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Purification and determination of 3-indole-butyric acid and alpha-napthaleneacetic acid from plant tissues using solid phase extraction and high performance liquid chromatography

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ABSTRACT

The method for separation and purification of auxin compounds based on reversed phase solid phase extraction and high performance liquid chromatography (RP-SPE-HPLC) was investigated. The auxins which were 3-indole-butyric acid (IBA) and alpha-napthaleneacetic acid (alpha-NAA) were separated using a reversed phase C_{18} column of 150 x 4.6 mm and 5 μm particle. IBA and alpha-NAA can be completely separated at the flow rate of 0.7 mL min^{-1} with methanol/1%formic acid in water (65:35 v/v) used as a mobile phase with the analysis time of 9.0 min. Solid phase extraction (SPE) was performed to pre-concentrate auxin compounds. Percent recoveries of the extraction method were found to be 89.0%. Plant tissues were extracted using solvent extraction followed by C_{18} SPE and then analyzed by HPLC. By spiking various concentrations of mixed standard into plant tissues, percent recoveries of both analyte were obtained in the range of 81.5-105. The detected IBA was found to be in the range of 0.65-2.84 mg mL^{-1} and 1.04-3.15 mg mL^{-1} for alpha -NAA. Due to its overall analytical performance, these SPE and HPLC methods are presently being proved to be simple and suitable for routine purposes such as those developed for plant extracts studies.

KEYWORDS

Alpha-napthaleneacetic acid;
Solid phase extraction;
3-indole-butyric acid.

INTRODUCTION

Auxins are well known for the plant growth hormones that are actually involved in a variety of biological processes in plants. Indole-3-acetic acid (IAA) is the primary auxin existed in plants which control regular processes such as cell elongation and division whereas 3-indole-butyric acid (IBA) also attributed rooting of cutting and generated new roots in cloning of plants through cutting. The greater ability of IBA to promoted rooting compared with IAA showed the higher effective than IAA and IBA also found naturally in a number of plant species. The other auxin-like substances present in plants such as alpha-napthaleneacetic acid (alpha-NAA) is known to stimulate adventitious rooting and callus induction[1-5].

Several methods for the determination of IBA and alpha-NAA have been reported such as high perfor-

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mance liquid chromatography (HPLC), gas chromatography coupled mass spectrometry (GC-MS), capillary electrophoresis (CE) and chemiluminescence (CL)[1, 6-7]. However, disadvantages with the most methods in case of GC-MS, the derivatization procedures required, particular the sensitivity must be reduced from the purification and derivatization processes. HPLC has emerged as a powerful technique for plant growth hormone analysis. This approach has been applied to qualitative and quantitative the class of auxin with the high sensitivity at room temperature and also the volatility of the auxin substances were ignored during the analysis.

Auxins are occurred in small amount in plant species with the interferences remained therefore solid phase extraction (SPE) is used for the sample preparation and purification procedure of plant extraction. It is usually used to clean up a sample before using a chromatographic or another analytical method to quantitate the amount of the analytes in the sample and also to extract or concentrate analytes. The advantage for the using of SPE is to reduce time consuming and minimize organic solvent with high obtained recovery[8-10].

No reports are available on callus induction and cell elongation in *Houttuynia cordata Thunb* and *Scindapsus aureus*. The aim of this research is to study the sample preparation by C_{18} SPE for the analysis of IBA and α-NAA in *Houttuynia cordata Thunb* and *Scindapsus aureus* samples and to determine the amount of these compounds. This method showed the capability of inducing extraordinarily rapid in vitro propagation of those plant tissues. The quantities of selected auxins may be useful for conservation of this threatened species as well as producing bulk quantities, e.g. gram, kilogram or more, of plant material for commercial production.

EXPERIMENTAL

Chemical and materials

Methanol was of HPLC grade (J. T. Baker SOLUSORB). All other chemicals which were ethanol, acetic acid and formic acid were analytical reagent grade. 3-indole-butyric acid (IBA) and alpha-napthaleneacetic acid (alpha-NAA) were from Sigma. Stock standard solution of IBA and alpha-NAA were prepared in 95%ethanol at 500 mg mL^{-1}. Each portion of working solution containing mixed standard was prepared from stock solution. Appropriate concentrations of standard solution were prepared by further dilution.

The mobile phase of HPLC consisted of methanol/ 1%formic acid in water (65:35 v/v). The solvent was filtered through a 0.45 μm filter membrane by a vacuum pump. After that the prepared solvent was degassed for 20 min in an ultrasonicator prior to its use.

Apparatus

High performance liquid chromatography was a LC-1100 (Hewlett packard) with variable wavelength detector set at 265 nm. Analysis was carried out at room temperature on a shim-pack ODS C_{18} column (i.d. 4.6 x 150 mm, particle size: 5 μm (GL Sciences Inc). Injections were made by Rheodyne G1313A (ALS) auto sample injector equipped with a 100 μL loop. The pH measurements were made with a PG 203-S (Mettler Toledo) using combined glass electrode.

Sample preparation procedure

Young leaves, old leaves and young shoots of *Houttuynia cordata Thunb* and young leaves and old leaves of *Scindapsus aureus* samples treatment was performed as follows. Fresh samples were dried, milled and weighed out accuracy for 25 g in an Erlenmeyer flask. A volume of 100 mL of ethanol was added to the flask which was left for 24 hr. Afterward the extract was filtered through the filter paper into the separation funnel, which was then added with 200 mL hexane. The extract solution was partitioned with hexane for 3 hr in order to remove a non-polar compound from the sample matrices. After the partition process, the hexane layer was discharge. Finally, the ethanolic extract was evaporated to approximately 5.0 mL by using evaporator with the temperature in water bath at 40.0°C[8].

Clean-up procedure

A C_{18} SPE column was conditioned with methanol : acetic acid (100:1 v/v), methanol : milli Q:acetic acid (50:50:1 v/v/v), methanol : milli Q : acetic acid (30:70:1 v/v/v) followed by 5.0 mL of milli-Q water, respectively[11]. The 5.0 mL of ethanolic extract was loaded into SPE column and then the SPE column was rinsed with 5.0 mL milli-Q water to remove the impurity from the sample matrices. The analytes were

eluted by using the concentration of 80/20 v/v methanol:1% acetic acid by gravity flow rate. The 10.0 mL of eluate was collected and then further evaporate to a small volume under a stream of nitrogen. The residue was dissolve in 1.0 mL of 95% ethanol and injected into HPLC system. The same experiments were done for 3 replicates.

Percent recovery of extraction method

The vairous concentrations of mixed standard solution was added into plant tissues extracted and extracted using the same procedure as described in section 2.4. The percent recovery of extraction method was calculated using the following relationship.

$$\% \text{ recovery} = \frac{\text{spiked sample response - unspiked sample response}}{\text{added standard response}} \times 100$$

Repeatability test of HPLC system

An aliquot of 40 mg mL^{-1} mixed standard was injected within intra-day by 13 replicates into HPLC system. Standard deviation (SD) and percent relative standard deviation (%RSD) of the retention time and peak area were calculated.

Linearity range

A volume of 20 μL of each standard solution at the concentration of 0.25, 0.50, 1.00, 2.50, 5.00, 10.00, 20.00, 30.00, 40.00, 60.00, 80.00, 100.00, 200.00, 300.00, 400.00 and 500.00 mg mL^{-1} was injected into HPLC system. The calibration curve of each mixed standard was constructed by plotting the detector response in terms of peak area against the concentration of each standard injected.

Limit of detection and limit of quantification

A mixture of standard containing 0.25, 0.50, 1.00, 2.50 and 5.00 mg mL^{-1} was injected into HPLC system to estimate the limit of detection based on the concentration. The limit of detection was calculated from the calibration curve by mean of the blank signal, which can be used as an estimation of the calculated value from the regression line.

Statistical analysis of data

Two-way ANOVA was used to test for significant differences between treatments.

RESULTS AND DISCUSSION

HPLC-UV separation

To separate individual plant growth hormones in a mixture, mixed standards were analyzed by C_{18} column reversed phase HPLC with UV detection in an isocratic mode. The results showed that IBA and alpha-NAA can be completely separated at the flow rate of 0.7 mL min^{-1} with methanol/1%formic acid in water (65:35 v/v) used as a mobile phase. The analysis time was 9.0 min. The retention times of IBA and alpha-NAA were 5.82 and 7.30 min, respectively (Figure 1).

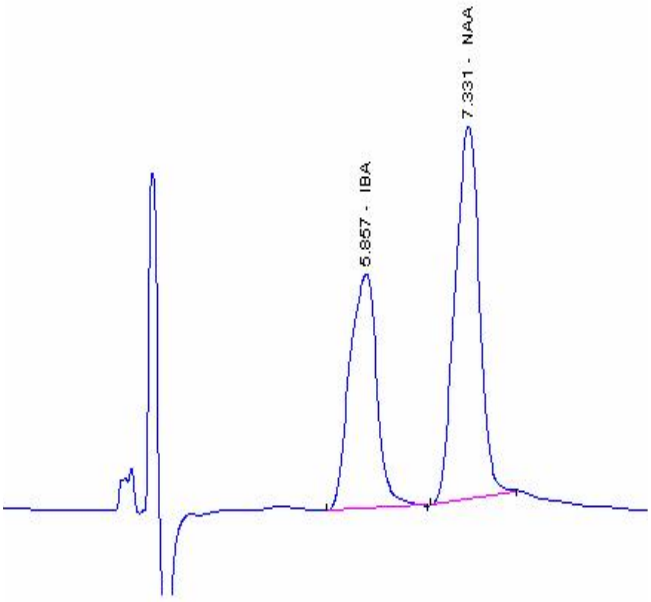

Figure 1 : Chromatogram of IBA and alpha-NAA when 65/35 v/v(0.1% pH 3.3) methanol : milli Q (formic acid buffer) was used as a mobile phase (flow rate 0.7 mL min^{-1})

Repeatability test of HPLC system

An aliquot of 40 mg mL^{-1} mixed standard was injected within intra-day by 13 replicates into HPLC system. Percent relative standard deviation (%RSD) of the retention time and peak area were in the range of 0.09-0.61 and 0.20-0.23, respectively.

Linearity range

An aliquot of 0.25, 0.50, 1.00, 2.50, 5.00, 10.00, 20.00, 30.00, 40.00, 60.00, 80.00, 100.00, 200.00, 300.00, 400.00 and 500.00 mg mL^{-1} was injected into HPLC system under the optimum conditions. It was found that the value of variation coefficient (r^2) of each linear plot of each standard were 0.9998 for both hormones.

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Limit of detection and limit of quantification

Linear regression analysis used for validation of linearity was performed under the assumption that the y-values are normally distributed around the regression line with a standard deviation $S_{y/x}$. The limit of detections of IBA and alpha-NAA were 0.096 and 0.127 mg mL^{-1}, respectively whereas the limit of quantitations of IBA and alpha-NAA were 0.320 and 0.420 mg mL^{-1}, respectively. The limit of detection and limit of quantification are presented in TABLE 1.

TABLE 1 : Calibration curves, limit of detection, limit of quantitative and precision of auxin

hormones	*LOD (mg mL^{-1})*	*LOQ (mg mL^{-1})*	*calibration equation (mg mL^{-1})*	*correlative coefficient*
IBA	0.096	0.320	y = 39.814x + 1.566	0.9978
alpha - NAA	0.127	0.420	y = 59.185x + 4.3589	0.9990

(n = 3 at each concentration)

Optimization of the SPE procedure

In optimizing the SPE procedure, many parameters affect on the efficiency of SPE, such as type and volume of eluent, sample flow rate, type of rinsing solvents, pH and volume of the sample. In this study, pH and volume of the sample, type of rinsing solvents and eluent were optimized and discussed in detail.

Influence of the sample volume

Sample volume is a parameter affected on the enrichment of extraction recoveries. In order to reduce the analysis time and increase high recoveries of extraction, this experiment was done with sample volume ranging from 1-30 ml. From TABLE 2, the recoveries were increased significantly to be 80.03-95.0% ($P < 0.05$) when 5 ml sample volume was used. One ml sample volume was inefficient because the recoveries were the lowest. Therefore, 5 ml of sample was selected for further study.

Influence of the pH sample

The pH of the sample is a crucial factor in the SPE pre-concentration procedure due to the pH indicates the extraction efficiency of the selected analytes. In order to increase high recoveries, this experiment was done with pH of the sample ranging from 2-9. The results show the pH was significant influence of the extraction ($P < 0.05$). The recoveries obtained for IBA and alpha–NAA with different pH are shown in TABLE 3. When the pH sample was 2, the recoveries were 85% for both hormones and increased to 87 and 92% for IBA and alpha–NAA, respectively, at pH 3. Then, the recoveries decreased as the sample pH ranging from 4-9. It is possible that the structure of both hormones contains acidic functional group and can not be eluted from the adsorbent when pH of the sample was adjusted to basic condition. Thus, the analyte remain on the adsorbent, resulted in low recoveries[12]. Based on this experiment, pH 3 was selected as the optimum condition for the extraction procedure.

TABLE 2 : Influence of the sample volume on the recoveries of IBA and alpha-NAA

pH of the sample	recoveries ± S.D.	
	IBA	alpha-NAA
2	85.0 ± 2.0a	85.0 ± 1.0b
3	87.0 ± 1.0a	92.0 ± 1.0a
4	80.0 ± 1.7b	76.0 ± 1.0c
5	77.0 ± 2.6c	74.0 ± 1.7c
6	61.0 ± 1.0d	72.0 ± 1.7c
7	55.0 ± 1.7e	53.0 ± 1.7d
8	40.0 ± 1.7f	45.0 ± 1.0e
9	39.0 ± 1.0f	44.0 ± 2.0e

(n = 3 at each concentration)

TABLE 3 : Influence of pH of the sample on the recoveries of IBA and alpha-NAA

pH of the sample	Recoveries ± S.D.	
	IBA	alpha-NAA
2	85.0 ± 2.0a	85.0 ± 1.0b
3	87.0 ± 1.0a	92.0 ± 1.0a
4	80.0 ± 1.7b	76.0 ± 1.0c
5	77.0 ± 2.6c	74.0 ± 1.7c
6	61.0 ± 1.0d	72.0 ± 1.7c
7	55.0 ± 1.7e	53.0 ± 1.7d
8	40.0 ± 1.7f	45.0 ± 1.0e
9	39.0 ± 1.0f	44.0 ± 2.0e

(n = 3 at each concentration)

Influence of type of rising solution

Type of rinsing solution is another factor affected the recoveries of extraction. The inefficient rinsing solution may elute some compounds in sample matrix with the analytes. In order to assess the applicability of the extraction method to the analysis of the real samples, the various type of the rinsing solution was used, that were 10 ml water, methanol and ethanol. The recover-

ies of IBA and alpha–NAA are shown in TABLE 4. The rinsing solutions have a significant effect on extraction efficiency ($P < 0.05$). The highest recovery (84%) was obtained when water was used as the rinsing solution. From our study, it is possible that the efficiency of the rinsing solution depended on the structure of adsorbent and analytes[8]. Therefore, water was chosen as the rinsing solution in subsequent method.

TABLE 4 : Influence of type of rising solutions on the recoveries of IBA and alpha-NAA

type of rinsing solutions	recoveries ± S.D.	
	IBA	alpha-NAA
water	84.0 ± 1.7a	78.0 ±1.0a
methanol	55.0 ± 2.6c	67.0 ±2.0b
ethanol	59.0 ± 1.0b	53.0 ±1.7c

(n = 3 at each concentration)

Influence of the eluent

The concentration of eluent is very important parameter in the SPE pre-concentration procedure. The recoveries of IBA and alpha–NAA for the SPE extraction was studied at four different ratios of methanol:water (1% v/v aceteic acid) as follows: 85:15, 80:20, 75:25 and 70:30. It is suspected that the adding of 1%v/v acetic acid could increase the recoveries of both hormones. Thus, the extraction of IBA and alpha–NAA using C_{18} adsorbent were possible with the reasonable recovery of auxin obtained by addition of 1%v/v acetic acid to organic eluent. The recoveries were significantly observed ($P<0.05$) with different concentration of eluent (TABLE 5). When the ratio of methanol:water was 80:20, the recoveries of IBA and α–NAA was 89.4 and 89.04%, respectively. However, the recoveries of both hormones decreased with increasing the polarity of the eluent, resulted retention on the adsorbent. It is possible that the auxin compound comprised of two main parts: aromatic or cyclic structure and acidic functional group. The polarity of eluent affects the retention of analytes on the absorbent. In order to obtain the most suitable recovery, the well-matched between the eluent and analyte together could consider in term of polarity and acidic condition[8, 13]. From our study, methanol:water at the ratio of 80:20 (1%v/v acetic acid) was the optimum condition for the SPE procedure.

Analysis of plant tissues

In this study, 3 parts of *Houttuynia cordata Thunb* (young leaves, old leaves and young shoots) and 2 parts of *Scindapsus aureus* (young leaves and old leaves) were selected as samples for the determination of IBA and alpha-NAA. Plant samples were extracted by solvent extraction method as described in section 2.3. Afterward, they were pre-treated and pre-concentrated with optimum SPE conditions as described in section 2.4 and then subjected onto HPLC system (Figure 2 and Figure 3). The quantity of IBA and alpha-NAA of *Houttuynia cordata Thunb* was found in the range of 1.73-2.84 mg mL^{-1}, and 2.43-3.15 mg mL^{-1}, respectively. The quantity of IBA and alpha-NAA of *Scindapsus aureus* was found in the range of 0.65-1.53 mg mL^{-1} and 1.04-1.86mg mL^{-1}, respectively. The results of quantitative analysis of plant extracted and %recovery were showed in TABLE 6.

TABLE 5 : Influence of the concentration of eluent on recoveries of IBA and alpha-NAA

methanol:water	recoveries ± S.D.	
	IBA	alpha-NAA
85 : 15	18.06 ± 1.88d	17.78 ± 1.43d
80 : 20	89.40 ± 1.88a	89.04 ± 1.43a
75 : 25	71.81 ± 1.63b	58.19 ± 1.97b
70 : 30	54.77 ± 1.63c	44.40 ± 1.97c

(n = 3 at each concentration)

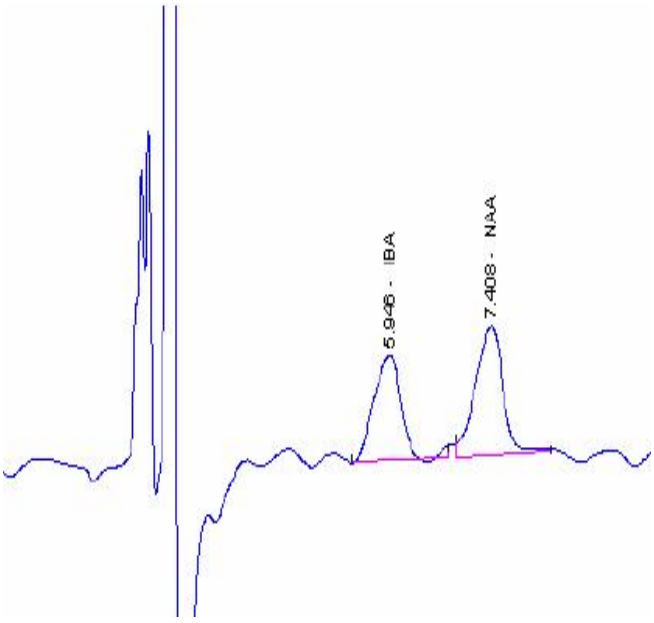

Figure 2 : Chromatogram of spkied IBA and alpha-NAA in *Houttuynia cordata Thunb* when 65/35 v/v(0.1% pH 3.3) methanol : milli Q (formic acid buffer) was used as a mobile phase (flow rate 0.7 mL min^{-1})

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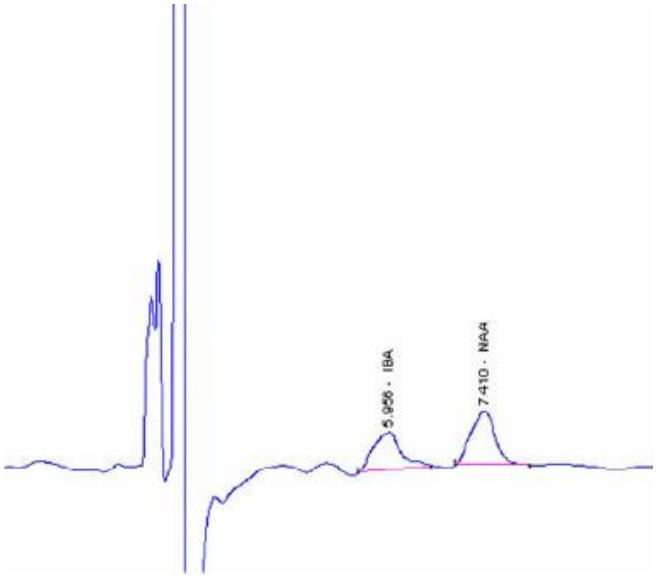

Figure 3 : Chromatogram of spkied IBA and alpha-NAA in *Scindapsus aureus* when 65/35 v/v(0.1% pH 3.3) methanol : milli Q (formic acid buffer) was used as a mobile phase (flow rate 0.7 mL min^{-1})

TABLE 6 : The detected of IBA and alpha -NAA in *Houttuynia cordata Thunb* and *Scindapsus aureus*

Plants	Tissues	Hormones	Detected (mg mL^{-1})	Recoveries (%)	RSD (%)
Houttuynia cordata Thunb	young leaves	IBA	1.91	105.0	3.3
		alpha-AA	2.50	102.0	2.4
	old leaves	IBA	1.73	95.5	4.8
		alpha-NAA	2.43	97.0	3.3
	young shoots	IBA	2.84	102.5	3.5
		alpha-NAA	3.15	99.5	2.0
Scindapsus aureus	young leaves	IBA	0.65	96.0	4.6
		alpha-NAA	1.04	81.55	2.1
	old leave	IBA	1.53	101.65	4.3
		alpha-NAA	1.86	92.24	3.5

(n = 3 at each concentration)

CONCLUDING REMARKS

In conclusion, HPLC-UV method for the detection and qualitative identification of auxin compounds is present. The treatments with C_{18} SPE enhance plant productivity resulting in high recoveries of extraction. The method provided chromatographic resolution for each analyte with superior sensitivity. The precision and accuracy of the assay are good without interferences. Thus this method is specific, quantitative and reproducibility enough to confirm the data obtained. Thus, the present method can be used for routine analysis of IBA and alpha-NAA in plant tissues.

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