Membrane-Supported Liquid–Liquid–Liquid Micro Extraction Combined With Anion-Selective Exhaustive Injection Capillary Electrophoresis-Ultraviolet Detection for Sensitive Analysis of Phytohormones

Selvi S R
Lecturer senior Grade, Department of Physics, Muthiah Polytechnic College, Chidambaram

***Abstract*- A novel method based on off-line membrane-supported liquid–liquid–liquid micro extraction (MS-LLLME) combined with on-column anion-selective exhaustive injection (ASEI) capillary electrophoresis–ultraviolet (CE–UV) detection was established for the analysis of seven phytohormones (abscisic acid (ABA), jasmine acid (JA), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), salicylic acid (SA) and gibberellic acid (GA)). In MS-LLLME, the target phytohormones were extracted from the acid donor phase to the alkaline acceptor phase, and the acceptor solutions were directly analyzed by ASEI-CE–UV. Under the optimal experimental conditions, the analytical performance of the method was evaluated. The limits of detection (LODs) of ABA, JA, 2,4-D, NAA, IAA, SA and GA were determined to be 1.00, 2.21, 0.33, 0.17, 0.67, 0.05 and 16.5 ng/mL, respectively. The relative standard deviations (RSDs, n = 7) ranged from 4.7% to 12.9%, and the enrichment factors were in the range of 307 to 20,160. The proposed method was successfully applied for the determination of multiple phytohormones in banana, cabbage and cucumber extracts, and ABA, IAA and SA were detected in these samples. The recoveries for the spiked samples were in the range of 79.0 to 116.4%. The proposed method was demonstrated to be suitable for the simultaneous quantification of multiple phytohormones with high sensitivity and good sample cleanup ability.**

***Index Terms*- Phytohormones, Membrane-supported liquid–liquid–liquid, Micro Extraction, Anion-selective Exhaustive Injection, Capillary Electrophoresis.**

I. INTRODUCTION

Phytohormones, a group of naturally occurring substances detected at extremely low concentrations, play important roles in a variety of regulation processes, such as growth, metabolism, cell division, cell differentiation and leaf and organ senescence, as well as the protective response to biotic and abiotic stresses [1–3]. The most widely recognized phytohormones include auxins, cytokinines, gibberellins, abscisic acid, salicylic acid and jasmonates [4]. Different phytohormones exert their functions additively, synergistically or antagonistically, and their regulation is greatly dependent on their levels in plants. Based on their physiological functions, different phytohormones can be utilized in agriculture to achieve various enhanced agricultural characteristics during critical growth stages. Phytohormones can also be found in mammals [5], stimulating the human body [6] or affecting immune cells [7]. Obviously phytohormones have its potential application value on agriculture and biomedicine, while a better understanding of the functions and interactions among them relies on the accurate quantitative method. Therefore, it is necessary to develop a fast, simple and sensitive method for the simultaneous analysis of multiple classes of trace phytohormones.

To date, several methods have been developed for the determination of phytohormones, including gas chromatography (GC) [8], high-performance liquid chromatography (HPLC) [9–11], capillary electrophoresis (CE) [12–14] and chromatography coupled with mass spectrometry (MS) [15–18]. Among these methods, CE is a powerful microanalytical technique, the fast speed of analysis, high resolution and sensitivity make it an attractive method to analyze phytohormones [12–14,18]. CE with UV [12,14], laserinduced fluorescence (LIF) [13] and MS [18] as the detector has been employed

for phytohormones analysis. However, MS is expensive, and the interface of CE–MS is extremely complicated, while a derivatization step is often required in LIF detection, which is time-consuming and can introduce error due to the complex matrix. UV is the most easily available and efficient detector for CE with low cost, and CE–UV is appropriate for the simultaneous analysis of multiple phytohormones without derivatization. However, the sensitivity of CE–UV is strictly limited by its sample injection volume (nL) and narrow optical path length. In addition, it is difficult to directly and accurately quantify trace phytohormones in complex plant matrices. Such limitations can be overcome by incorporating both on-column concentration techniques or/and off-line sample pretreatment techniques with CE–UV [19]. Oncolumn preconcentration techniques, such as stacking, sweeping and dynamic pH junctions, are powerful approaches for substantially improving the sensitivity of CE–UV [19] but have not been fully applied to the analysis of phytohormones. Only Chen et al. [20] have developed a method involving microemulsion electrokinetic capillary chromatography (MEKC) coupled with large volume sample stacking (LVSS) for the analysis of six phytohormones. This method has a high enrichment factor but a low sample cleanup capacity. Due to the existence form of many phytohormones as anions under alkaline conditions, anion-selective exhaustive injection (ASEI) is expected to be a powerful stacking technique that can selectively, electrokinetically and efficiently inject phytohormones and enhance sensitivity simultaneously.

To improve the matrix resistance and sensitivity of the method, an appropriate pretreatment technique is required prior to instrument analysis. Solid phase extraction (SPE) [21–23] and liquid–liquid extraction (LLE) [24,25], have been extensively utilized for phytohormones, while poor selectivity and lengthy SPE time as well as solvent consumption and low enrichment factors in LLE are their drawbacks. To develop a more economical and automated analytical method, green and miniaturized sample pretreatment techniques, such as solid phase microextraction (SPME) and liquid phase microextraction (LPME), have rapidly evolved in recent decades. LPME is an attractive pretreatment technique for combining extraction and enrichment into a single step and is a fast and simple method with a large enrichment factor and good cleanup ability. A hollow-fiber-based liquid–liquid–liquid microextraction system for the extraction of several phytohormones was developed in our previous study [9], and this system has been successfully applied to the analysis of complex samples. A method that combines single drop liquid–liquid–liquid phase microextraction (SD-LLLME) with direct analysis in real-time mass spectrometry (DART-MS) was proposed by Bai et al. [4], but the stability of the method needs improvement. It can be concluded that three-phase liquid–liquid–liquid microextraction can efficiently remove the sample matrix while simultaneously enriching the target phytohormones. To improve the enrichment factor and the stability of LPME, a membrane-supported liquid–liquid–liquid microextraction (MS-LLLME) [23] technique with a larger sample volume and good reproducibility is a very promising method for extracting target phytohormones from acidic sample solutions via organic solvent extraction into an alkaline acceptor solution using a pH gradient and distribution in three phases. In this system, the acceptor phase volume matches the CE injection volume well.

The aim of this work is to develop a rapid, inexpensive, sensitive, efficient method based on CE–UV for the simultaneous quantification of multiple phytohormones in fruits and vegetable samples. For this purpose, an MS-LLLLME system with strong matrix resistance and high extraction efficiency was combined with an automatic, sensitive ASEI-CE–UV system for the analysis of seven target phytohormones. The factors that affect extraction and ASEI were optimized and the analytical performance was evaluated. The proposed method was applied for the determination of multiple phytohormones in banana, cabbage and cucumber extracts for validation.

II. EXPERIMENTAL

2.1. Reagents and materials

The chemical structures of the target phytohormones as well as some of their chemical properties. Indole-3-acetic acid (IAA) and (±) jasmonic acid (JA) were purchased from SigmaAldrich (Sigma-Aldrich Company, USA), and gibberellic acid (GA), (±) abscisic acid (ABA), salicylic acid (SA), 1-naphthalene acetic acid (NAA) and 2,4-

dichlorophenoxyacetic acid (2,4-D) were obtained from Aladdin Reagent Database Inc. (Shanghai, China). Stock solutions (1 mg/mL of each analyte) were prepared by dissolving each phytohormone in methanol. All of the stock solutions were stored at 4 ◦C in a refrigerator. The working standard solutions were prepared daily by stepwise dilution of the corresponding stock standard solutions with high-purity water. High-purity deionized water was obtained from a Milli-Q water purification system (18.2 M/cm, Millipore, Bedford, MA, USA).

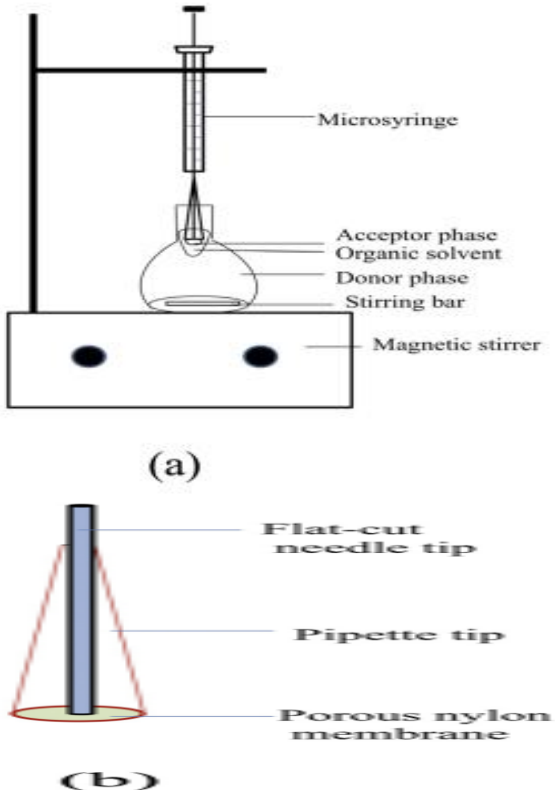
2.2. CE system

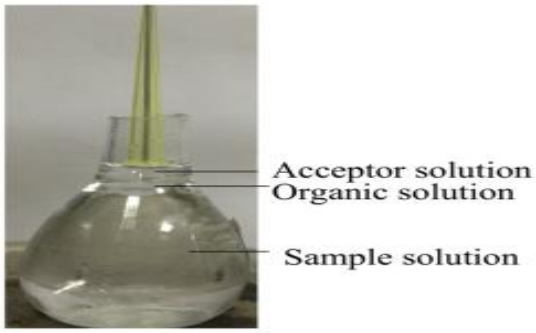
All of the separation experiments were performed by a G1600AX CE system (Agilent, Waldron, Germany) equipped with a programmable, multi wavelength UV–Visible detector. The wavelengths employed for the determination of target phytohormones were 200 nm for GA, JA, 2,4-D and SA; 220 nm for IAA and 2,4-

D; and 254 nm for SA, respectively. The capillary temperature for the separation of target phytohormones was maintained at 25 ◦C. The new capillary was conditioned by successive washings with 1 mol/L NaOH (30 min), water (10 min), 0.1 mol/L HCl (10 min) and water (30 min). Between runs, the capillary was rinsed with running buffer for 5 min. The separation conditions for the CE standard injection were as follows: fused-silica capillary with dimensions of 58.5 cm (50 cm to the detector) × 50 m i.d. ×360 m o.d. (Yong-nian Optical Fiber, Hebei, China), background electrolyte (BGE) containing 35 mmol/L sodium borate buffer and 0.1% (m/V) CD (pH 10.0, adjusted by NaOH and filtered through a membrane filter with a pore size of 0.45 m), separation voltage of 25 kV and hydrodynamic injection of the samples at 50 mbar for 5 s

2.3. MS-LLLLME procedure

The MS-LLLME unit (shown in Fig. 1) was set up as indicated in our previous study [26]. The porous-nylon-membrane-supported extraction tip is shown in Fig. 1b. Briefly, the nylon membrane (0.8 m pore size,) was sealed on the slightly flamed larger end of pipette tip (200 L scale). Then, the smaller end of pipette tip was manually cut to a proper length so that the flat-end tip of a 25-L micro syringe could be inserted into the pipette tip tightly. In the MS-LLLME system, the sample solution volume was 9.1 mL, containing an appropriate amount of NaCl and HCl. This solution was transferred to a flask, and 250 L of an organic solvent (phenetole:octanol = 6:4) was carefully added to the sample solution to form a supernatant solvent layer by pipetting. Then, a 25-L micro syringe with a flat-end needle tip filled with 15 L of ammonia acceptor solution was tightly inserted from the small end of the flamed pipette tip until the flat needle tip was in close contact with the nylon membrane, and 5 L of the acceptor solution was injected to wet the nylon membrane. Next, the micro syringe along with the porous-nylon-membrane supported extraction tips were immersed in the organic solvent layer and the remaining acceptor solution was pushed out from the micro syringe to form a large droplet under the nylon membrane. An 85-2A constant-temperature magnetic stirrer was used to stir the sample solution at 800 rpm for 50 min. When the extraction process was completed, the analytic-enriched acceptor solution was slowly withdrawn into the micro syringe and injected into the insert, which was fixed in the sample vial for further CE analysis. To avoid cross-contamination, the inexpensive and easy-to-prepare extraction tip was disposable after use.





(c)

Figure. 1 (a) The scheme of MS-LLLME. (b) The homemade membrane assisted extraction tip. (c) Photograph of MS-LLLME

2.4. ASEI Pre Concentration

A typical procedure for ASEI was performed as follows (Fig. S2): The separation capillary was filled with BGE, and a 24-cm water plug (41% length of separation capillary) was injected into the capillary using pressure The extracts of MS-LLLME were electro kinetically injected into the capillary by applying a negative voltage of −10 kV, and the water plug was simultaneously removed from the inlet (Fig. S2B). When the current reached 95% of the initial current value that was obtained by thoroughly filling the capillary with BGE at −10 kV the applied voltage was switched to a positive voltage (+25 kV) to initiate the subsequent separation.

III. RESULTS & DISCUSSION

3.1. Optimization of MS-LLLME parameters

In MS-LLLME, phytohormones are extracted from the acid donor phase to the organic solvent, followed by extraction back into the alkaline acceptor phase via a pH gradient and hydrophobic forces. The transport mechanism of MS-LLLME is similar to that of other three-phase LPME techniques [9]. The factors affecting MS-LLLME, such as the organic solvent, acid concentration of the donor phase, acceptor phase concentration, stirring rate, extraction time and salt effect, were investigated.

3.1.1. Selection of organic solvent

An appropriate organic solvent is of great importance in determining the extraction efficiency of MS-LLLME. A suitable organic solvent should satisfy the following criteria: low density, good extraction efficiency, good stability and reproducibility. Based on these factors, five organic solvents with different polarities (i.e., 1-octanol, toluene, phenetole, o-xylene and cyclohexane) were evaluated for the extraction of seven phytohormones by MS-LLLME under the same conditions. As shown in Fig. 2, phenetole exhibited the highest extraction efficiency among all of the phytohormones, except GA. Among these solvents, only 1-octanol was able to extract GA, but the extraction efficiencies of the other six phytohormones were inferior to the extraction efficiency of phenetole. Therefore, the co-solvent mixture of 1-octanol and phenetole was selected as the extraction solvent, and the volume ratio of the two solvents was investigated in detail. The experimental results in Fig. 3 indicated that the mixed solvent resulted in higher extraction efficiencies, and a volume ratio of 6:4 (phenetole:1-octanol) was selected for use in subsequent experiments. The intermediate phase volume affects the formation of droplet as well as the extraction efficiency. It was found that the drop of acceptor phase was unstable under agitation when mixed solvent of phenetole and 1-octanol (6:4) less than 200 L was used as the intermediate phase, and the stability and reproducibility was improved obviously when 250 L mixed solvent was used as the intermediate phase. On the other hand, with a limited contact area between the sample solution and intermediate phase at the bottleneck (Fig. 1), the thickness of intermediate phase would increase with the increase of the intermediate phase volume, making the mass transfer route longer, and the extraction efficiency would decrease with a relative large intermediate phase in a prescribed time. Therefore, 250 L of mixed solvent were used as the intermediate phase.

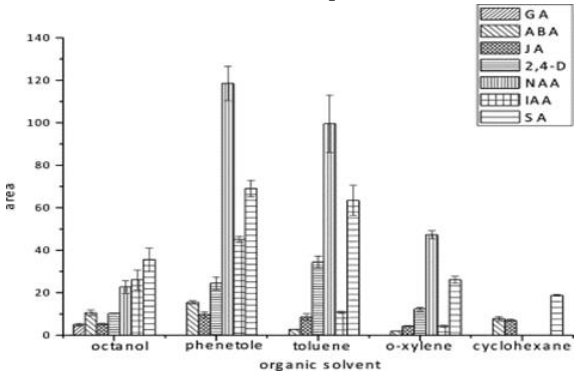


Figure 2:The effect of organic solvent on MS-LLME

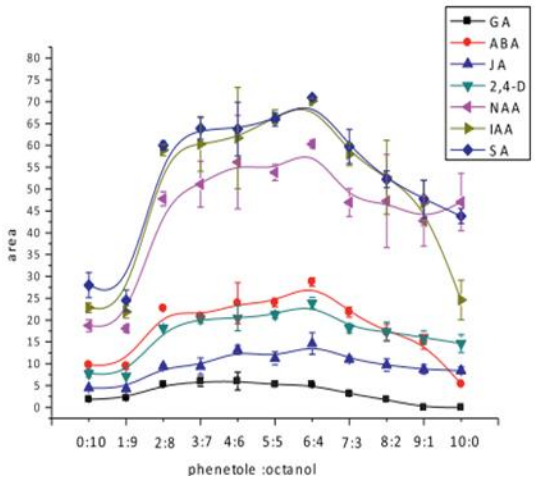


Figure 3: The effect of the ratio of phenetole and octanol on MS-LLLME

3.1.2. Effect of donor phase and acceptor phase in MS-LLLME

The donor and acceptor phases play important roles in determining the extraction efficiency. In this work, HCl was used to acidify the sample solution. To examine the effect of HCl concentration on the extraction efficiency, experiments were performed with the HCl concentration in sample solution varying from 0 to 0.5 mol/L. The experimental results demonstrated that the extraction efficiency of all seven phytohormones was improved as the concentration of HCl was initially increased, and then, a plateau was reached at an HCl concentration above 0.05 mol/L (Fig. 4). Therefore, 0.1 mol/L HCl was selected as the donor phase for subsequent experiments.

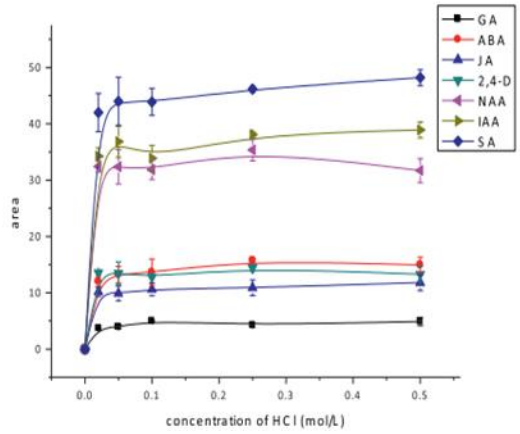


Figure 4: The effect of HCl concentration on MS-LLLME.

The effect of the NH3•H2O concentration on the extraction efficiency of target phytohormones was investigated. The results indicated that the signal intensity of target phytohormones first mushroomed as the NH3•H2O concentration increased and then remained constant as the NH3•H2O concentration was increased to 0.05 mol/L (Fig. S3). Because a higher concentration of NH3•H2O may lead to a decrease in the enrichment factors in ASEI, 0.05 mol/L NH3•H2O was selected as the acceptor solution for subsequent experiments.

Analytes	Linear Range (ng/ml)	Linear Equation	R^2	RSD[a] n=7	LOD(ng/ml)		EF[c]
					SI[b]	ASEI	
GA	500-20000	y= 0.013x ± 2.82	0.9999	1.7	5070	168	30.2
ABS	150-5000	y = 0.0.7x – 2.68	0.9993	5.1	1204	40	30.1
JA	800-20000	y= 0.010x ± 3.4	0.9992	5.2	12500	333	37.5
2.4D	75-5000	Y= 0.0137x -7.92	0.9988	2.3	800	22.5	35.6
NAA	50-5000	Y=0.230x -10.3	0.9996	5.9	646	13.9	46.5
IAA	50-5000	y= 0.139x-11.0	0.9993	6.1	723	20	36.2
SA	40-5000	y= 0.39x± 4.28	0.9996	1.0	1008	13.3	75.8

Table 1: Analytical performance of ASEI-CE–UV for target phytohormones

a) RSD: GA 1 ng/mL, ABA 400 ng/mL, JA 1 ng/mL, 2,4-D 200 ng/mL, NAA 200 ng/mL, IAA 200 ng/mL, SA 100 ng/mL.

b)SI: standard injection, 50 mbar 5 s.

c)EF = LOD(CE–UV)/LOD(ASEI-CE–UV).

Analytes	Linear Range (ng/ml)	Linear Equation	R^2	RSD[a] n=7	LOD(ng/ml)		EF[c]
					SI[b]	ASEI	
GA	50-2000	y= 0.095x ± 8.52	0.9993	9.1	5070	16.5	307
ABS	3-50	y = 2.10 x- 4.25	0.9995	11.4	1204	1.00	1204
JA	6-2000	y= 0.867 x-4.48	0.9994	6.9	12500	2.21	5650
2.4D	1-500	Y= 0.7.12x±14.2	0.9989	11.1	800	0.33	2424
NAA	0.5-500	y= 17.3 x - 8.1	0.9999	12.9	646	0.17	3800
IAA	2-500	y= 8.33 x- 9.13	0.9978	12.0	723	0.67	1079
SA	0-2500	y= 28 x ± 19	0.9999	4.7	1008	0.05	20160

Table 2: Analytical performance of MS-LLLME-ASEI-CE–UV for phytohormones

3.2. Optimization of ASEI conditions

ASEI, a powerful on-column pre concentration technique, was utilized to further enrich the seven phytohormones for CE. To obtain a high sensitivity, the parameters affecting the enrichment factors including the injection voltage and the length of the water plug were investigated in detail.

3.3. Analytical performance of ASEI-CE–UV

Under the optimal conditions of ASEI, the analytical performance of the proposed MS-LLLME-CE–UV method was evaluated, and the results are listed in Table 2. Good correlation coefficients (R2) ranging from 0.9988 to 0.9999 for ASEI were achieved over the studied concentration range. The reproducibility, which is expressed by the relative standard deviations

(RSDs) of the corrected peak areas that were obtained from seven consecutive injections, was less than 6.1%. Based on an S/N of 3,

The LODs for seven target phytohormones were in the range of 13.3–333.3 ng/mL. The enrichment factor (EF) was evaluated, which is defined as the ratio of LODs obtained by standard injection (at 50 mbar for 5 s) to those obtained by ASEI-CE–UV. The EFs were in the range of 30.1 to 75.8. The results demonstrated that the EF of SA was much higher than that for the other phytohormones, which was most likely due to the high electro migration rate achieved by SA with smaller molecular and higher charge number.

3.4. Analytical performance of MS-LLLME-ASEI-CE–UV

Therefore, the optimized MS-LLLME-ASEI-CE–UV conditions were as follows: 9.1 mL of the sample solution containing 10% (m/v) NaCl and 0.1 mol/L HCl were used as the donor phase. Two-hundred microliters of a mixture of phenetole and 1-octanol (6:4(v/v)) was used as the extraction solvent. Ten microliters of 0.05 mol/L $NH_3 \cdot H_2O$ was used as the acceptor phase. In addition, the stirring rate was set to 800 rpm, with an extraction time of 50 min, an injection voltage of −10 kV and a 24-cm-long water plug for ASEI. For the MS-LLLME-ASEI-CE–UV analysis of the seven phytohormones, the extraction process was performed as described in the MSLLLME procedure section. The acceptor phase for MS-LLLME was directly injected into the capillary for further ASEI-CE–UV analysis. All of the validation data for the MS-LLLME-ASEI-CE–UV method are shown in Table 3. Good linearity, with correlation coefficients ranging from 0.9978 to 0.9999, was obtained for the seven target phytohormones. The precision of this method was determined by consecutively analyzing a standard solution with a fixed concentration for seven times, and the RSDs were determined to be 4.7–12.9% for the target phytohormones. The LODs based on an S/N of 3 were in the range of 0.05–16.5 ng/mL. The enrichment factors, calculated as the ratio of LODs obtained from direct CE–UV analysis with standard injection mode (at 50 mbar for 5 s) and that from the MS-LLLME-ASEI-CE–UV, were in the range of 307 to 20,160. To further evaluate the effectiveness of the proposed method, the analytical performance obtained in this work was compared with that of several other approaches for phytohormone analysis. As shown in Table 4, the LODs obtained by the proposed method are lower than those previously reported [4,29], which were obtained using an expensive, sensitive MS detector. In addition, the LODs obtained by the proposed method are better than those obtained using HPLC–UV [9,30] and comparable to those obtained with CE-LIF [13]. Compared with the LVSS-MEKC-UV method without micro extraction [20], the proposed method is more sensitive and can be applied to complex samples. It is important to note that the proposed method, which uses an inexpensive commercial UV detector, exhibited low LODs and good sample cleanup ability.

IV.CONCLUSION

In the current study, a novel, inexpensive, efficient and effective method involving a CE–UV instrument for the analysis of multiple phytohormones was developed. The MS-LLLME pre concentration technique combined with an online ASEI enhancement procedure provides an attractive alternative to ultrasensitive detectors, which are relatively expensive due to their high instrumental operating costs. The described approach exhibits high sensitivity and good sample cleanup ability and is highly suitable for the determination of phytohormones in samples with complex matrices.

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