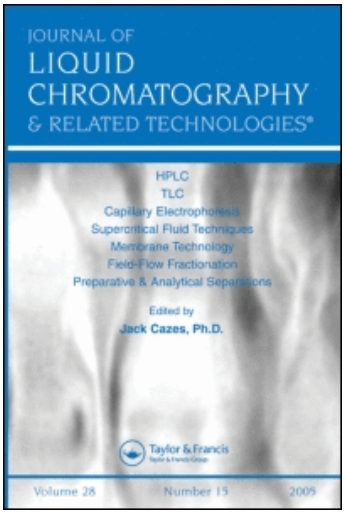

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High-Performance Liquid Chromatographic Analysis of Cytokinins Using Isatoic Anhydride as a Fluorescent Derivatizing Reagent

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CYTOKININS USING ISATOIC ANHYDRIDE AS A FLUORESCENT DERIVATIZING REAGENT

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ABSTRACT

A method for quantitative determination of cytokinins containing ribose such as N^6-isopentenyladenosine (i^6Ado) and zeatin riboside (zR) has been developed. This based on the preparation of fluorescent anthraniloyl derivatives of cytokinins by the reaction of isatoic an hydride with hydroxyl groups of the ribose, and then the resolution of fluorescent derivatives by high-performance liquid chromatography with a spectrofluoro monitor. The fluorescent anthraniloyl derivatives were separated more sharply and symmetrically from each other compared with the separation of the non-derivatized i^6Ado and zR. The detection limits for anthraniloyl-i^6Ado and anthraniloyl-zR were 1.1 and 1.2 pmol per 10 μl injection, respectively, and these values indicate that the fluorimetric analyses were approximately 70- to 150-times more sensitive than the UV-monitoring method. The simplicity, speed, sensitivity and selectivity make this method an attractive alternative to established cytokinin assay systems.

INTRODUCTION

Cytokinins are a group of plant hormones which promote cell division and differentiation (1, 2). To define their regulatory role more precisely, it is necessary to identify and quantify cytokinins in plant tissues during develop-

ment. From earlier works on analysing cytokinins, their occurrence and levels have been measured by bioassays. The most widely used is the tobacco callus assay which measures the ability to promote cell division. These bioassay procedures, however, have some disadvantages with respect to the time required for the assay, specificity and quantitation range. On the other hand, instrumental analyses such as gas chromatography and gas chromatography-mass spectrometry have been used to identify molecular species of cytokinins precisely and specifically (3 12). These procedures using analytical instruments, however, have also some disadvantages regarding the sensitivity and the requirement of expensive apparetuses. Staiger and Miller (13) have reported the reaction of isatoic anhydride (IA) with the nucleophilic reagents such as primary and secondary alcohols, phenols, thiophenols and mercaptanes, yielding anthraniloyl derivatives. Hiratsuka (14. 15) also has reported the syhthesis of fluorescent derivatives of adenine and guanine nucleotides by reaction with IA. These reports suggested to us that it should be possible to prepare the fluorescent anthraniloyl derivatives of cytokinins containing available hydroxyl groups of the ribose such as N^6-isopentenyladenosine (i^6Ado) and zeatin riboside (zR). We report here the synthesis of fluorescent anthraniloyl derivatives of cytokinins containing the ribose and the resolution of fluorescent derivatives of cytokinins precisely and sensitively by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Apparatus

A Japan Spectroscopic (JASCO) Model 800-MP-15 high-performance liquid chromatograph with a JASCO FP-210 spectrofluoro monitor was used. Chromatograms were recorded on a JASCO Model 805-GI graphic integrator, while fluorescence spectra were obtained on a JASCO FP-770 spectrofluorometer. Reversed phase octadecylsilane-bonded silica gel column, Finepak Sil C18T-5 (250 mm × 4.6 mm: JASCO) was used for the separation of anthraniloyl derivatives.

Chemicals

N^6-isopentenyladenosine (i^6Ado) and zeatin riboside (zR) were obtained from Sigma (St.Louis, MO, U.S.A.). Isatoic anhydride was purchased from

Molecular Probes Inc. (Junction city, OR, U.S.A.). All other chemicals used were of analytical grade from commercial sources. Standard solutions were freshly prepared by dissolving the carefully weighed i^6Ado or zR in 70% methanol to a concentration of 1 mg/ml. These standard solutions were diluted to the target concentrations in 70% methanol and stored at 4°C.

Chromatographic Conditions

Separations were performed with the linear gradient elution from 10mM K_2HPO_4, pH 8.2-acetonitrile (80:20, v/v) to 10mM K_2HPO_4, pH 8.2-acetonitrile (40/60, v/v) in 30 min at a flow-rate of 1.0 ml/min at 40°C. The column effluent was monitored fluorometrically at an excitation wavelength of 268 nm and at an emission wavelength of 425 nm.

Preparation of Anthraniloyl Derivatives

A 0.1M IA solution was prepared by dissolving a commercial IA in dimethylsulfoxide. Unless specified otherwise, the following procedure was used. The proper volume of biological sample or diluted standard solutions in a reaction tube was dried under a dryer, then to the dried sample, 20 μl of 0.1M IA and 0.98 ml of 10mM K_2HPO_4, pH 8.2-acetonitrile (80/20, v/v) were added. The reaction tube was stoppered and left for 20 hr at 28°C. A 5 10 μl aliquot was injected into the HPLC system.

RESULTS

Fluorescence Spectra of Anthraniloyl Derivatives

After the derivatization of dried standards containing 50 μg each of the i^6Ado and zR, 100 μl aliquot was injected into the HPLC system. Then, the column effluent corresponding to each anthraniloyl-i^6Ado, anthraniloyl-zR and the fluorescent side product was collected and analyzed, respectively. The fluorescence spectra are given in Figure 1. As can be seen, anthraniloyl-i^6Ado and anthraniloyl-zR exhibited similar fluorescence excitation and emission spectra patterns, however the side product had a different fluorescence excitation spectra pattern. The synthesis of Anthraniloyl-i^6Ado was proposed as in Scheme I. To eliminate the disturbance by the fluorescent side product in HPLC anal-

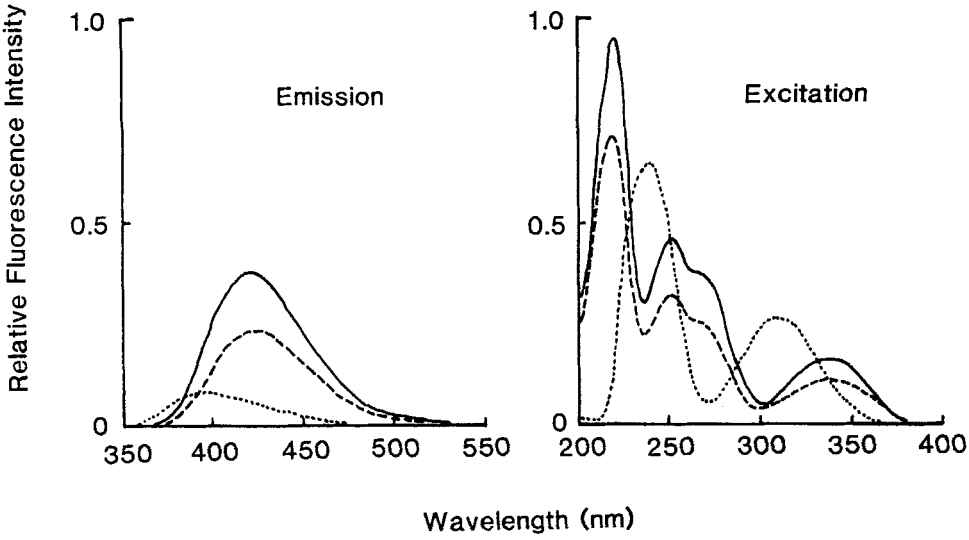


FIGURE 1 Fluorescence spectra of anthraniloyl-i^{6}Ado(———), anthraniloyl-zR (– – –) and the side product (- - - -). Emission was measured at an excitation wavelength of 268nm, while excitation was measured at an emission wavelength of 425nm.

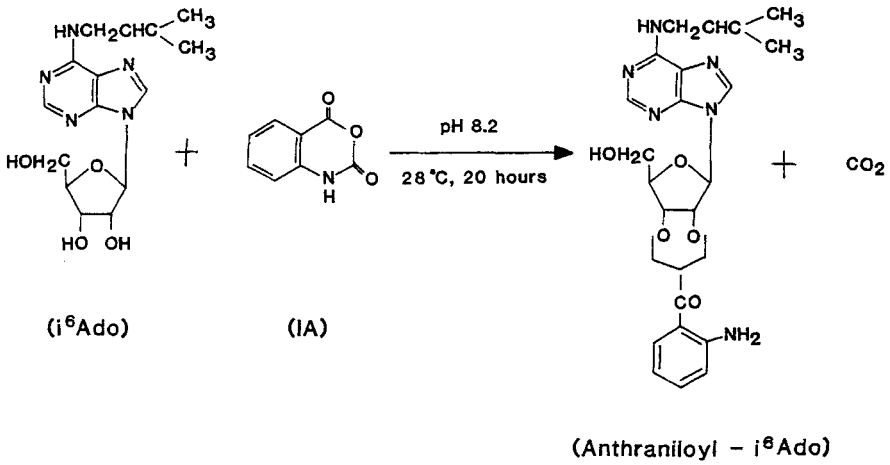
ysis, the fluorescence intensity was measured using excitation at 268 nm and emission at 425 nm for simultaneous analysis of the anthraniloyl derivatives of i^{6}Ado and zR.

Separation of Anthraniloyl Derivatives by HPLC

The separation of the anthraniloyl derivatives of i^{6}Ado and zR is shown in Figure 2A, while Figure 2B shows the separation of non-derivatized i^{6}Ado and zR as monitored by UV absorbance at 254 nm. The anthraniloyl derivatives were separated more sharply and symmetrically from each other compared with the separation of the non-derivatized i^{6}Ado and zR.

Assay Linearity and Detection Limit

The fluorescence intensities were linear over a range of detection limits up to 120 pmol per 10-μl injection. The detection limits for anthraniloyl-i^{6}Ado



Scheme I Synthesis of proposed anthraniloyl derivative of N^6-isopentenyladenosine

and anthraniloyl-zR were 1.1 and 1.2 pmol per 10-μl injection respectively, at a signal-to-noise ratio of about five. Conversely, the detection limits for non-derivatized i^{6}Ado and zR were 150 and 71 pmol per 10-μl injection respectively. This is taken to indicate that the fluorimetric analyses were approximately 70- to 150-times more sensitive than the UV-monitoring method.

Assay Precision

Relative standard deviation obtained in each of 6 measurements for 11.4 pmol / 10 μl of anthraniloyl-i^{6}Ado and 12.0 pmol / 10 μl of anthraniloyl-zR was 1.23% and 4.82%, respectively, and 1.81% and 3.08% for 114 pmol / 10 μl of anthraniloyl-i^{6}Ado and 120 pmol / 10 μl of anthraniloyl-zR, respectively.

Effect of pH on the Derivatization

To examine the effect of the pH in the reaction mixture on the reaction yield, standard samples containing 2 μg of i^{6}Ado and zR were derivatized using 0.2M Na_2HPO_4/NaH_2PO_4(pH 6–8) or 0.2M $Na_2CO_3/NaHCO_3$(pH 9–10) for pH adjustments. As shown in Figure 3, pH 9 in the reaction mixture was

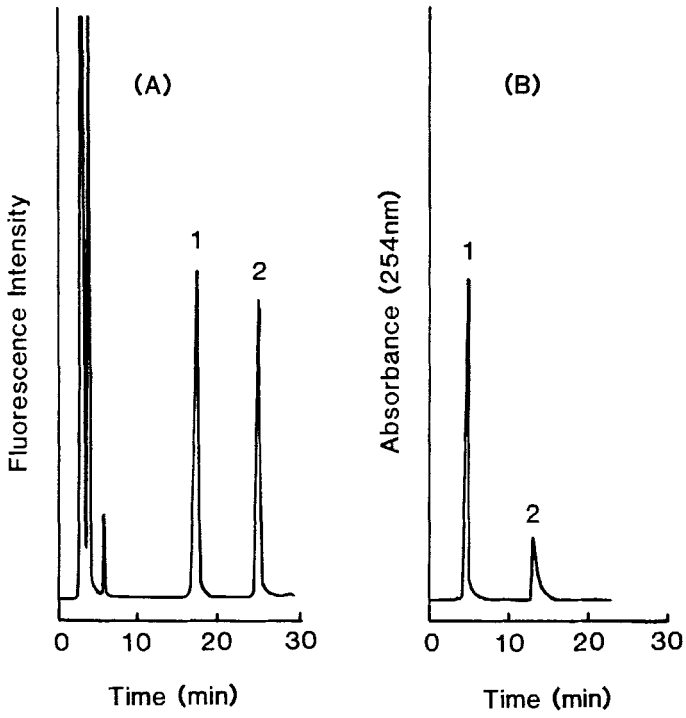


FIGURE 2 Chromatograms of anthraniloyl derivatives of i^6Ado and zR(40ng of each per 10 μl injection) (A) and of non-derivatized i^6Ado and zR(2μg of each per 10 μl injection) monitored by UV absorbance at 254nm (B). Peaks: (A) 1 = anthraniloyl-zR; 2 = anthraniloyl-i^6Ado; (B) 1 = zR; 2 = i^6Ado.

found to be most effective on the derivatization, however the fluorescence intensities decreased markedly above pH 9. Tris(hydroxymethyl)aminomethane or sodium borate as a constituent of the buffer inhibited the derivatization.

Effect of Reaction Temperature and Reaction Time on The Derivatization Yield

The derivatization with IA was carried out using the procedure described in the MATERIALS AND METHODS, except that the reaction temperature was varied from room temperature to 100°C. A mild reaction at 28°C

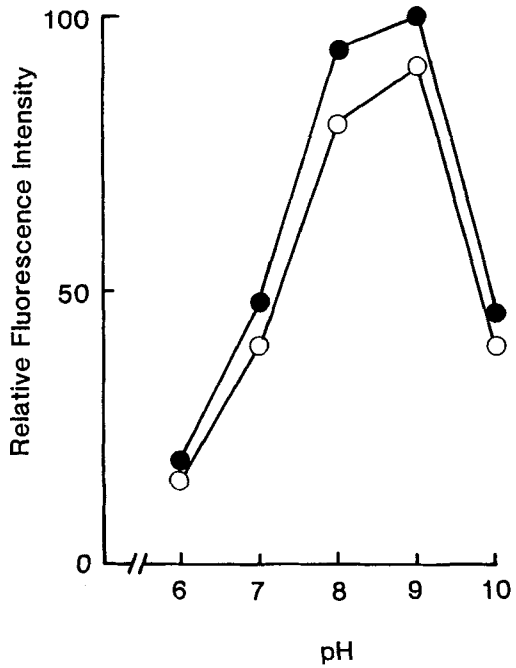


FIGURE 3 Effect of the pH in the reaction mixture on isatoic anhydride derivatization of i^6Ado and zR. ●, anthraniloyl-i^6Ado; ○, anthraniloyl-zR. Each point represents the mean of triplicate determinations of the fluorescence intensity of each peak of the anthraniloyl derivative separated on the Asahipak GS-320H column.

was found to be the most effective. The effect of the reaction time at 28°C was examined over a period of 48 hr. The results shown in Figure4 indicate that the fluorescence intensities of anthraniloyl derivatives increased with reaction time, then reached the plateau in 7 hr.

Effect of IA Concentration on Quantitative Analysis

The molar ratio of IA to total amount of i^6Ado and zR was varied from 1 to 350. The total amount of i^6Ado and zR present was always 11.7 nmol. A minimum molar ratio of 175 of IA to i^6Ado and zR was required for quantitative analysis.

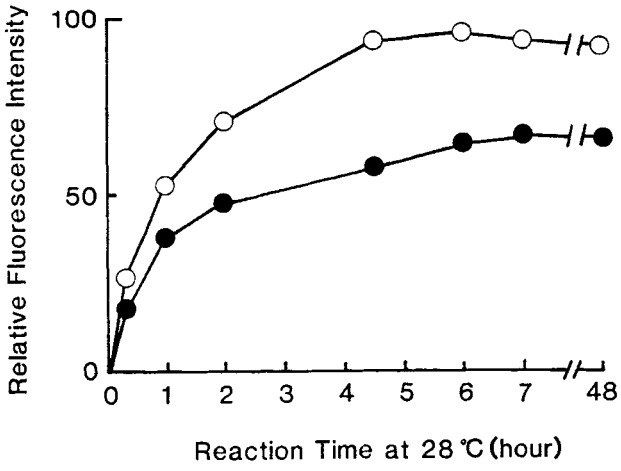


FIGURE 4 Effect of reaction time at 28°C on isatoic anhydride derivatization of i^6Ado and zR. Details as in Figure 3.

Stability of Anthraniloyl Derivatives

The stability of the anthraniloyl derivatives in the reaction mixture was examined. Each derivative was stable without any decomposition within 2 days. Anthraniloyl-i^6Ado underwent up to 13% decomposition within 2 weeks. During this time, the fluorescence intensity of anthraniloyl-zR decreased by 20%.

Application to the Biological Materials

The root exudate of sweet potato obtained from a cut end of root was offered to analyse i^6Ado and zR. The concentrations of i^6Ado and zR detected in the root exudate were 1.05×10^{-8} mol/l and 0.95×10^{-8} mol/l, respectively.

DISCUSSION

To realize the phenomena of growth, development and differentiation in plant, the microanalysis of plant hormones with high sensitivity and selectivity is required. Cytokinins are a group of plant hormones which promote cell divi-

sion and exert other growth regulatory functions. Although several bioassays based on in vitro growth of plant tissue have been used for the detection and attempt at quantitative estimation of cytokinins in biological materials, those bioassay procedures have some defects from the viewpoint of specificity, rapidity and quantitation range. Instrumental analyses such as gas chromatography, gas chromatography-mass spectrometry and HPLC by monitoring the ultraviolet absorbance have also some disadvantages with respect to the sensitivity and the requirement of expensive apparatus.

In the present study, we attempted to develop the procedure with which cytokinins are detected precisely, sensitively, specifically and rapidly. The essential features of this procedure are summarized below. [1] The ribosyl cytokinins such as i^6Ado and zR were transformed to the fluorescent anthraniloyl compounds by the action of IA as the fluorimetric labelling reagent. These derivatives were readily prepared under the mild conditions. [2] The fluorescent derivatives of i^6Ado and zR were distinctly separated from each other by HPLC and were distinguished from non-ribosyl cytokinins such as isopentenyladenine and zeatin which seemed to be freed from the IA derivatization. The detection limit of anthraniloyl-zR, 1.2 pmol per 10-μl injection, was almost same as that of anthraniloyl-i^6Ado, 1.1 pmol per 10-μl injection, which means that the molar ratio of IA attached to zR is equal to that to i^6Ado. This fact seems to show that the hydroxyl group in isopentenyl chain of zR is not derivatized with IA. In fact, the hydroxyl group in isopentenyl chain of zeatin failed to react with IA by the derivatization method used in this study. Staiger and Miller (13) has reported that the primary and secondary alcohols react with IA, however, the alcoholic hydroxyl group in isopentenyl chain of zR seems to fail to react with IA in this study. This discrepancy may be due to differences in the reaction conditions such as temperature, pH and IA concentration.

The incubation of the anthraniloyl derivative in 0.1M NaOH for 90 min at 25°C resulted in the complete disappearance of the original material, and the appearance of anthranilic acid together with the parent cytokinin, suggesting that the hydroxyl groups in ribosyl moiety were derivatized. Hiratsuka (14) has reported that the 2'-hydroxyl groups of AMP and GMP were more open to the reaction with IA than the 3'-hydroxyl groups. Cremo *et al.* (16) have recently reported that the (N-methylanthraniloyl) adenine 5'-diphosphate consists of a mixture of the 2'-isomer (35%) and the 3'-isomer (65%). The synthesis

of Anthraniloyl-i^{6}Ado is proposed as in Scheme I, however, the configuration of resultant isomers of anthraniloyl-i^{6}Ado and anthraniloyl-zR could not be specified in this study.

This study was primarily undertaken to develop an analytical method for measuring ribosyl cytokinins such as i^{6}Ado and zR in biological materials. The significance of structure-function relationships between the *cis* and the *trans* forms of zR has been discussed, therefore the precise quantitative analysis of each geometrical isomers, which remains to be settled, are required in the next study.

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