DOI:10.1021/jf1000138

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Selective Differentiation of Indoleacetic Acid and Indolebutyric Acid Using Colorimetric Recognition after Ehrlich Reaction

Jun-Min Guo, You-Ying Xin, and Xue-Bo Yin*

Research Center for Analytical Sciences, College of Chemistry, Nankai University, Tianjin 300071, China

A simple colorimetric method for the differentiation of indoleacetic acid (IAA) and indolebutyric acid (IBA) in plant samples is described. The color change is based upon the reaction between the auxins and *p*-(dimethylamino)benzaldehyde (PDAB, Ehrlich reagent) following the electrophilic substitution reaction mechanism at the indole ring. Using their different response to reaction temperature and time, the selective determination of IBA in the presence of IAA is achieved by controlling the incubation time of 40 min at 25 °C. The total absorbance of IAA and IBA is determined after they react to PDAB for 150 min at 70 °C. The concentration of IAA can then be calculated using the difference between their total absorbance and the calculated absorbance of IBA. The detection limits (3σ) of IAA and IBA were 0.10 μM and 0.28 μM, respectively. The precisions for five replicate measurements of 10 μM IAA and IBA were less than 5% (RSD). The recovery from mung bean sprout samples varied from 87.5% to 108% for the two auxins. Moreover, the Ehrlich reaction conditions are compatible with the methanol−hydrochloric acid extraction procedure. All of the above results indicate that this protocol provides a rapid, simple, convenient and practical method for detection and differentiation of IAA and IBA. From the color changes of IAA and IBA after Ehrlich reaction, the identification of auxin at the μM level can be achieved even with the naked eye. The method was successfully used to investigate the auxin changes of mung bean sprout during the growth procedure.

KEYWORDS: Colorimetric method; Ehrlich's reaction; indoleacetic acid; indolebutyric acid

INTRODUCTION

Phytohormones, as a kind of trace compounds in plant tissues, regulate the growth and development of the plants at their low concentrations (*1*). A group of phytohormones termed auxins are regarded to regulate the division, elongation and differentiation of cells (*1*, *2*). Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are two kinds of important auxins, and their determination attracts much interest because of their importance in the plant development. Some techniques with high sensitivity are successfully developed for the quantification of the two auxins.

Enzyme immunoassay, with excellent specificity to the target analytes, was used as a selective method for estimating auxin levels (*3*, *4*). However, enzyme immunoassay suffers from some drawbacks, such as the cross-reactivity between antibodies and the interfering substances and the need of the artificial synthesis of auxin antibody (*4*). The flow injection fluorimetric method for the determination of IAA shows high sensitivity (*5*). High resolution separation techniques, such as capillary electrophoresis (CE) (*6*−*8*) and high performance liquid chromatography (HPLC) (*3*, *9*−*13*), in combination of highly sensitive detection techniques (including mass spectrometry, fluorescence and chemiluminescence), are still the mainstream methods for auxin determination.

As discussed above, most of the previous methods for auxin determination need expensive instrumentation and complex sample pretreatment. In recent year, the development of colorimetric methods has drawn much attention due to the decrease in the experimental cost and the simplification of the sensing process (*14*, *15*). Those methods even only utilize the naked eye instead of complex instruments (*14*, *15*). A good colorimetric method should meet the rapid, simple and specific criteria (*16*). It is well-known that *p*-(dimethylamino)benzaldehyde (PDAB), named as Ehrlich reagent, can be used to indicate the compounds containing an indole ring under wild reaction conditions and Lambert−Beer's law was obeyed over a wide analyte concentration range (*16*−*18*). However, no method for determination of IAA and IBA based on the Ehrlich reaction was found, to the best of our knowledge. Herein, an attempt was made to develop a colorimetric recognition method for differentiation of IAA and IBA using the Ehrlich reaction. To achieve the goal, IAA, IBA or their mixture was reacted with PDAB under acidic conditions. Using their different response to reaction temperature, the selective determination of IBA in the presence of IAA was achieved by incubation of their mixture at 25 °C for 40 min. Their total absorbance was determined after their mixture reacted to PDAB at 70 °C for 150 min. The concentration of IAA can then be calculated using the difference between their total absorbance and the calculated absorbance of IBA. The results are reported from the evaluation of solvent composition, reaction time, temperature and PDAB concentration. The ultimate resulting methods estimate the auxin change in mung bean sprout during its growth. The accuracy of the proposed method was

*Corresponding author. E-mail: xbyin@nankai.edu.cn. Fax: 86-22-23502458.

 Published on Web 05/13/2010

illustrated by the recovery test for the real samples and the comparison of the results obtained by the present method and the previous CE-electrochemiluminescent method (*19*).

MATERIALS AND METHODS

Apparatus and Reagents. The auxin standard solutions were prepared by diluting 1.0 mg mL^{-1} methanol solutions of indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (both from Dingguo Biotechnology Co., Beijing, China). A stock methanol solution containing 3.0 mg mL^{-1} *p*-(dimethylamino)benzaldehyde (PDAB, Ehrlich reagent, Kermel Chemicals Co., Tianjin, China) was prepared. Sodium dodecyl sulfonate (SDS), hydrochloric acid, methanol and other chemicals used in this study were of analytical grade and obtained from Tianjin Chemicals Co., Tianjin, China. Doubly distilled water was used all through this work. All spectral measurements were performed on a Shimadzu UV-2450 UV−visible spectrophotometer (Shimadzu Corporation, assembled in China) in the wavelength range of 700−200 nm with 1 cm path length cell.

Procedures for Determination of IAA and IBA. The auxin standard solutions were prepared by stepwise dilution of the stock standard solution with methanol. An aliquot of 0.5 mL of IAA and IBA standard solution was transferred into a centrifuge tube, and then 1 mL of 3.0 mg mL^{-1} Ehrlich reagent solution and 0.45 mL of 36% hydrochloric acid were added into the tube. After being shaken to achieve mixture homogeneity and reacted at 25 °C for 40 min, the mixtures were measured using a 1 cm cell at 583 nm for quantification of IBA. Subsequently, the reaction mixtures were heated to 70 °C using a water bath for 2.5 h, and the absorbance at 583 nm for the mixtures was determined. A reagent blank, prepared in the same manner as the reaction mixtures except for the IAA and IBA solution being replaced with an equal volume of methanol, was used as the reference solution. Before each determination, SDS was added into the determination solution to maintain the concentration of 1% for the improvement of the detection sensitivity.

Sample Preparation and Pretreatment. The proposed method was used to evaluate the auxin changes along with the development procedure of bean sprout. Briefly, mung beans were soaked in tap water for 12 h and then germinated in a tray with a plastic membrane to give a suitable temperature and humidity. The water was refreshed periodically throughout the growth period. Every day from the first day to the sixth day, the bean sprout samples were collected and the content of auxins was determined as followed. The samples were washed with tap water and doubly distilled water (DDW) successively. Then they were exposed to air for 1 h at room temperature to evaporate the adsorbed water. After being flash frozen in liquid nitrogen, the samples were skived in a bowl with agate.

Colorimetric Determination of IAA and IBA in Real Samples. About 4.000 g of the sample was combined with 15 mL of methanol containing 1.5 mL of 1 M HCl, and maintained overnight at 4 °C in darkness to extract the auxins (*3*, *8*). Acidic methanol−water was used as extractant because it is also compatible with that used in the Ehrlich reaction. After being centrifuged for 15 min at 7000 rpm, the supernatant was collected and condensed to 3 mL by blowing with N_2 gas (*20*, *21*). One milliliter of the acidic methanol−water solution containing the extracted auxins was added into PDAB solution for Ehrlich reaction as described in Procedures for Determination of IAA and IBA. The recovery was tested after the auxins were added into the sprout samples. In addition, another 1 mL of the above solution prepared in the same manner as the reaction mixtures except for the PDAB solution being replaced with an equal volume of methanol was used as the reference solution.

RESULTS AND DISCUSSION

Spectrum Change of the Two Auxins before and after Reacting to PDAB. Figure 1 shows the spectrum changes before and after IAA and IBA react to PDAB. The absorbance peaks of IAA, IBA and PDAB exist mainly in the ultraviolet region, and they have no absorbance in the visible region. Therefore, their solutions are transparent and colorless. However, once two auxins react to PDAB and form their PDAB derivatives, some new absorbance peaks appear, such as those at 296 nm, 583 and 628.4 nm. Although those peaks were different for IAA and IBA in intensity, the peak positions were identical for the two auxin derivatives, showing the same sources of the absorbance peaks. The obvious absorption occurring at the visible region makes colorimetric recognition of IAA and IBA possible. Although the absorbance at 296 nm is much stronger, the absorption at 583 and 628.4 nm can be used to eliminate the background influence from the auxins and PDAB (as shown in **Figure 1**). To simplify the experimental procedure, 583 nm was selected as the detection wavelength for the determination of IAA and IBA. The apparent molar extinction coefficients are 5214 and 14597 mol^{-1} L cm^{-1} at 583 nm IAA- and IBA-PDAB derivatives, respectively.

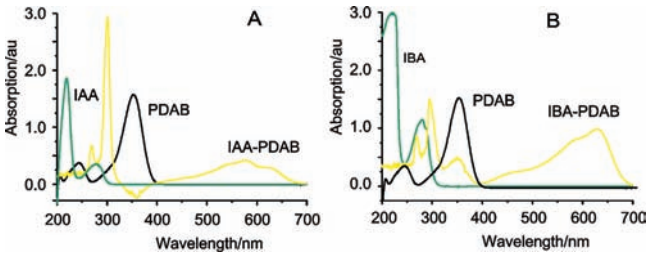

Figure 1. Spectrogram change of IAA (**A**) and IBA (**B**) before and after reacting to PDAB. The concentrations of different species in (**A**) are 1.0×10^{-4} M, 5.0×10^{-5} M, and 5.0×10^{-5} M for IAA−PDAB derivative, IAA, and PDAB and (**B**) 1.0×10^{-4} M, 1.0×10^{-4} M, and 5.0×10^{-5} M for IBA−PDAB derivative, IBA, and PDAB. The conditions of Ehrlich reaction for preparation of IAA−PDAB and IBA−PDAB derivatives: 1.35 M HCl and 3 mg mL^{-1} PDAB.

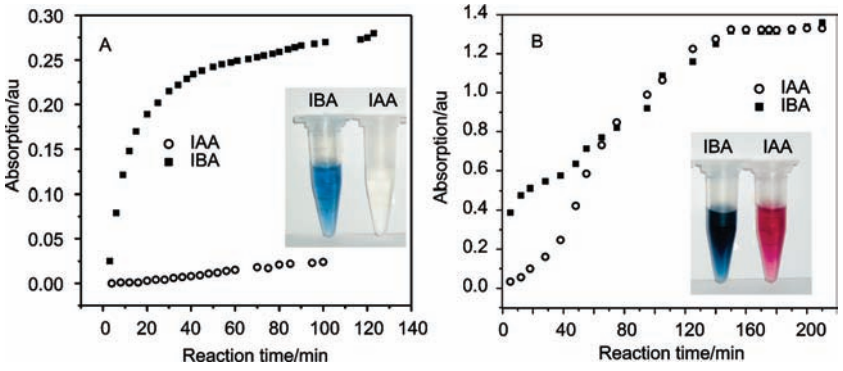

Figure 2. Influence of reaction time on the absorbance (at 583 nm) of IAA and IBA after Ehrlich reaction at 25 °C (**A**) and 70 °C (**B**). Inset in (**A**): the photos of IAA (2.0×10^{-4} M) and IBA (2.0×10^{-4} M) after reaction for 40 min at 25 °C and (**B**) the photos of IAA (2.0×10^{-4} M) and IBA (1.0×10^{-4} M) after reaction for 150 min at 70 °C. The conditions for Ehrlich reaction are as shown in **Figure 1**.

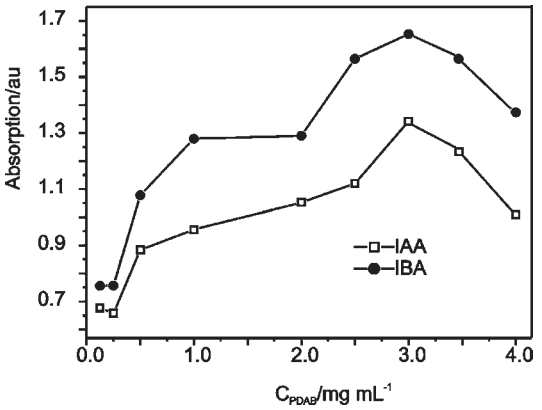

Figure 3. Influence of PDAB concentration on the signal (at 583 nm) for 2.5×10^{-4} M IAA and 1.2×10^{-4} M IBA after Ehrlich reaction at the incubation temperature of 70 °C for 150 min. The other conditions for Ehrlich reaction are as shown in **Figure 1**.

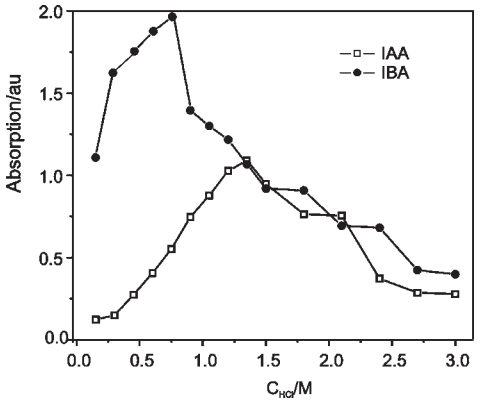

Figure 4. Influence of hydrochloric acid concentration on the signal (at 583 nm) of 2.0×10^{-4} M IAA and 1.0×10^{-4} M IBA after Ehrlich reaction at the incubation temperature of 70 °C for 150 min. The other conditions for Ehrlich reaction are as shown in **Figure 1**.

Selective Determination of IBA in the Presence of IAA. To assess the potential to differentiate IAA and IBA, it is important to investigate the factors influencing the formation of the two auxin−PDAB derivatives. The previous work (*18*) validated the dependence of the detection signal on the reaction temperature, whereas higher temperatures provide faster color reaction. The reaction concerning the auxins and PDAB was found to have different response to the reaction time at different temperature. **Figure 2** shows the influence of the reaction time on the signal intensity (at 583 nm) of the PDAB derivatives of IBA and IAA at different incubation temperatures (25 and 70 °C). IBA has a faster reaction to PDAB than IAA generally. However, IAA does not react to PDAB almost at 25 °C within 60 min, such that no IAA can be detected at 583 nm under this condition. In contrast, the signal of IBA−PDAB increases quickly from 0 to 40 min and remained nearly constant up to 60 min at 25 °C. However, the incubation temperature of 70 °C makes the absorption signals of the two PDAB derivatives increase sharply to the maximum as the incubation time increased from 0 to 150 min and then remained constant until 200 min.

The previous study indicated that PDAB can react to indole at the unsubstituted α-position to form a colored condensation production (*22*). However, because of the excess PDAB, the colored products can react with PDAB to form a colorless byproduct with increased incubation time and hence the reaction time should be short (*22*). Therefore, selective determination of IBA in the presence of IAA is proposed by controlling the incubation time of 40 min at 25 °C. The total absorbance of IAA and IBA is obtained after they react to PDAB for 150 min at 70 °C. The concentration of IAA is then obtained using the difference between their total absorbance and the calculated absorbance of IBA.

Effects of PDAB Concentration, Sample Acidity, and Surfactants. Ehrlich reaction concerning IAA or IBA and PDAB involves an electrophilic aromatic substitution process, where the positively charged carbonyl group in PDAB reacts with the electron-rich ring of indole derivatives to form the intermediate. The intermediate is converted into a colored cation stabilized by electron delocalization within the π system (*16*, *23*). Moreover, the previous studies indicated that Ehrlich reaction occurs under an acidic condition because it requires the protonation of nitrogen in the ring and then the delocalization of the charges on the indole ring (*16*, *22*−*24*). The acid concentration governs the rate of color reaction. At low acid concentrations, the colored products form slowly. Although the products form rapidly at high acid concentrations, the relatively colorless diprotonated salt forms easily (*23*). Therefore, the concentration of PDAB and sample acidity should be investigated for the optimal reaction conditions.

The effect of PDAB concentration on the determination of two auxins was investigated with the incubation time of 150 min at 70 °C, as shown in **Figure 3**. The absorbance of two auxin−PDAB derivatives increases sharply from 0.2 to 3.0 mg mL^{-1} PDAB and then decreases. The PDAB concentration should be enough to ensure a complete electrophilic substitution reaction, but the high concentration of PDAB would lead to a decrease in the signal and poor precision because of the effect of ionic strength and reagent blank (*18*). Therefore, a PDAB concentration of 3.0 mg mL^{-1} was employed throughout this work.

Various acidic systems, such as acetic acid/HCl (*22*, *23*), perchloric acid/HCl (*16*, *23*) or acetic acid/perchloric acid/HCl (*23*), have been used to provide the acidic media for Ehrlich reaction. The use of acetic acid in place of water slowed the rate of Ehrlich reaction but increased the optical intensity of the product (*23*). The intensity of the color was somewhat greater when perchloric acid was used (*23*). HCl medium can also provide a high efficiency (*24*), and therefore, HCl was solely used to provide the acidic medium being compatible with the sample pretreatment in this work. The effect of HCl concentration on the absorbance (at 583 nm) of IAA and IBA at 70 °C was studied varying from 0.15 to 3.0 mol L^{-1} and is shown in **Figure 4**. The absorption intensity of IAA and IBA was increased with increasing HCl concentration at the first and then decreased. The value of IBA showed maximum intensity at 0.75 mol L^{-1} and then decreased, while the value of IAA gets to maximum at 1.35 mol L^{-1}. As a compromise between reaction rate and color intensity, 0.75 mol L^{-1} HCl was selected for the reaction to detect IBA at 25 °C and 1.35 mol L^{-1} HCl was selected at 70 °C to detect total absorbance of IAA and IBA and therefore used to obtain the content of IAA eventually.

The addition of surfactant was considered to enhance the absorbance of analytes. The effect of Triton X-114 and SDS was investigated. We found the addition of Triton X-114 had no effect on the absorbance at 583 nm. Different from Triton X-114, the signals of two auxins increased with the increasing content of SDS as shown in **Figure 5**. However, the content of SDS exceeded 1% resulting in serious effervescence, leading to a poor reproducibility. Therefore, the optimal concentration of SDS was chosen as 1% in all the experiments. The 1.5- and 1.3-fold improvements on detection signal are achieved for IAA and IBA keeping the SDS concentration of 1% simply in the determination solution after Ehrlich reaction.

Interference in Ehrlich Colorimetric Reaction for the Determination of IAA and IBA. There are two types of potential interferences in the present colorimetric recognition of IAA and IBA. One is the interference from the coexisting auxins in the plant tissues, and the other is that from other indole derivatives. Because a water bath at 70 °C is needed for the Ehrlich reaction, the stability of IAA and IBA at 70 °C was investigated via the spectrogram change of IAA and IBA before and after heating for 2.5 h. As shown in Figure S1 in the Supporting Information, the consistency between the spectrograms of the auxins before and after being heating indicated that they are stable during the period of Ehrlich's reaction. We also investigated the mutual interference between IAA and IBA. As shown in **Table 1**, the same slopes in the calibration function of IAA (or IBA) in presence of IBA (or IAA) demonstrated that IAA and IBA in their mixture have same reaction response to that of the single IAA or IBA possibly because of the excess of Ehrlich's reagent, PDAB.

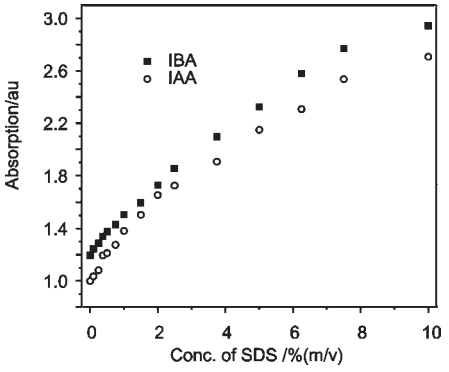

Figure 5. Effect of surfactant concentration on the signal (at 583 nm) of 2.0×10^{-4} M IAA and 1.0×10^{-4} M IBA after Ehrlich reaction at the incubation temperature of 70 °C for 150 min. The conditions for Ehrlich reaction are as shown in **Figure 1**.

Two auxins, 1-naphthylacetic acid (NAA) and phenylacetic acid (PAA) as the potential interferents, were used to investigate the specificity of the Ehrlich reaction for IAA and IBA. **Figure 6** shows the changes of the absorbance after IAA (at 1.0×10^{-5} M level) and IBA (at 2.5×10^{-5} M level) reacting to PDAB in presence of 2.5×10^{-3} M NAA or PAA, and the results are shown in **Table 2**. The effect is expressed as the recovery in the presence of the two interfering species relative to the interference-free response. As shown in **Table 2**, 250-fold and 100-fold of the interfering species had no influence on the signal intensity of IAA and IBA with the recovery of 97.3−105%. Moreover, the presence of the interfering species has no influence on the reproducibility for the determination of the two auxins, and the RSDs are less than 5.4%, similar to those of the standard samples. The anti-interference capacity of the proposed method is reasonable because of the specificity of the Ehrlich reagent to the indole group.

Tryptophan and indole-3-carboxylic acid (ICA) were selected to investigate the influence of indole derivatives on the determination of IAA and IBA. As shown in Figure S2A in the Supporting Information, tryptophan does not react to PDAB almost at 25 °C within 60 min, therefore tryptophan has no interference to the determination of IBA at its concentration level of 1.0×10^{-3} M. At 70 °C, a low absorption was observed after reacting to PDAB for 150 min but with an obvious low reaction efficiency compared with IAA and IBA. Figure S2B in the Supporting Information presents the spectra of 1.0×10^{-5} M IAA after addition of tryptophan with different concentrations. We can find at least 50-fold of tryptophan has no influence on the signal intensity of IAA. Figure S3 in the Supporting Information shows the spectra of ICA after Ehrlich reaction at 25 and 70 °C.

Table 1. Analytical Performance for IAA and IBA after Ehrlich Reaction

	IAA	IBA
detection limits (3σ)	1.0×10^{-7} M	2.8×10^{-7} M
calibration function[a] (C, mol L^{-1})	$A = 0.010 + 5214C$ (70 °C)[b]	$A = 0.0015 + 1017C$ (25 °C for 40 min)[b]
	$A = 0.156 + 5201C$ (70 °C)[c]	$A = 0.0041 + 1070C$ (25 °C for 40 min)[d]
		$A = -0.013 + 14597C$ (70 °C for 150 min)[b]
calibration ranges, mol L^{-1}	5.6×10^{-5} to 2.8×10^{-7}	4.2×10^{-5} to 8.4×10^{-7}
corr coeff	0.9984 (70 °C)[b]	0.9997 (25 °C for 40 min)[b]
		0.9977 (70 °C for 150 min)[b]
precision (RSD, $n = 5$), %		
10 μM	4.5	2.8
20 μM	2.6	3.0

[a] With 12 concentration points. [b] Single auxin. [c] In the presence of 1×10^{-5} M IBA. [d] In the presence of 1×10^{-5} M IAA.

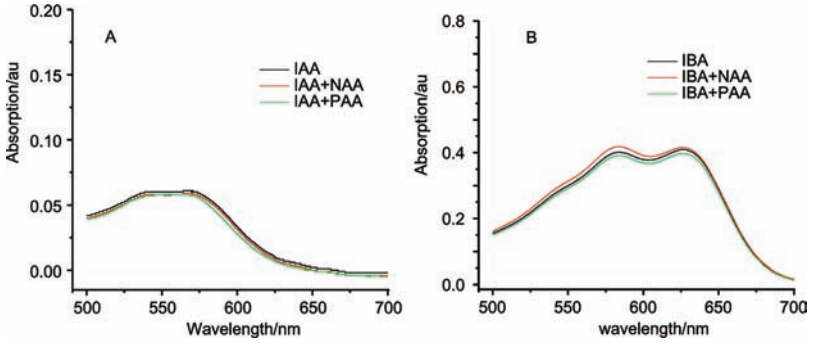

Figure 6. (**A**) Spectrogram of 1.0×10^{-5} M IAA and 1.0×10^{-5} M IAA with added 2.5×10^{-3} M NAA or PAA after Ehrlich reaction at the incubation temperature of 70 °C for 150 min. (**B**) Spectrogram of 2.5×10^{-5} M IBA and 2.5×10^{-5} M IBA with added 2.5×10^{-3} M NAA or PAA after Ehrlich reaction at the incubation temperature of 70 °C for 150 min. The conditions for Ehrlich reaction are as shown in **Figure 1**.

Table 2. Effect of Interfering Species (NAA and PAA) on the Determination of 1×10^{-5} M IAA and 2.5×10^{-5} M IBA

	IAA	IBA
recovery, %		
NAA[a]	98.2	105
PAA[a]	97.3	97.7
precision (RSD, $n = 4$), %		
NAA[a]	3.6	4.3
PAA[a]	5.4	2.3

[a] In the presence of 2.5×10^{-3} M NAA or PAA as interfering species.

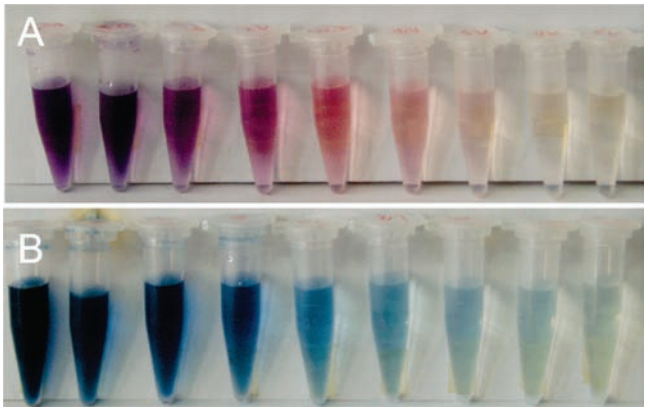

Figure 7. Photos of IAA (**A**) and IBA (**B**) with different concentrations after Ehrlich reaction at the incubation temperature of 70 °C for 150 min. The conditions for Ehrlich reaction are as shown in **Figure 1**. (**A**) Concentrations of IAA are 4.0×10^{-4}, 2.0×10^{-4}, 1.0×10^{-4}, 5×10^{-5}, 2.0×10^{-5}, 1.0×10^{-5}, 5.0×10^{-6}, 2.5×10^{-6}, and 2×10^{-6} M, respectively. (**B**) Concentrations of IBA are 2.0×10^{-4}, 1.0×10^{-4}, 5.0×10^{-5}, 2.5×10^{-5}, 2.0×10^{-5}, 1.0×10^{-5}, 5.0×10^{-6}, 2.0×10^{-6}, and 1.0×10^{-6} M, respectively.

The maximum absorption wavelength was transferred from 564.4 to 537.6 nm. Under the two conditions, ICA has high reactivity to Ehrlich reagent, PDAB. Therefore, if ICA exists, it will affect the determination of IAA and IBA. We searched the papers relating to the content of ICA and tryptophan in the fresh plant sample, but no paper was found to the best of our abilities. That means it should be difficult to determine ICA and tryptophan possibly due to their low contents in fresh plant samples.

Analytical Performance of the Ehrlich Colorimetric Reaction for IAA and IBA. The analytical characteristic data of colorimetric determination of IAA and IBA are summarized in **Table 1**. The detection limits (3σ) of IAA and IBA were 0.10 μM and 0.28 μM, respectively. The relative standard deviations (RSD) for five replicate determinations of the two auxins are less than 5% at 10.0 μM level. The linear ranges of IAA and IBA were found to be 0.28−56 μM and 0.84−42 μM with the correlation coefficient (R) exceeding 0.997 by the use of 12 standard point separated in the ranges. **Figure 7** presents the photos of IAA and IBA at different concentration level after Ehrlich reaction. We find the identification of micromolar auxin can be achieved even with the naked eye. A comparison of the detection limits for auxin determination by the use of different methods is presented in Table S1 in the Supporting Information. Even when the simple colorimetric procedure was used, the proposed method had much lower detection limits over the HPLC−tandem mass spectrometry (HPLC−MS/MS) (*3*).

Because of the lack of suitable certified reference materials available for auxin analysis, the accuracy of the present colorimetric method for IAA and IBA was checked and demonstrated by analyzing the recovery of the auxin-spiked plant samples and the comparison between the results obtained by the present method and the previous capillary electrophoresis−electrochemiluminescence (CE−ECL) (*19*). The recovery was tested for spike of two auxins in the studied samples at the 2.0 μg g^{-1} level. As shown in **Table 3**, the recovery ranged from 87.5 to 108%, indicating no interference encountered from the sample matrices. The sample preparation for CE−ECL detection can be found in our previous work (*19*). The contents in whole bean sprouts after being cultured 3 days obtained by the present method and the CE−ECL method were 1.11 and 0.964 μg g^{-1} as listed in **Table 4**. No IBA is detected in whole bean sprout sample by the use of either of the two methods, and the IAA levels obtained by the two methods show a good agreement.

Table 3. Content and Recovery Results for IAA and IBA in Whole Mung Bean Sprouts

	auxin	detected/ μg g^{-1}	added/ μg g^{-1}	found/ μg g^{-1}	recovery/%	RSD/$n = 5$, %
1st	IAA	0.95	2.0	2.70	87.5	4.5
	IBA	nd[a]	2.0	2.16	108	3.1
2nd	IAA	1.50	2.0	3.32	91.0	4.2
	IBA	nd	2.0	2.10	105	3.2
3rd	IAA	1.11	2.0	2.92	90.5	5.4
	IBA	nd	2.0	1.82	91.0	3.5
4th	IAA	0.81	2.0	2.65	92.0	4.8
	IBA	nd	2.0	2.05	102.5	3.6
5th	IAA	0.75	2.0	2.54	89.5	3.7
	IBA	nd	2.0	2.10	105	2.8
6th	IAA	0.60	2.0	2.38	89.0	3.4
	IBA	nd	2.0	1.82	91.0	2.9

[a] Not detectable.

Table 4. Determined Content of IAA in Whole Bean Sprouts and Sprout Cotyledon at Different Periods

	IAA (μg g^{-1})		
time	whole bean sprouts	sprout cotyledon	IBA
1st day	0.95	3.9	nd[a]
2nd day	1.50	3.2	nd
3rd day	1.11	2.4	nd
	0.964[b]		nd[b]
4th day	0.81		nd
5th day	0.75		nd
6th day	0.60		nd

[a] Not detectable. [b] Capillary electrophoresis−electrochemiluminescence-based method (*19*).

Analysis of Real Samples. The proposed method was applied to determine auxin changes in mung bean sprouts during the growth procedure. The sample preparation was presented in Materials and Methods. Methanol−hydrochloric acid was used as extracting agent to be compatible with the Ehrlich reaction. The spectrum changes of auxin in mung bean sprouts after Ehrlich reaction are shown in **Figure 8**. IAA was detected in mung bean sprouts, but IBA was not found or its content was too low to be detected. Moreover, the content of IAA in mung bean sprouts varied along the growth procedure as shown in **Table 4** and Figure S4 in the Supporting Information. The IAA content in the whole bean sprouts increased from the first day to the second day and then decreased with an increased growth time. It may be due to the increased total weight for the sprout, but the IAA exists mainly in the growth active sites, such as the sprout cotyledon and the bud root. This was proved by the IAA content in sprout cotyledon, in which the content of IAA was found 2-fold higher than the average content in the sprout. As shown in **Table 3**, the recovery was from 87.5 to 108% for the two auxins, the RSDs for

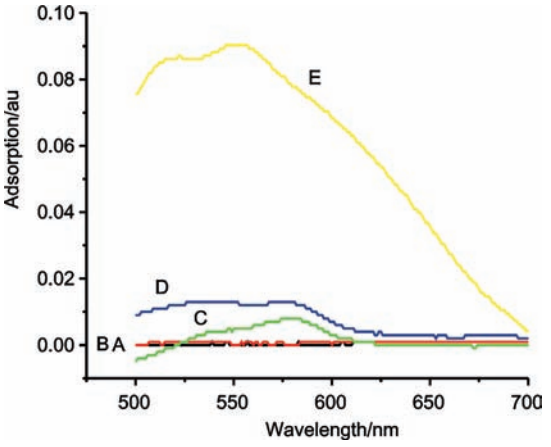

Figure 8. Spectrogram of the extract of mung bean sprout cultured 3 days after Ehrlich reaction. (**A**) The blank reagent. (**B**). The extract of the sample after Ehrlich reaction at 25 °C for 40 min. (**C**) The extract of the sample added 5 μM IAA and IBA after Ehrlich reaction at 25 °C for 40 min. (**D**) The extract of the sample after Ehrlich reaction at 70 °C for 2.5 h. (**E**) The extract of the sample added 5 μM IAA and IBA after Ehrlich reaction at 70 °C for 2.5 h. The conditions for Ehrlich reaction are as shown in **Figure 1**.

the sample analysis were below 5.4% with five replicate measurements. It can be concluded that this method was suitable for routine detection and differentiation of IAA and IBA.

The results demonstrated the feasibility of the colorimetric method for detection and selective differentiation of indoleacetic acid (IAA) and indolebutyric acid (IBA). Compared to other methods, such as HPLC (*3*, *9*−*13*), immunoassay (*3*, *4*), and capillary electrophoresis (*6*−*8*), the present colorimetric method needs no complex instrumentations, and the determination procedure is compatible with the sample pretreatment, leading to a simplified experimental process. The low cost and easy operation of the present system make it very attractive for routine determination of the two auxins in plant samples.

Supporting Information Available: Additional figures and a table as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Chen, J. G.; Du, X. M.; Zhao, H. Y.; Zhou, X. Fluctuation in levels of endogenous plant hormones in ovules of normal and mutant cotton during flowering and their relation to fiber development. *J. Plant Growth Regul.* **1996**, *15*, 173–177.

(2) Olsson, J.; Claeson, K.; Karlberg, B.; Nordström, A.-C. Determination of indole-3-acetic acid and indole-3-acetylaspartic acid in pea plant with capillary electrophoresis and fluorescence detection. *J. Chromatogr., A* **1998**, *824*, 231–239.

(3) Ma, Z.; Ge, L.; Lee, A. S. Y; Yong, J. W. H.; Tan, S. N.; Ong, E. S. Simultaneous analysis of different classes of phytohormones in coconut (*Cocos nucifera L.*) water using high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry after solid-phase extraction. *Anal. Chim. Acta* **2008**, *610*, 274–281.

(4) Li, J.; Xiao, L.-T.; Zeng, G.-M.; Huang, G.-H.; Shen, G.-L.; Yu, R.-Q. A renewable amperometric immunosensor for phytohormone beta-indole acetic acid assay. *Anal. Chim. Acta* **2003**, *494*, 177–185.

(5) Calatayud., J. M.; de Ascenção, J. G.; Albert-García, J. R. FIA-fluorimetric determination of the pesticide 3-indolyl acetic acid. *J. Fluorescence* **2006**, *16*, 61–67.

(6) Liu, B. F.; Zhong, X. H.; Lu, Y. T. Analysis of plant hormones in tobacco flowers by micellar electrokinetic capillary chromatography coupled with on-line large volume sample stacking. *J. Chromatogr., A* **2002**, *945*, 257–265.

(7) Yin, X.-B.; Liu, D.-Y. Polydopamine-based permanent coating capillary electrochromatography for auxin determination. *J. Chromatogr., A* **2008**, *1212*, 130–136.

(8) Zheng, B.; Yang, H. X.; He, J. L. Quantitative analysis of plant hormones with capillary electrophoresis. *Chin. J. Anal. Chem.* **1999**, *27*, 704–707.

(9) Hou, S. J.; Zhu, J.; Ding, M. Y.; Lv, G. H. Simultaneous determination of gibberellic acid, indole-3-acetic acid and abscisic acid in wheat extracts by solid-phase extraction and liquid chromatography-electrospray tandem mass spectrometry. *Talanta* **2008**, *69*, 1773–1781.

(10) Liu, H.-T.; Li, Y.-F.; Luan, T.-G.; Lan, C.-Y.; Shu, W.-S. Simultaneous determination of Phytohormones in plant extracts using SPME and HPLC. *Chromatographia* **2007**, *66*, 515–520.

(11) Mattivi, F.; Vrhovšek, U.; Versini, G. Determination of indole-3-acetic acid, tryptophan and other indoles in must and wine by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr., A* **1999**, *855*, 227–235.

(12) Pan, X. Q.; Wang, X. M. Profiling of plant hormones by mass spectrometry. *J. Chromatogr., B* **2009**, *26*, 2806–2813.

(13) Xi, Z. J.; Zhang, Z. J.; Sun, Y. H.; Shi, Z. L.; Tian, W. Determination of indole-3-acetic acid and indole-3-butyric acid in mung bean sprouts using high performance liquid chromatography with immobilized $Ru(bpy)_3^{2+}$-KMnO4 chemiluminescence detection. *Talanta* **2009**, *79*, 216–221.

(14) Li, B. L.; Du, Y.; Dong, S. J. DNA based gold nanoparticles colorimetric sensors for sensitive and selective detection of Ag(I) ions. *Anal. Chim. Acta* **2009**, *644*, 78–82.

(15) Li, T.; Li, B. L.; Wang, E. K.; Dong, S. J. G-quadruplex-based DNAzyme for sensitive mercury detection with the naked eye. *Chem. Commun.* **2009**, 3551–3553.

(16) Breksa, A. P., III; Ibarra, P., Jr. Colorimetric method for the estimation of total limonoid aglycones and glucoside contents in citrus juices. *J. Agric. Food Chem.* **2007**, *55*, 5013–5017.

(17) Kohno, Y.; Kamiya, J.; Okubo, T.; Matsushima, R. Color sensors for indole vapors based on Ehrlich-type reactions. *Anal. Sci.* **2009**, *25*, 129–132.

(18) Jin, Q. Z.; Shan, L.; Yue, J. H.; Wang, X. G. Spectrophotometric determination of total serotonin derivatives in the safflower seeds with Ehrlich's reagent and the underlying color reaction mechanism. *Food Chem.* **2008**, *108*, 779–783.

(19) Yin, X.-B.; Guo, J.-M.; Wei, W. Dual-cloud point extraction and tertiary amine labeling for selective and sensitive capillary electrophoresis-electrochemiluminescent detection of auxins. *J. Chromatogr., A* **2010**, *1217*, 1399–1406.

(20) Liu, C. Q.; Xia, X. L.; Yin, W. L.; Zhou, J. H.; Tang, H. R. Direct somatic embryogenesis from leaves, cotyledons and hypocotyls of Hippophae rhamnoides. *Biol. Plant.* **2007**, *51*, 635–640.

(21) Barkawi, L. S.; Tam, Y. Y.; Tillman, J. A.; Pederson, B.; Calio, J.; Al-Amier, H.; Emerick, M.; Normanly, J.; Cohen, J. D. A high-throughput method for the quantitative analysis of indole-3-acetic acid and other auxins from plant tissue. *Anal. Biochem.* **2008**, *372*, 177–188.

(22) Šatínskỳ, D.; Sklenářová, H.; Huclová, J.; Karlíček, R. Determination of bopindolol by sequential injection technique with spectrophotometric detection. *Il Farmaco* **2003**, *58*, 1057–1062.

(23) Mauzerall, D.; Granick, S. The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. *J. Biol. Chem.* **1956**, *219*, 435–446.

(24) Hidalgo, F. J.; Alaiz, M.; Zamora, R. Effect of pH and temperature on comparative nonenzymatic browning of proteins produced by oxidized lipids and carbohydrates. *J. Agric. Food Chem.* **1999**, *47*, 742–747.

Received for review September 28, 2009. Revised manuscript received March 5, 2010. Accepted March 8, 2010. This work is supported by National Nature Science Funding of China (No. 90717104) and the Program for New Century Excellent Talents in University (NCET-06-0214), Chinese Ministry of Education.