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Ultrasound Wave Assisted-Matrix Solid Phase Extraction for Determination of Synthetic Plant Growth Regulators Residues in Fruits and Vegetables by Ultra-High Performance Liquid Chromatography

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An effective method using ultra-high performance liquid chromatography (UHPLC) was developed and optimized to obtain a complete separation of four representative synthetic plant growth regulators *e.g.*, [2,4-dichlorophenoxyacetic acid (2,4-D), thidiazuron, forchlorfenuron and paclobutrazol] in fruits and vegetables. Extraction was performed with acetonitrile containing 0.5 % (v/v) acetic acid + 5 g sodium chloride (pH 4) under 3 min with an ultrasound wave assisted bath (40 kHz, 50 W), then 2 mL organic extraction and 1 g sorbents mixed, purified, analyzed by UHPLC and HPLC. The performance of chromatography was assessed by UHPLC and HPLC. Good recoveries and relative standard deviation were found for all analytes in fruits and vegetables.

Keywords: Synthetic plant growth regulators, Ultrasound wave assisted extraction, Matrix solid phase extraction, UHPLC.

INTRODUCTION

Synthetic plant growth regulators (SPGRs) are a class of chemical substance, with similar physiological activity to phytohormones, that play crucial roles in the regulation of plant growth and development, including the regulation of root growth[1], shoot branching[2], nodulation[3], meristem functionality[4] and modulation of fruit set[5].

Synthetic plant growth regulators have appeared to be extensively used in edible plants in many countries. The residue level of SPGRs in foods, especially in fruits, received more and more attention. Zhang *et al.*[6] reported that the residue of forchlorfenuron was detected in fruit and vegetable from 2.2 to 23 μg/kg, Shi *et al.*[7] reported that the concentrations of 2,4-dichlorophenoxyacetic acid (5.1-1503 μg/kg) and paclobutrazol (1-1381 μg/kg) found in orange and peach, respectively in China. Owing to the potential risks posed by SPGRs, a rapid and convenient multi-residue analysis method of SPGRs for pursuing routine analysis was clearly needed.

Gas chromatography-mass spectrometry (GC-MS) for SPGRs was being used less, because of laborious and time-consuming derivative pretreatment[8,9]. The trend for SPGRs analysis was the use of high performance liquid chromatography (HPLC) or liquid chromatography-mass (LC-MS). Some methods were established for determination single-SPGR by HPLC, for example, paclobutrazol in apple[10,11], 2,4-dichlorophenoxyacetic acid in fertilizer[12]. Others developed methods to quantify multi-SPGRs in grape[13] and tomato[14] by LC-nMS or LC-Tof[15]. However, the strong polarity of most of the SPGRs caused them to be difficult separated in traditional analytical methods[16].

Traditional purification procedures such as liquid-liquid extraction[11], solid-phase extraction[10] had been used for the purification of SPGRs in plants and the large solvents were harm to environment.

To eliminate some of the difficulties associated with solvent extraction of SPGRs residues, a rapid and simple method based on a modified version of solid-phase extraction called matrix solid-phase dispersion (MSPD) were present[17,18]. The main benefits of MSPD include flexibility, selectivity and the possibility of minimizing extraction and cleanup steps, resulting in a drastic shortening of the analysis time and lower solvent consumption[19].

On the other hand, sonication in an ultrasonic bath, or using other devices such as cylindrical probes, provides an efficient contact between the solid and the extractant, which typically result in higher recovery rates of the target analytes[18]. So far, no methods have been published regarding the application of ultrasound-assisted MSPD to the determination of SPGRs.

The aim of the present work was to develop and validate ultra-high performance liquid chromatography (UHPLC) method for the simultaneous determination of SPGRs (2,4-dichlorophenoxyacetic acid, thidiazuron, forchlorfenuron and paclobutrazol) in

fruit and vegetable, taking advantage of all the benefits from the hyphenation of ultrasonic-assisted MSPD for sample preparation without a complex pretreatment procedure.

EXPERIMENTAL

All reagents were of analytical grade, except for acetonitrile (HPLC-grade) and methanol (HPLC-grade) from Merck (Darmstadt, Germany) and dichloromethane, ethanol and isopropyl alcohol from Shanghai Chemical Reagent (Shanghai, P.R. China). Organic free water was obtained from Milli-Q A10, Millipore (Bedford, MA, USA). Solid phase extraction cartridge: Primary secondary amine (PSA, 20-45 μm), amino-NH_2 (20-45 μm), florisil (100-200 μm) and Base alumimun oxide -Al_2O_3 (45-100 μm) were obtained from Agilent Co. Ltd. (America).

Certified standards: 2,4-Dichlorophenoxyacetic acid (2,4-D), thidiazuron, forchlorfenuron and paclobutrazol (purity higher than 95 %) were purchased from Dr. Ehrenstofer GmbH (Augsburg, Germany) and their chemical structures were shown in Fig. 1. Stock solution was prepared at 1000 mg/L in methanol. Mixed standard solutions were prepared by dilution of the stock solutions with methanol. All of the solutions were stored at -20 °C.

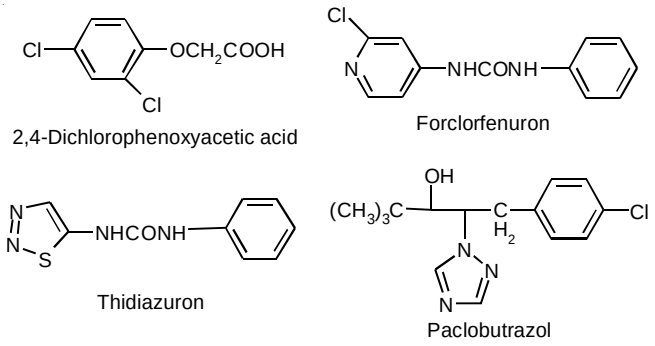

Fig. 1. Structure of four plant growth regulators

Sample extraction and cleaning: A 10 g portion sample, 5 g of sodium chloride and 10 mL of 0.5 % acetic acid-acetonitrile were added and homogenization was enhanced by sonication for 3 min in an ultrasonic bath (40 kHz, 50 W). Afterward, 2 mL organic phase is typically mixed with 1 g of the dispersant sorbents in a mortar with a pestle in fume hood until the solvents were evaporated, then the matrix was transferred into an empty cartridge reservoir and coelution was removed by washing the sorbent bed with 4 mL petroleum ether and finally the target analytes were eluted with 5 mL of 5 % methanol in dichloride. The samples were subsequently evaporated to dryness under a nitrogen stream in a water bath at 45 ºC and the residues were dissolved in 1 mL mobile phase solution and analyzed by UHPLC or HPLC.

Chromatographic condition

UHPLC condition: Agilent 1290 UHPLC system equipped with an auto-sample, a quaternary pump system and a DAD detector, thermostated column compartment, degasser and data software. The analytical column Zorbax RRHD 300SB (Agilent Co. Ltd., USA) with 1.8 μm octadecylsilane chemically bonded silica C18 (50 mm × 2.1 mm) was used. The mobile phase consisted of methanol and 20 mM ammonium acetate (52: 48 v/v), which was pumped at 0.4 mL min^{-1} and run at 30 ºC. The injection volume was 1 μL.

HPLC condition: A Waters Alliance 2695 system equipped with an auto-sample, a quaternary pump system and a 996 detectors, thermostated column compartment, degasser and Empower software. The analytical column was Symmetry (250 mm × 4.6 mm, 5 μm) from Waters Co. Ltd. There were the same conditions with UPLC, except the flow rate and injection volume. The higher flow rate (1.0 mL min^{-1}) the larger injection volume (10 μL) was set in HPLC than in UHPLC.

The two chromatographic systems were compared by determining the following parameters: resolution, repeatability and reproducibility of retention time and peak area and sensitivity.

The resolution (R) allowed defining the quality of the separation of two neighbouring peaks. For two peaks (i and j), R was defined as $2 \times (Rt_j - Rt_i)/(w_i + w_j)$, where w was peak width at baseline. Repeatability of Rt and of peak areas was calculated from the repetition of six injections of a mix of pure standards performed the same day and reproducibility was calculated from the repetition of six injections of a mix of pure standards performed on different days spread evenly over 3 months. Repeatability and reproducibility are expressed as residual standard deviations (RSD %).

The instrument detection limit (LOD) and limit of quantification (LOQ) were estimated through ten repetitive injections of standard mix solution, which can detect at a signal-to-noise ratio (S/N) of three multiples and ten multiples, respectively.

RESULTS AND DISCUSSION

Optimization of sample preparation: Various organic solvents[10-12] were succeeded to extract SPRGs, but it was difficult to extract 2,4-dichlorophenoxyacetic acid from neutral organic solvents (pKa < 4)[20,21]. So, the pH condition of the extraction was critical for developing a multi-residue extraction method. Fig. 2 showed the extract efficiencies of target SPGRs at different pH conditions. As expected, the recoveries of 2,4-dichlorophenoxyacetic acid were greatly improved when the pH value of extraction was decreased from 7 to 4, which can be attributed to the dissociation equilibrium moving toward the neutral forms with the acidity increasing. For thidiazuron, forchlorfenuron and paclobutrazol, the recoveries were slightly increased from 78 to 92 %.

The effect of ultrasound wave-assisted extraction was studied at different times under three setting conditions (40 kHz, 50 W, 100 W and 250 W). With a rise in the setting powder, the recoveries increased slowly in 3 min. Thus, acetonitrile added to 0.5 % acetic acid (pH 4) under 3 min with an ultrasound wave assisted bath (40 kHz, 50 W) was chosen as the extraction solution.

Optimization of MSPE sorbents: To reduce the matrix affection, MSPE was used to further purify the SPRGs from vegetable and fruit. In the study, the sorbents of primary secondary amine, NH_2, base-Al_2O_3, florisil was evaluated by sample spiked with 0.1 mg/kg of target SPGRs. As shown in

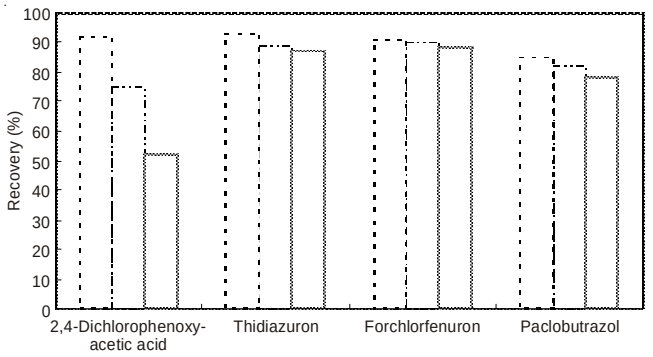

Fig. 2. Effect of pH of the extraction on the recoveries of the SPGRs spiked at 0.1 mg/kg: (a) 0.5 % (v/v) acetic acid in acetonitrile (pH 4), (b) 1 g of ammonium acetate in acetonitrile (pH 6.5), (c) acetonitrile (pH 7) (n = 3)

Fig. 3, the resolution of thidiazuron was good in primary secondary amine and NH_2, but 2,4-dichlorophenoxyacetic acid, thidiazuron and co-extraction was not separated with florisil and base- Al_2O_3, which used as normal phase sorbent. Comparatively, it was provided good recoveries (80-110 %) for SPGRs by primary secondary amine and thus was selected as sorbent in this study.

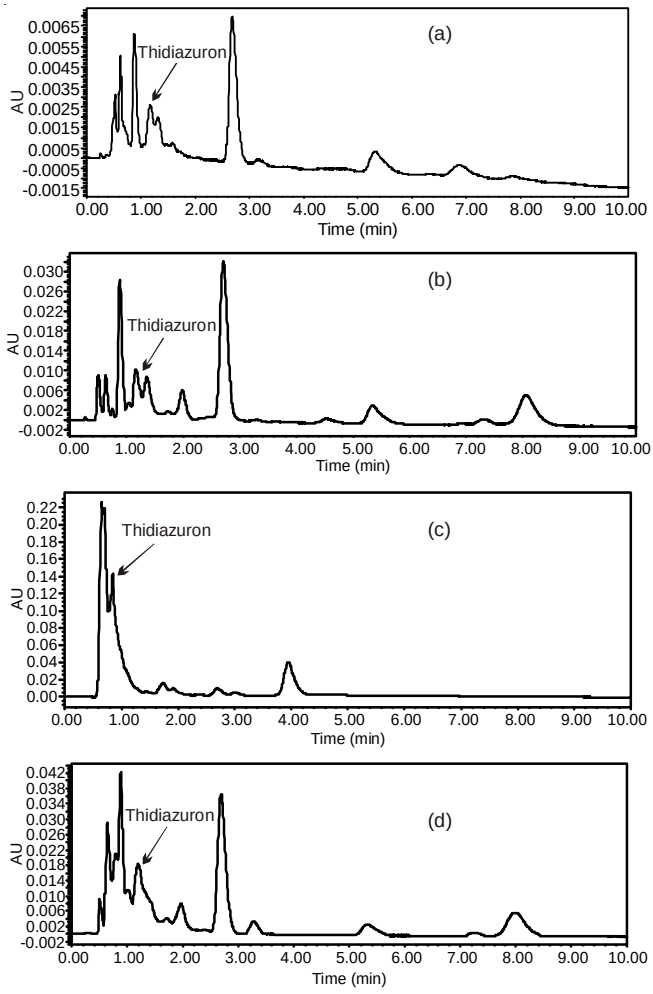

Fig. 3. UHPLC chromatograms of four plant growth regulators under MSPD sorbents: (a) primary secondary amine elution with 95 % dichloromethane: 5 % methanol; (b) NH_2 elution with 95 % dichloromethane: 5 % methanol; (C) Florisil elution with 30 % acetone: 70 % hexane; (d) base-Al_2O_3 elution with 30 % acetone: 70 % hexane

Resolution: The Purnell equation for resolution in chromatography[22] showed that improvements in column efficiency that may be had by reducing the particle size. Therefore increasing the column length and flow rate by the same factor will enable separations to be achieved in a much reduced run time without a loss of resolution. But which factors were it more important? The key then was to exploit the physical robustness of with 1.7 µm particles in UHPLC, will brought about an increase in k to resolve the critical most difficult to separate peaks. Meanwhile, having achieved the desired separations, the linear velocity was increased to a point where the working pressure was high but was still comfortably below the maximum operating pressure of 16000 psi. Fig. 4 showed chromatograms of SPGRs, obtained with HPLC (A) and UHPLC (B). However, in studying the effect of efficiency and selectivity on resolution, retention time must first be considered. The resolution was achieved in a four-fold run time in HPLC than UHPLC.

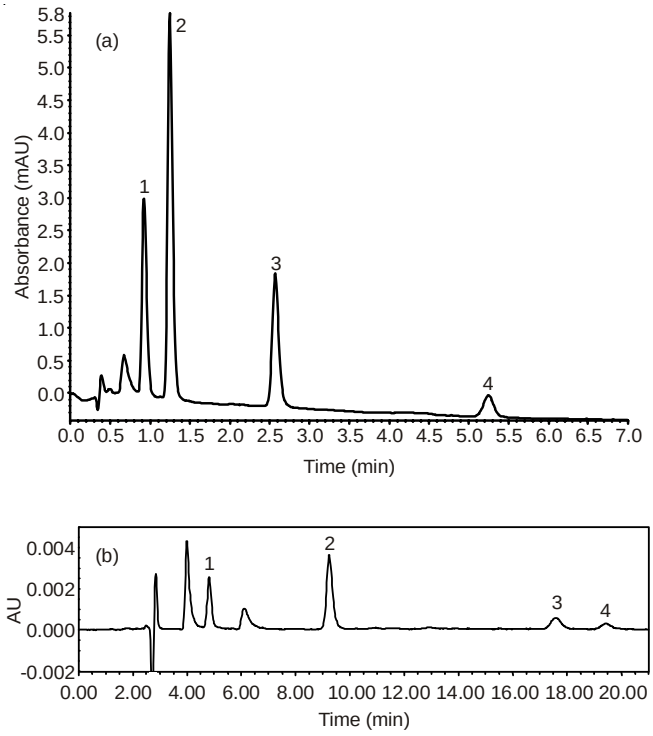

Fig. 4. Chromatogram of four plant growth regulators (1, 2,4-dichlorophenoxyacetic acid, 2, thidiazuron, 3, forchlorfenuron, 4 paclobutrazol), a), isocratic mobile phase in UPLC; 52 % methanol, 48 % 20 mM ammonium acetate in water on Zorbax RRHD 300SB-C18 (1.8 µm, 50 mm × 2.1 mm ID); 8955 psi. pressure at 0.4 mL/min, 1 µL injection; b), Symmetry C18 (5 µm, 250 mm × 4.6 mm ID) in HPLC with same mobile phase; 2650 psi pressure at 1.0 mL/min, 10 µL injection

Maximum absorbing wavelength: The maximum absorbing wavelength was different with SPGRs in Fig. 5. As shown in Fig. 5a, SPGRs were recorded at 230 nm; however, forchlorfenuron was more sensitive at 270 nm. It can be validated the peak's purity on 270 nm, excepting paclobutrazol.

Reproducibility and repeatability of retention times and peak areas: Reproducibility and repeatability of Rt (Table-1) was similar between UHPLC system and HPLC system. The lower performance of the HPLC system on

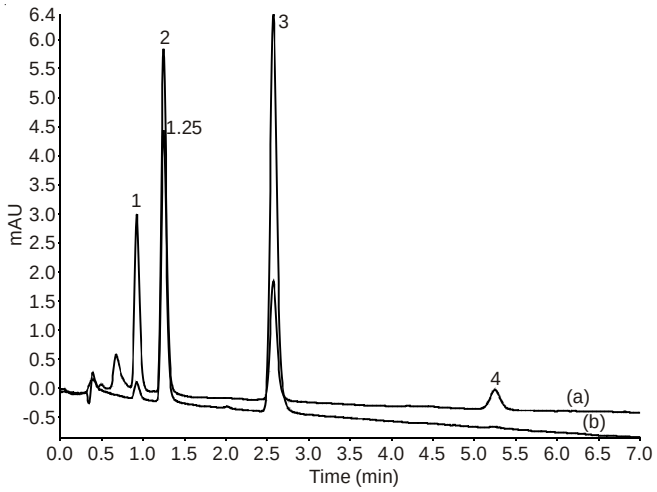

Fig. 5. UHPLC chromatograms of four plant growth regulators (**1**, 2,4-dichlorophenoxyacetic acid, **2**, thidiazuron, **3**, forchlorfenuron, **4** paclobutrazol) under two wavelengths: (a) 230 nm, (b) 270 nm

reproducibility and repeatability of peak area can be explained by the lower resolution and longer retention time making peak integration difficult to perform. For the same reasons, repeatability of peak areas was better with UHPLC than HPLC.

Limit of detection (LOD) and limit of quantity (LOQ): To obtain alike LOD and LOQ, the larger injection volume (10 µL) was used by HPLC than UPLC. The results showed that the sensitivity for SPGRs was threefold higher with UHPLC than with HPLC (Table-2). This was primarily due to the better resolution and shorter retention in UHPLC. Table-2 also showed the calibration equation obtained as a result of the triplicate injection of five standard concentrations with UHPLC system and HPLC system. The calibration graph of coefficient (R^2) was better with UHPLC system than with HPLC system.

Recovery study: Accuracy of the whole method was evaluated by the development of a recovery study carried out at three concentration levels (0.01, 0.05 and 0.1 mg/kg). All experiments were carried out in quintuplicate at each level (results are shown in Table-3). As it can be seen in Table-3, recovery values were satisfactory, ranging between 78 and 110 % with RSD lower than 12 %. As it can be seen from the RSD values, the method was reproducible and applicable to the analysis of SPRGs in fruits and vegetables.

Conclusion

In this study, there were significantly improved conditions that meet the requirements for the residue analysis of four SPGRs concerning speed and economy. The optimal chromatographic separation and sensitivity was successfully applied to the analysis of large amounts of samples.

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TABLE-1
RETENTION TIMES (Rt), REPRODUCIBILITIES, REPEATABILITIES WITH THE TWO SYSTEMS

Component	HPLC					UHPLC				
	Rt (min)	Reproducibility (RSD %)		Repeatability (RSD %)		Rt (min)	Reproducibility (RSD %)		Repeatability (RSD %)	
		Tr	Area	Tr	Area		Tr	Area	Tr	Area
2,4-D	4.863	2.68	6.19	2.17	3.87	1.152	2.52	2.67	0.23	0.91
Thidiazuron	9.324	3.17	7.23	1.05	5.11	1.536	2.16	2.21	1.47	1.76
Forchlorfenuron	17.831	2.97	8.27	1.21	7.62	2.732	2.17	1.65	1.11	1.65
Paclobutrazol	19.042	2.51	9.03	0.98	6.21	3.862	2.48	3.06	1.35	1.97

2,4-D = 2,4-Dichlorophenoxyacetic acid

TABLE-2
LIMITS OF DETECTION, QUANTIFICATION, CALIBRATION EQUATION

	2,4-Dichlorophenoxyacetic acid	Thidiazuron	Forchlorfenuron	Paclobutrazol
		HPLC		
LOD (mg/kg)	0.01	0.01	0.03	0.1
LOQ (mg/kg)	0.004	0.004	0.01	0.03
Calibration equation (n = 5)	$Y= 1.79 \times 10^3X + 2.83 \times 10^3$	$Y= 3.59 \times 10^3X - 2.09 \times 10^4$	$Y= 3.59 \times 10^3X - 2.09 \times 10^4$	$Y= 1.07 \times 10^3X - 3.35 \times 10^4$
Determination coefficient (R^2)	0.998	0.997	0.996	0.996
Linear range tested (µg/mL)	0.1-2	0.1-2	0.2-4	0.5-10
		UHPLC		
LOD (mg/kg)	0.005	0.003	0.005	0.03
LOQ (mg/kg)	0.001	0.0005	0.001	0.01
Calibration equation (n = 5)	$Y= 1.16 \times 10^4X + 2.32 \times 10^3$	$Y= 1.91 \times 10^4X + 2.02 \times 10^3$	$Y= 2.53 \times 10^4X + 6.07 \times 10^3$	$Y= 2.91 \times 10^4X + 1.02 \times 10^3$
Determination coefficient (R^2)	0.9996	0.9998.	0.9989	0.9996
Linear range tested (µg/mL)	0.05-2	0.05-2	0.05-2	0.2-5

TABLE-3
RECOVERIES FROM TWO SAMPLES (FIVE REPLICATES)

Sample	Spiked value (mg/kg)	2,4-Dichlorophenoxy-acetic acid		Thidiazuron		Forchlorfenuron		Paclobutrazol	
		RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
Strawberry	0.03	7.89	78.7	3.51	90.6	4.17	102.6	4.81	106.3
	0.06	6.54	82.9	4.98	79.3	3.56	95.4	8.91	87.5
	0.10	3.62	88.7	8.75	85.4	1.13	92.3	6.95	93.0
Grape	0.03	10.2	83.9	4.16	92.2	9.87	99.5	3.86	104.9
	0.06	6.51	105.6	7.67	80.7	3.79	89.5	11.2	82.9
	0.10	8.76	92.3	8.35	87.4	7.66	88.3	6.05	83.4
Tomato	0.03	12.1	89.7	11.3	90.6	10.3	80.6	10.3	97.3
	0.06	10.7	93.6	2.91	101.2	8.54	82.7	3.91	89.7
	0.10	8.97	79.2	9.22	93.4	3.62	106.3	9.23	88.6
Cucumber	0.03	9.12	110.2	8.54	87.6	8.32	90.3	7.66	78.9
	0.06	5.23	89.7	5.72	93.2	9.13	96.4	3.44	102.1
	0.10	11.6	92.3	3.97	95.4	5.66	92.3	4.27	99.8

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