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ARTICLE

Liquid Chromatographic Determination of Endogenous Phytohormones in Vegetable Samples Based on Chemical Derivatization with 6-Oxy(acetylpiperazine) Fluorescein

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In phytohormone analysis, mass spectrometry (MS)-based methods are primary and powerful tools. However, complex sample preparation and high cost are problems for their application. As a complement for MS-based methods, a new fluorescent labeling reagent for carboxylic acids, 6-oxy(acetylpiperazine) fluorescein (APF), has been used for the determination of endogenous phytohormones, including indolebutyric acid, 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. The derivatization yield was maximized by optimizing derivatization conditions in detail, and the derivatives of three phytohormones could be separated completely in 15 min on a C18 column with fluorescence detection at $\lambda_{ex}/\lambda_{em}$ = 467/512 nm. The derivatization limits could reach 0.1 μM, and the detection limits (signal-to-noise ratio = 3) were 4.43−14.2 nM. The proposed method has been applied to the determination of the exogenous phytohormones in the crude extracts of vegetable samples without extra purification and enrichment with recoveries of 94.2−102.4%.

KEYWORDS: Phytohormones; 6-oxy(acetylpiperazine) fluorescein; derivatization; high-performance liquid chromatography

INTRODUCTION

To regulate the growth of plants artificially and get high yield in agriculture, many compounds that have structures or functional groups similar to the endogenous phytohormones are applied exogenously to plants as low-cost alternatives, such as indolebutyric acid (IBA), 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Usually, the concentrations used of these exogenous phytohormones as plant growth regulators are up to 1 or 10 μM (*1*), which are much higher than the levels of endogenous phytohormones in plants. To standardize the application of these hormones applied exogenously to crops, many countries have classified the most used plant growth regulators as pesticides and developed standards for the residue limits. For example, U.S. Environmental Protection Agency regulations state that 2,4-D residues in the apples, oranges, and pears should not exceed 5 mg/kg (*2*). The General Administration of Quality Supervision Inspection and Quarantine of the People's Republic of China has clearly defined the maximum residue limit of 2,4-D in leaf vegetable as 0.1 mg/kg (*3*). Because IBA, NAA, and 2,4-D are the most widely used auxins in agriculture (**Figure 1**), the exploitation of their sensitive, selective, and simultaneous determination is of great importance in planting management and food safety.

To date, various analytical techniques have been developed for the determination of exogenous phytohormones, such as gas chromatography−mass spectrometry (GC-MS) (*4*−*6*), liquid chromatography−mass spectrometry (LC-MS) (*7*−*10*), capillary electrophoresis with UV detection (CE-UV) (*11*, *12*), capillary electrophoresis with fluorescence detection (CE-FD) (*13*), high-performance liquid chromatography with UV detection (HPLC-UV) (*14*−*17*), high-performance liquid chromatography with chemiluminescence detection (HPLC-CL) (*18*), and enzyme-linked immunosorbent assay (ELISA) (*19*). Although these methods make great contributions to the analysis of phytohormones, some limitations are still found in the application of these methods. For example, when GC or GC-MS is employed, derivatization of phytohormones to more volatile methyl esters is required (*4*). The ELISA method exhibits cross-reactivity of structurally related compounds in the same sample (*19*). The LC-MS method is too expensive for large amounts of real samples (*10*). In addition, most of the above-mentioned techniques need complicated and intensive purification for phytohormones in plant samples.

Chemical derivatization is thought to be a feasible way to improve the detection sensitivity and selectivity of chromatographic methods including HPLC, CE, etc. An excellent derivatizing reagent used here can provide not only a sensitively detected chromophore or fluorophore but also a reactive moiety to react with a specific substituent selectively. Therefore, chemical derivatization coupled to HPLC or CE is advantageous in the achievement of both high sensitivity and high selectivity. For phytohormone analysis in plant samples, there are a few derivatizing reagents reported, such as 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) for 1-aminocyclopropane-1-carboxylic acid

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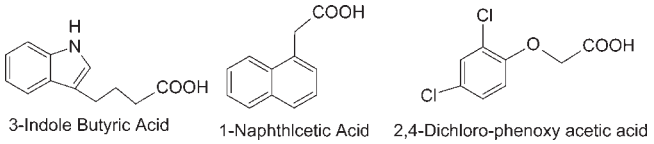

Figure 1. Molecular structures of indolebutyric acid (IBA), 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D).

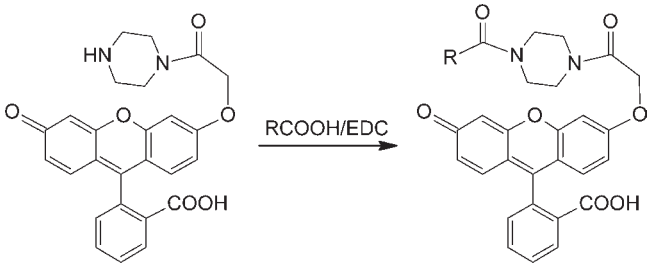

Figure 2. Derivatization reaction of APF and carboxylic compounds.

(ACC) (*20*), 8-aminopyrene-1,3,6-trisulfonate for abscisic acid (ABA) (*21*), and 9-anthryldiazomethane and 5-bromomethyl fluorescein for jasmonic acid (JA) (*22*). Although these methods have been used for the determination of endogenous phytohormones, the application of derivatizing reagents for the analysis of exogenous phytohormones was rarely reported. Considering the great importance and increasing application of exogenous phytohormones in fields, the development of new methods and derivatizing reagents with better analytical performance is still required.

In our previous work, 6-oxy(acetylpiperazine) fluorescein (APF) has been synthesized in our laboratory and used for the labeling of free fatty acids in human serum samples (*23*). Here, the feasibility of APF in the determination of exogenous phytohormones, such as IBA, NAA, and 2,4-D with HPLC−fluorescence detection has been well verified. Practical sample analysis with the proposed method has been assessed using vegetable samples, including cucumber, lettuce, and tomato.

EXPERIMENTAL PROCEDURES

Chemicals. IBA, NAA, 2,4-D, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich (St. Louis, MO). Na_2HPO_4 and citric acid were purchased from Shanghai Chemicals Co. (Shanghai, China). Stock solutions of exogenous phytohormones and EDC were prepared by dissolving them in anhydrous acetonitrile to a concentration of 1.0×10^{-4} M and 1.0×10^{-2} M, respectively. Citric acid−Na_2HPO_4 buffer was prepared by mixing 0.1 M citric acid solution and 0.1 M Na_2HPO_4 solution to the required pH. APF was synthesized in our laboratory (*23*), and a 1.0×10^{-3} M solution was prepared in anhydrous acetonitrile. Water used in the experiment was double distilled. All of the stock solutions except buffers were stored in a refrigerator prior to use.

Instrument. The Agilent 1100 series HPLC system (Agilent Technologies, Böblingen, Germany) was used in the experiments. It comprised the following modules: a high-pressure gradient quaternary pump, an online vacuum degasser, a 20 μL manual injection, and a fluorescence detection (FLD) system. The column used was a 250 mm × 4.6 mm i.d., 5 μm, Kromasil C18 (Eka Chemicals, Bohus, Sweden). A Delta-320 pH-meter (Mettler Toledo, Shanghai, China) was used for measuring the pH of solutions.

Derivatization Procedure. The reaction of APF with phytohormones is shown in **Figure 2**. Portions of mixed phytohormones, 80 μL of 1×10^{-2} M EDC and 18 μL of 1×10^{-3} M APF, were added to a vial. The whole solution was diluted to 200 μL with acetonitrile, capped, vortex-mixed, and then kept at 60 °C for 1 h in the dark. Before analysis, the resulting mixture was diluted to 1 mL with the mobile phase.

Chromatographic Method. Before separation work, the column was preequilibrated by the mobile phase for 30 min. A 20 μL aliquot of sample solution was injected into the C18 column. The solvent flow rate was 0.7 mL/min with working pressures around 130 bar, and the detection wavelength was set as $\lambda_{ex}/\lambda_{em}$ = 467/512 nm. All of the solvents were filtered with a 0.45 μm membrane filter before use.

Sample Preparation. Samples were treated according to the method of Liu et al. (*20*) with slight changes. Cucumber, lettuce, and tomato samples were purchased from a local market. Pieces of vegetable samples (0.5 g) were weighed, ground into fine powder in the presence of liquid nitrogen, and placed in a vial filled with 10 mL of cold acetonitrile. After high-speed blending, the mixture was maintained overnight at 4 °C and then centrifuged at 4 °C (4000*g* for 10 min). Filtered through a 0.45 μm membrane, the supernatant was collected. The sample solution of 200 μL was dried under vacuum and redissolved in 100 μL of anhydrous acetonitrile. The redissolved solution was derivatized via the procedure described under Derivatization Procedure.

The recoveries of exogenous phytohormone analysis were obtained by standard addition method, which is described as follows: an appropriate portion of each phytohormone was spiked after the vegetable samples were weighed, and the resulting mixture was treated according to the same procedure as for sample preparation.

RESULTS AND DISCUSSION

Optimization of Derivatization Conditions. Although the optimum derivatization conditions of APF with free fatty acids have been obtained in our previous work (*23*), to achieve the best efficiency of derivatization reaction of APF with IBA, NAA, and 2,4-D, several parameters affecting the derivatization reaction were studied around the optimum conditions of fatty acids, including the amount of labeling reagent, condensing reagent, reaction time, and temperature (**Figure 3**).

EDC is a widely used condensing reagent in the reaction of carboxylic compounds with amines and also adopted in our experiment. According to the mechanism of carbodiimide-mediated coupling reactions (*24*), the carboxylic group in APF can also react with EDC to produce some byproducts. It was found that APF in acetonitrile presented only one peak in the chromatogram; however, the addition of EDC brought about three additional peaks. Therefore, the amounts of APF and EDC should be carefully controlled in the derivatization with respect to high derivatization yields and low byproduct interference.

The influence of APF amount on the derivatization was investigated within the concentration range of $(6-9.5) \times 10^{-5}$ M, as shown in **Figure 3A**. When the APF concentration is 9×10^{-5} M, the peak areas of the derivatives reach a maximum, and 9×10^{-5} M was chosen as the optimal concentration of APF. The effect of various EDC concentrations on the peak areas of phytohormone derivatives was also studied from 3.5×10^{-3} to 6×10^{-3} M (**Figure 3B**). It was found that the largest peak areas appeared at an EDC concentration of 4×10^{-3} M. Therefore, the optimum EDC concentration was determined as 4×10^{-3} M from the results obtained.

High temperatures could accelerate the derivatization reaction, and thus the temperature influence was investigated. **Figure 3C** shows the change of the peak areas of the derivatives varied with the reaction temperature. For the higher yields of all the derivatives, 60 °C was chosen as the best reaction temperature. The effect of the reaction time from 30 to 70 min at 60 °C has also been examined, which is shown in **Figure 3D**. In the tested range, the peak areas of all three phytohormone derivatives are nearly constant throughout, and the derivatization was carried out for 40 min.

Separation of APF Derivatives. The effect of the methanol concentration and pH in the mobile phase on the capacity factors (K) of the derivatives is shown in **Figure 4**.

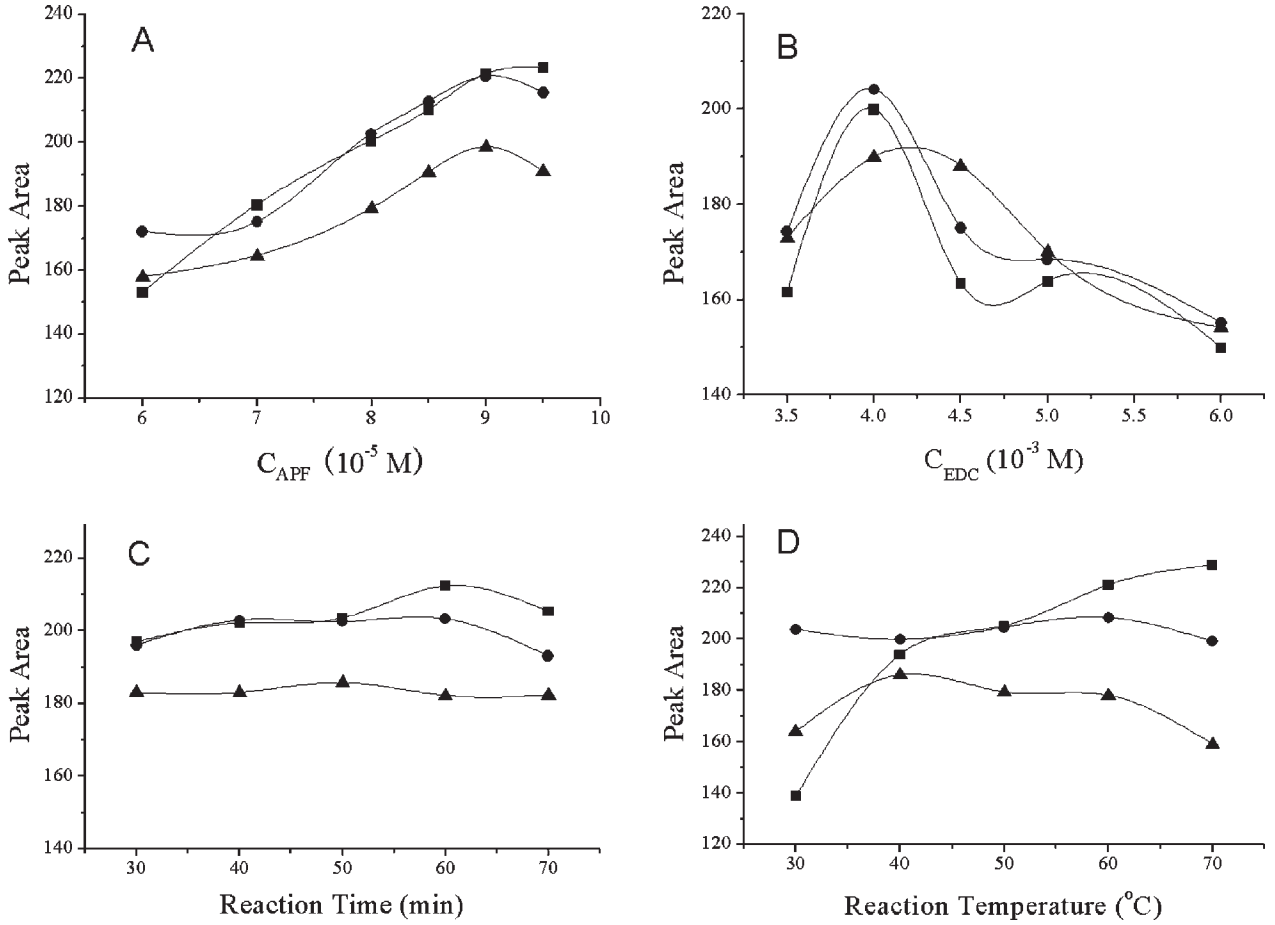

Figure 3. Effects of (**A**) APF concentration, (**B**) EDC concentration, (**C**) reaction time, and (**D**) reaction temperature on the peak areas of APF derivatives: (■) IBA; (●) NAA; (▲) 2,4-D. Standard phytohormone concentration = 1 μM.

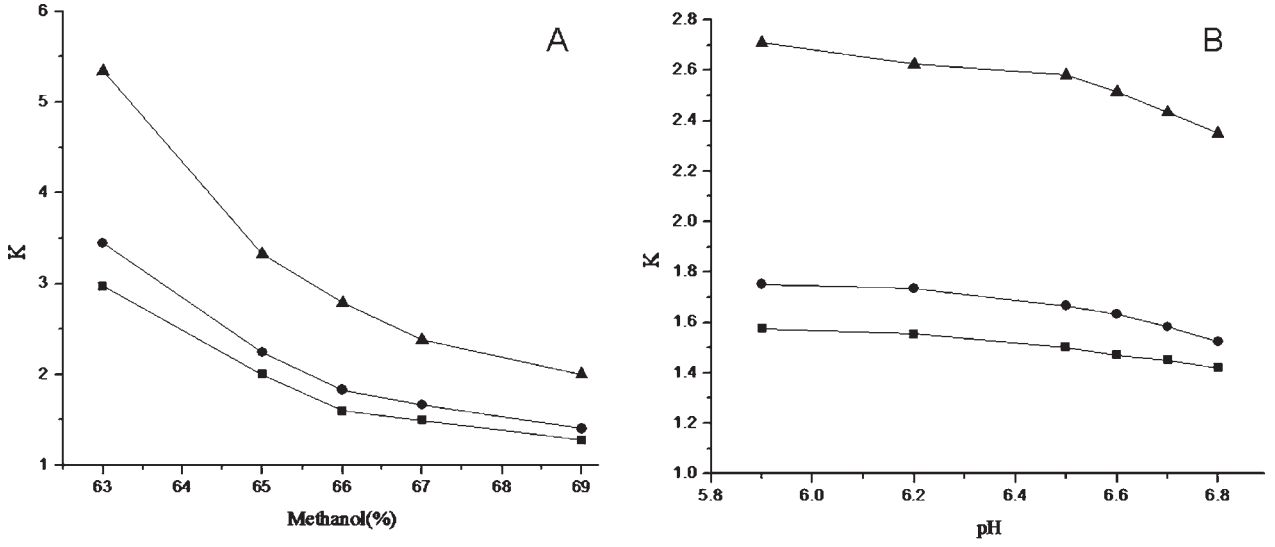

Figure 4. Effect of (**A**) different methanol concentration containing 10 mM pH 6.6 citric acid-Na_2HPO_4 buffer, and (**B**) pH with 10 mM citric acid-Na_2HPO_4 buffer in methanol-water on *K* (capacity factor): (■) IBA; (●) NAA; (▲) 2, 4-D. Phytohormones concentration: 1 μM.

The solubility of the three phytohormone derivatives is relatively good due to the labeling APF. Correspondingly, the organic solvent in the mobile phase can be used in low content. Using a methanol/water system as the basic eluate, the influence of the methanol concentration has been studied. When the methanol content is above 68% (v/v), the peaks of IBA and NAA derivatives overlap. If methanol content is lower than 63%, the separation time is prolonged. When methanol content is in the range of 63−68%, all peaks could be separated on the baseline. Thus, 66% was employed as the optimum methanol content.

Because the fluorescence intensities of the phytohormone derivatives are greatly dependent on the medium pH owing to

the existence of fluorescein fluorophore, according to the chemistry of fluorescein, the pH value of the mobile phase may influence the derivative fluorescence and thus the detection sensitivity (*23*). Therefore, citric acid−Na_2HPO_4 buffer was used to adjust the pH value of the mobile phase from 5.9 to 6.8. It was found that the peaks of IBA and APF overlapped in part at pH 6.7. As a result, pH 6.6 was selected in the following experiment.

The effect of citric acid−Na_2HPO_4 buffer content in the mobile phase on the separation has also been examined. When the content is lower than 6%, the reproducibility of retention times is poor. When the content is higher than 11%, the salt could easily precipitate and the separation is time-consuming. Therefore, 10% citric acid−Na_2HPO_4 buffer was adopted in this experiment.

Under the optimum conditions, the typical chromatogram of the derivatives of APF with three exogenous phytohormones is given in **Figure 5**. The separation was complete within 15 min.

Analytical Calibration. A test mixture of three exogenous phytohormones with different concentrations (1×10^{-7}−1×10^{-5} M) was analyzed under the optimized derivatization procedure and separation conditions for the determination of the linearity. The detection limits were performed as the concentration of analyte providing a signal equal to 3 times the noise. The precision study was performed by repeating six sequential runs within-day and between-days using 1 μM standard phytohormone solution, which is expressed with relative standard deviations (RSDs). The within-day RSDs for APF phytohormone derivatives were found to be less than 2.06% and those between-days were less than 2.20% for peak areas. The calibration ranges were 0.05−10 μM for 2,4-D and 0.1−10 μM for IBA and NAA. The detection limits for IBA, NAA, and 2,4-D were 14.8, 7.24, and 4.43 nM, respectively, which were lower than or equivalent to those of phytohormones obtained by other methods (*9*−*18*).

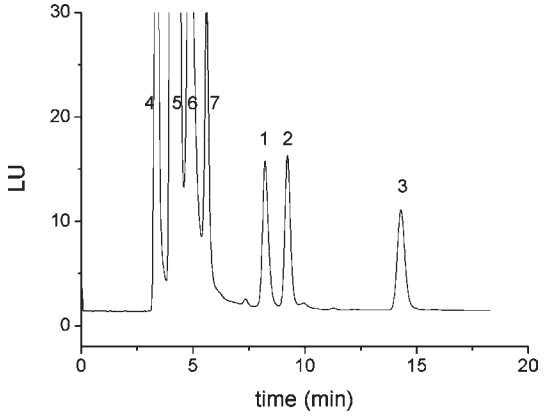

Figure 5. Typical chromatogram of APF and APF-phytohormone derivatives. Standard phytohormone concentration = 1 μM. Peaks: (1) IBA; (2) NAA; (3) 2,4-D; (5) APF; (4, 6, 7) byproducts of APF-EDC.

Interference. Under the chosen derivatization conditions, APF can also react with amino acids, carboxylic acids, and the endogenous phytohormones, which may interfere with the determination of exogenous phytohormones. Therefore, the chromatographic behaviors of C1−C18 fatty acids and common amino acids have been investigated systematically. Under the optimum separation conditions, the peaks of labeled carboxylic acid are eluted far behind the peak of 2,4-D derivative, which matches the fact that the APF−carboxylic acid derivatives are separated with the mobile phase containing 88% (v/v) methanol on a C18 column (*23*). For amino acids, there is no difference of chromatographic peaks between the samples with and without the addition of saturated solution of 16 common amino acids in crude extracts of vegetables under the chosen conditions, because amino acids can hardly dissolve in acetonitrile or APF could not react with amino acids in our chosen condition.

The interference of endogenous phytohormones was evaluated by spiking 1×10^{-5} M abscisic acid (ABA), indole-3-acetic acid (IAA), and gibberellic acid (GA_3) to the solution of the exogenous phytohormone mixture (5×10^{-6} M each). Using the mobile phase containing 63% methanol, the peak of GA_3 overlapped with the reagent peaks, and the peaks of IAA and ABA are both eluted at 6.1 min, whereas the retention time of the APF−IBA derivative is 10.4 min. Under the optimized separation conditions, the derivatives of ABA, IAA, and GA_3 were all coeluted with the reagent peaks.

Sample Analysis. The proposed method has been applied to the determination of exogenous phytohormones in vegetables. Because the condensation of APF with the exogenous phytohormones of interest is a reaction that releases water molecules, water in the supernatant of the sample extracts will influence the derivatization reaction and reduce the yield, which affects the accuracy of sample analysis. Accordingly, the supernatant of the sample extracts should be dried under vacuum and redissolved in anhydrous acetonitrile.

The chromatograms of the samples unspiked and spiked with the standard solutions are shown in **Figure 6**. The recoveries range from 94.2 to 102.4%, and the RSDs vary from 1.05 to 3.55%. The three exogenous phytohormones of interest have not been found in the vegetable samples tested, probably because these three exogenous phytohormones have not been applied to the examined samples.

In summary, an HPLC method has been developed for the simultaneous determination of exogenous phytohormones with a carboxyl group using 6-oxy(acetylpiperazine) fluorescein (APF)

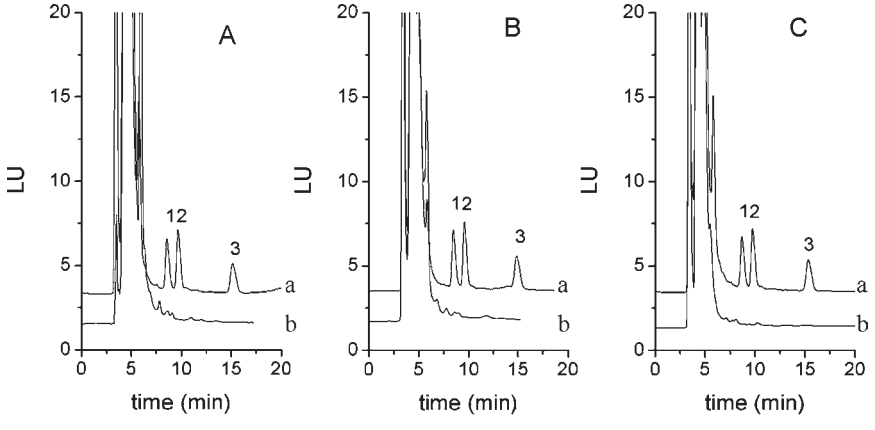

Figure 6. Chromatograms obtained from (**A**) cucumber, (**B**) lettuce, and (**C**) tomato samples (n = 6): (b) vegetable samples and (a) the same samples spiked with standard solutions. Chromatographic conditions are the same as in **Figure 3**. Peaks: (1) IBA; (2) NAA; (3) 2,4-D.

as a precolumn fluorescent derivatizing reagent. The application of chemical derivatization using this method does not require further purification. Under the optimum derivatization and separation conditions of our method, the detection limit of 2,4-D can reach 4.43 nM, which is equivalent to or better than those of phytohormones obtained by other methods. The proposed method has good selectivity and sensitivity in the determination of phytohormones, and the analytes are easily prepared and derivatized as well. Our work shows that the APF-based HPLC method should have powerful potential in the analysis of endogenous phytohormones with a carboxyl group in many complex samples. Further investigation of APF-based capillary electrophoresis with laser-induced fluorescence detection for endogenous phytohormones continues.

Supporting Information Available: Tables for analytical calibration and analytical results of real samples in detail. This material is available free of charge via the Internet at http://pubs.acs.org.

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