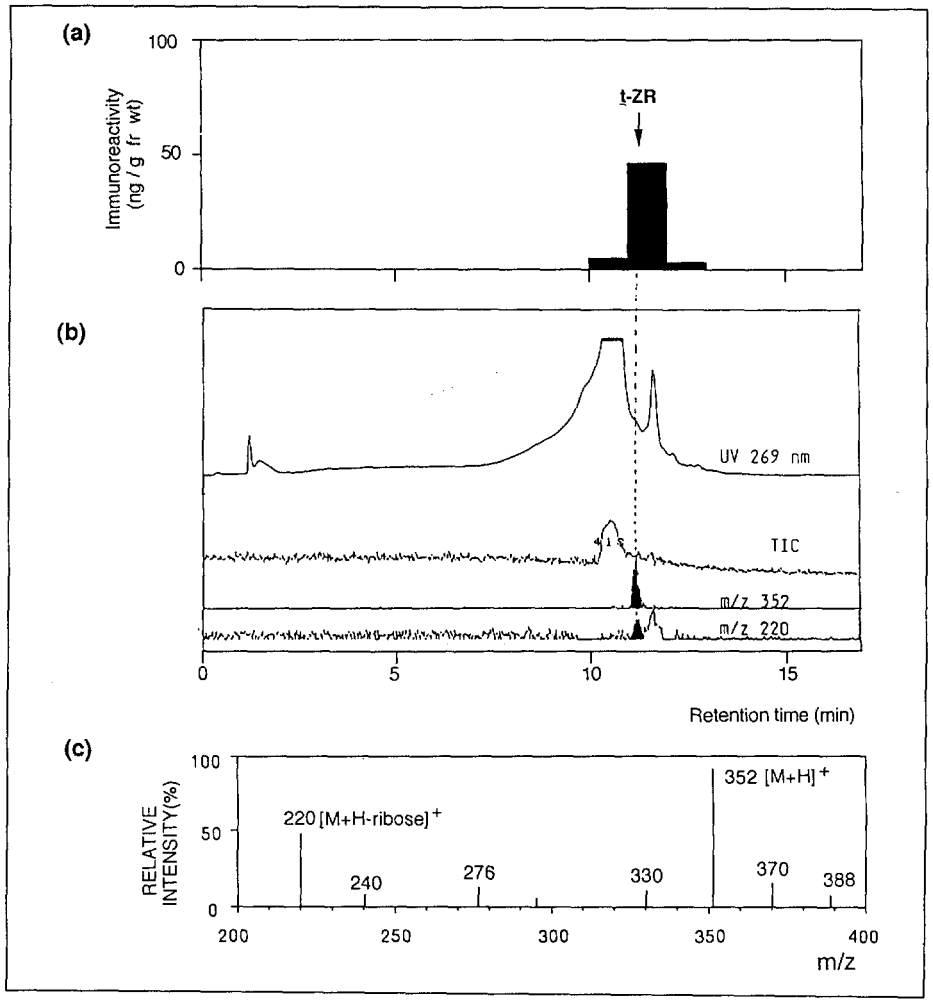

Fig. 5. LC/APCI-MS analysis of *t*-ZR in the teratoma of *Nicotiana tobacum*. **(a)** Histogram of *t*-RZ detected by ELISA after the final HPLC. HPLC conditions: column, Asahipak ODP; mobile phase, 0–5 min 20% aqueous acetonitrile isocratic, 5–30 min 20–50% aqueous acetonitrile linear gradient; flow rate, 1 ml/min. **(b)** The profiles of UV absorption and the mass chromatogram in the final HPLC. HPLC conditions as in **(a)**. **(c)** The full-scan mass spectrum determined at the t_R corresponding to that of *t*-ZR.

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