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Development of a Method for the Analysis of Four Plant Growth Regulators (PGRs) Residues in Soybean Sprouts and Mung Bean Sprouts by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract A method has been developed for the simultaneous determination of four plant growth regulators (PGRs) residues in soybean sprouts and mung bean sprouts. The sample preparation procedure was based on a QuEChERS method. The method showed excellent linearity ($r^2 \geq 0.9985$) and precision (RSDs $\leq$ 13.0 %). Average recoveries of four PGRs ranged between 74.9 % and 106.3 % at spiking levels 0.05, 0.5 and 1 mg kg^{-1}. The LODs and LOQs were in the ranges of 0.27–9.3 μg kg^{-1} and 0.90–31 μg kg^{-1}, respectively. The procedure was applied to 18 bean sprout samples, and benzyladenine was found in some of the analyzed samples.

Keywords PGRs · Bean sprouts · Residue · LC–MS/MS

The soybean sprout and mung bean sprout, which become mature after 6–7 days germination, have been important traditional vegetable in China. Plant growth regulators (PGRs) are important synthetic phytohormones analogues and play a crucial role at different stages of plant development. They are present at low concentrations and regulate the processes in a plant's life cycle, such as growth and metabolism (Wu and Hu 2009). Some PGRs such as 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellins A_3 (GA), 4-chlorophenoxyacetic acid (4-CPA) and benzyladenine (BA) are widely applied for better growth in the process of the production of bean sprouts. Thus, sprouts produced contain chemical residues that could be hazardous to human health in the long term. In recent years, some poisoned bean sprouts have been reported several times in China. For this reason, the maximum residue limits (MRLs) of some PGRs in bean sprouts, i.e. 2,4-D (0.1 mg kg^{-1}), GA (0.5 mg kg^{-1}), 4-CPA (1 mg kg^{-1}), BA (0.2 mg kg^{-1}), have been established by China government agencies. There has been no MRL of these four PGRs established in bean sprouts by other agencies yet.

In order to monitor and control the compliance of the tolerance level of PGRs and phytohormones, sensitive, accurate and robust analytical methods are needed. Traditionally, gas chromatography (GC) and high performance liquid chromatography were utilized for the analysis of phytohormones (Zhang et al. 2010). But for the confirmation of suspected positive samples, mass spectrometry coupled with the adequate chromatographic separation was one of the most powerful techniques for the residue analysis. Perrine et al (Perrine et al. 2004) reported a method to determine phytohormones using gas chromatography with mass spectrometry (GC–MS). However, complicated derivatization steps prior to GC analysis are necessary to enhance the volatility and sensitivity, and some thermally labile phytohormones could be decomposed for the GC analysis (Hou et al. 2008). Moreover, liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry under multiple-reaction monitoring (MRM) were applied for the determination of phytohormones (López-Carbonell et al. 2009; Giannarelli et al. 2010). Due to its high sensitivity, selectivity, and good linearity, HPLC–MS/MS allows a reliable quantification of compounds at trace levels (Fan et al. 2011). Some

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literatures reported the use of this technique in the comprehensive analysis of several groups of phytohormones (Flores et al. 2011). Furthermore, some analytical techniques using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) have been reported for determining phytohormones in plant in recent years (Hussain et al. 2010). Up to now, little literature was reported on the analysis of PGRs residues in bean sprouts. This paper presents a simple, rapid and sensitive method which allows the simultaneous determination of four PGRs in soybean sprouts and mung bean sprouts, using QuEChERS–LC–MS/MS analysis. The most representative and practically used PGRs were selected to study. Further, it can be applied in routine analysis to provide a large amount of data related to the presence of PGRs in agricultural commodities.

Materials and Methods

Certified pesticide standards (≥95.0 %) for 2,4-D, GA, 4-CPA and BA were purchased from Pesticide Analysis Laboratory, China Agricultural University (Beijing, China). Common names and chemical structures of four PGRs evaluated here are shown in Fig. 1. Acetonitrile and methanol of HPLC grade were obtained from Fisher Chemicals (Fair Lawn, NJ, USA). Ultra-pure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Magnesium sulfate anhydrous (98 %) and sodium chloride (99.5 %) of analytical grade were purchased from Sinopharm Chemical Reagent (Beijing, China). The 0.22 μm nylon syringe filters were used to filter the extracts.

Individual stock standard solutions of 1,000 mg L^{-1} were prepared by exact weighing of the powder and dissolved in 50 mL of acetonitrile and stored at −20°C in the dark. They were stable over a period of at least 3 months. Multicomponent working standard solutions were prepared by diluting each primary standard solution with acetonitrile and were used for spiking samples, preparing matrix-matched calibration standards and studying the linear dynamic range of the LC–MS/MS analysis. Matrix-matched calibration standards, which prepared by adding the extract of blank samples, were in the range of 0.005–0.5 mg L^{-1} for BA and 0.05–2 mg L^{-1} for the other three PGRs. The working solutions were stored in refrigerator at 4°C and in darkness.

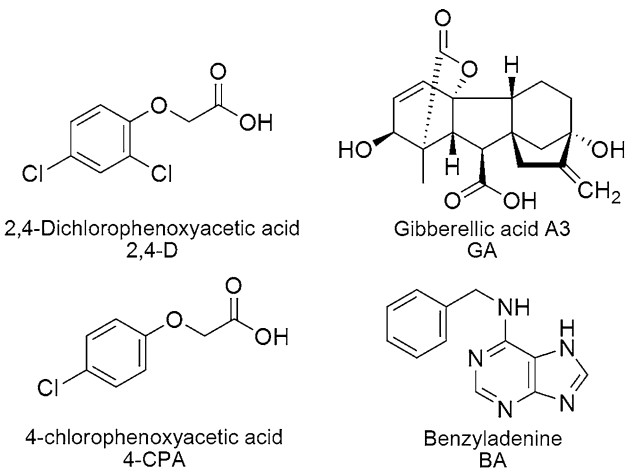

Fig. 1 Chemical structures of the four PGRs investigated

All soybean sprout and mung bean sprout samples were purchased from local supermarkets in Beijing, China. About 500 g of roughly chopped sample was mixed in a conventional blender (A11-basic, IKA, Germany) to obtain thoroughly mixed homogenates. After confirmation with LC–MS/MS analysis that they did not contain the target PGRs, each matrix was used for each experiment. These matrixes were stored at −20°C prior to use.

An amount of 10 g previously homogenized samples, which spiked with standard multicomponent working solution at three concentration levels (0.05, 0.5 and 1 mg kg^{-1}), were placed in a 50 mL centrifuge tube. After 2 mL water and 5 mL acetonitrile added, the centrifuge tube was ultrasound-assisted extracted for 5 min (KQ-50B Ultrasonic cleaner, Kunshan Ultrasonic Instrument Co., Ltd., Jiangsu, China), vortex for 1 min (QL-901, Kylin-bell Lab Instruments Co., Ltd, Jiangsu, China) and then centrifuged for 5 min at 3,800 r min^{-1} (TDL-40B, Anke, China). The supernatants were collected and the residues were re-extracted with 5 mL acetonitrile as the first time. After 4 g anhydrous magnesium sulfate and 1 g sodium chloride added, the merged supernatants were shaken and centrifuged again for 5 min at 3,800 r min^{-1}. Acetonitrile layer was filtered through a 0.22 μm filter membrane and transferred into autosampler vial for LC–MS/MS analysis.

An Agilent 1200 HPLC (Agilent Technologies, USA), equipped with a degasser and an autosampler, was used for the chromatographic analysis. Separation was achieved on a ZORBAX C_{18} column, 50 mm × 2.1 mm, 1.8 μm (Agilent), with a flow rate of 0.3 mL min^{-1}. The isocratic elution condition employed a mobile phase of methanol (mobile phase A) and water (mobile phase B). The elution started at 15 % A and was linearly increased up to 75 % in 8 min. The composition was held for 2 min before being returned to the initial conditions in 2 min, hold for 3 min, before next injection. The inject volume was 5 μL and column temperature was maintained at 30°C. Mass spectrometry was carried out using an Agilent 6410 Triple Quadrupole LC/MS (Agilent). The instrument was operated using ESI ionization in positive and negative ion modes separately. Nitrogen was used for both nebulizer and collision gas. The drying gas temperature was 350°C

with the flow rate 8.0 L min^{-1}. The nebulizing gas pressure was 35 psi.

Results and Discussion

In order to identify and quantify the analytes in real samples at trace levels, the MRM transitions and associated acquisition parameters were optimized for the maximum abundance of fragmented ions under ESI positive and negative mode conditions by infusing standard solutions of the target compounds into the LC–MS/MS. Two individual injections were set for positive and negative mode avoiding alternative scanning, which would cause loss of data points. Identification of the parent ion as well as the choice of the ionization mode for each analyte was performed in the full scan mode by recording mass spectra from m/z 50–400. BA was analyzed in the positive scan mode, and the precursor ion was selected as $[M + H]^+$ ions; while 2,4-D, GA and 4-CPA were analyzed in the negative scan mode, and the precursor ion was selected as $[M - H]^-$ ions. Different fragmentor voltages were optimized in single MS in order to obtain the most abundant precursor ions. Then dissociation was induced and different collision energies were tested in order to select the most sensitive transition for quantification purposes. The most sensitive transition in MRM mode was selected for quantification in the screening method. The optimum values for each condition for each compound are summarized in Table 1, and the most intense characteristic MRM transitions were chosen for each analyte. Figures 2 and 3 show the typical total ion chromatogram and extracted ion chromatogram of MRM mode for four PGRs at concentration 0.5 mg kg^{-1}.

The method was validated for linearity, matrix effect (ME), detection and quantification limits, selectivity, accuracy and precision. The calibration was performed by the external standard method for quantification in the experiment. The matrix-matched calibration standards prepared as described in experimental section in order to compensate for the ME. Molecules originating from the sample matrix that coelute with the compounds of interest can interfere with the ionization process in the mass spectrometer, causing ionization suppression or enhancement, which is the so-called ME. The ME of the present method was investigated by comparing standards in solvent with matrix-matched standards. 2,4-D, GA and 4-CPA calibration curves were constructed at the concentrations of 0.05, 0.1, 0.5, 1 and 2 mg L^{-1} in soybean sprouts and mung bean sprouts. BA calibration curves were constructed at the concentrations of 0.005, 0.01, 0.05, 0.1 and 0.5 mg L^{-1}. Table 2 summarizes the analytical results including slope, r^2 and ME values calibrated by using slope ratio method in two matrixes. Good linearity of the response was found for all PGRs at concentrations within the tested interval, with linear determination coefficients above 0.9985. The ME values of four PGRs in soybean sprouts and mung bean sprouts are between 0.060 and 0.703.

The LODs and LOQs were defined as the concentration with a signal-to-noise ratio (S/N) of three and ten using the less intensive ion transition for each analyte. This parameter was determined by analysis of a series of decreasing concentrations of the spiked sample in multiple replicates. The LODs and LOQs values obtained for four PGRs were in the ranges of 0.27–9.3 μg kg^{-1} and 0.90–31 μg kg^{-1}, respectively. The LODs and LOQs results are shown in Table 2. The selectivity was evaluated by the analysis of four blank samples. Specificity was found to be satisfactory, with no chromatographic interference being observed around the retention time of the target compounds. The accuracy and precision of the method were assessed using soybean sprout and mung bean sprout samples fortified with three different levels (0.05, 0.5, and 1 mg kg^{-1}). The recovery, repeatability and reproducibility were determined by the relative standard deviation. The repeatability RSD_r (intra-day precision) was measured by comparing standard deviation of the recovery percentages spiked samples run the same day. The reproducibility RSD_R (inter-day precision)

Table 1 Retention times and MS/MS parameters of the selected PGRs

PGRs	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)	Ionization mode
2,4-D	10.39	219.0	125	80	20	−
			161[a]	80	10	−
GA	7.12	345.1	143[a]	120	20	−
			239	120	5	−
4-CPA	6.57	184.9	127[a]	80	10	−
			141	80	8	−
BA	10.96	226.1	91[a]	120	20	+
			148	120	15	+

[a] MS transition used for quantification

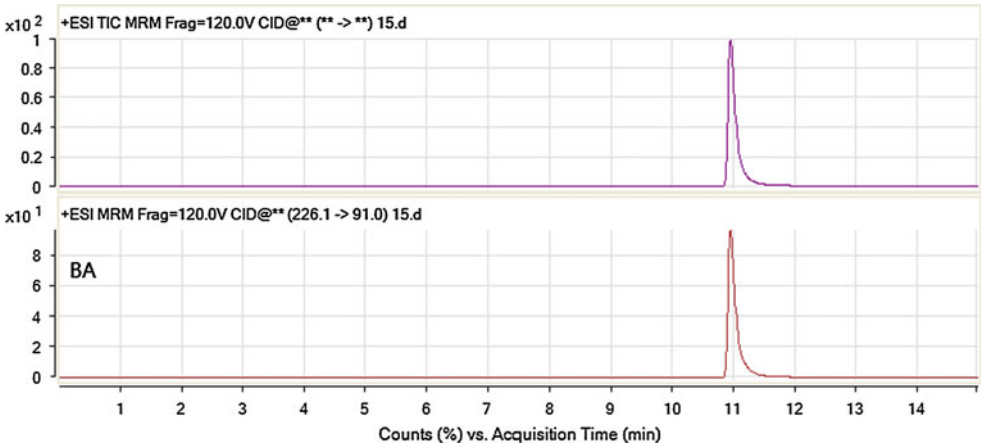

Fig. 2 LC–MS/MS total ion chromatogram and extracted ion chromatogram (MRM positive mode) of BA, 0.5 mg kg^{-1}

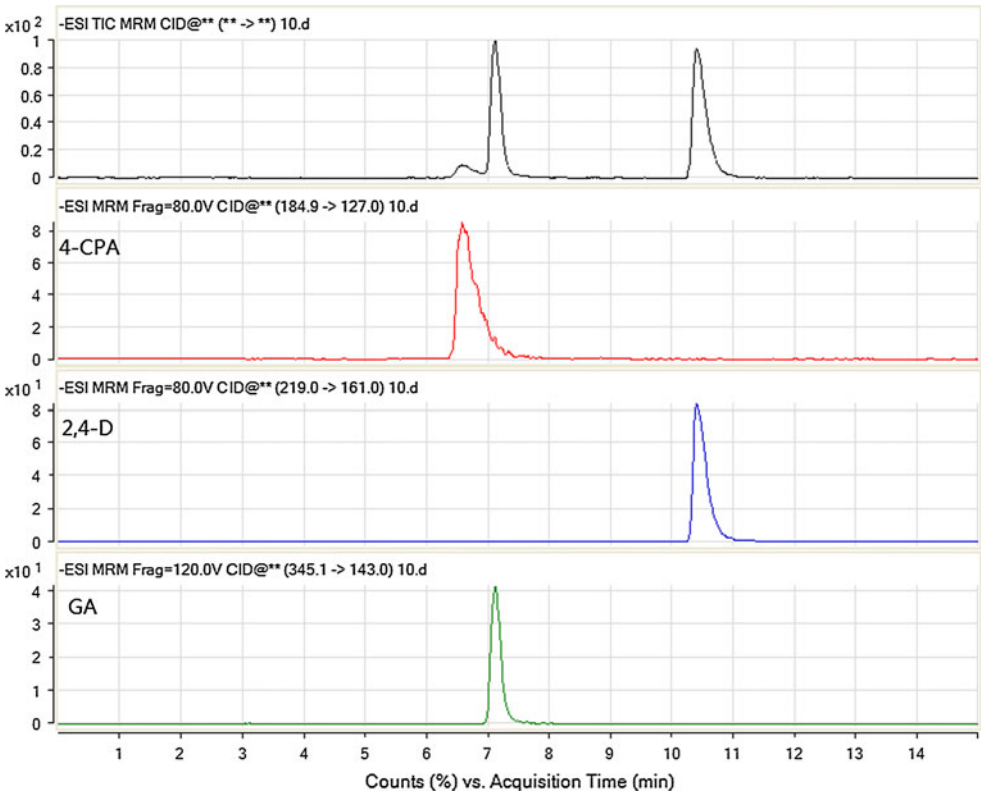

Fig. 3 LC–MS/MS total ion chromatogram and extracted ion chromatogram (MRM negative mode) of GA, 2,4-D and 4-CPA, 0.5 mg kg^{-1}

was determined by analyzing spiked samples for three alternate days. Good corrected recoveries were obtained for each of the four PGRs at all fortification levels as shown in Table 3. The average recoveries ranged between 74.9 and 106.3 % with intra-day RSD_r values between 0.8 % and 8.2 %. Inter-day repeatability was found satisfactory for the four compounds under survey ($RSD_R \leq 13.0$ %).

The effectiveness of this method in measuring trace levels of these four PGRs was checked by analyzing 18 bean sprout samples (some bean sprout manufacturers in China). Seven soybean sprout and four mung bean sprout positive samples were detected, which contained 16–47 μg kg^{-1} and 10–47 μg kg^{-1} of BA, respectively. However, none of the samples analyzed showed residues of the other three PGRs at detectable levels.

In summary, a simple, rapid and sensitive method in which QuEChERS–LC–MS/MS in positive and negative MRM mode has been successfully applied to the determination of four PGRs residues (2,4-D, GA, 4-CPA and BA) in soybean sprout and mung bean sprout samples. The method developed shows satisfactory validation parameters in terms of linearity, low limits, accuracy and precision. The average recovery in all matrixes for each PGR ranged between 74.9 % and 106.3 %. The uncertainty associated to the

Table 2 Analytical data for the four PGRs using the proposed method

PGRs	Matrix	Slope	r^2	Matrix effect[a]	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
2,4-D	Solvent	44,381	0.9998			
	Soybean sprout	27,278	0.9993	0.615	9.3	31
	Mung bean sprout	30,767	0.9992	0.693	8.7	29
GA	Solvent	8,551.3	0.9999			
	Soybean sprout	1,568.1	0.9999	0.183	8.4	28
	Mung bean sprout	1,721.8	0.9997	0.201	7.5	25
4-CPA	Solvent	33,736	0.9998			
	Soybean sprout	22,623	0.9998	0.671	5.4	18
	Mung bean sprout	23,720	0.9998	0.703	3.9	13
BA	Solvent	2,775,691	1.0000			
	Soybean sprout	165,985	0.999	0.060	0.60	2.0
	Mung bean sprout	342,713	0.9985	0.123	0.27	0.90

[a] Slope matrix/slope solvent

Table 3 Recoveries, repeatability (RSD_r) and reproducibility (RSD_R) values of the four PGRs at spiking levels 0.05, 0.5 and 1 mg kg^{-1}(n = 5)

PGRs	Spike level (mg kg^{-1})	Soybean sprout			Mung bean sprout		
		Mean (%)	RSD_r (%)	RSD_R (%)	Mean (%)	RSD_r (%)	RSD_R (%)
2,4-D	0.05	85.4	3.1	8.7	83.4	1.8	8.5
	0.5	85.8	1.7	4.1	86.7	2.5	3.5
	1	89.2	1.1	6.4	89.5	4.4	11.8
GA	0.05	106.3	8.2	4.8	96.2	8.1	5.9
	0.5	102.8	3.4	2.7	100.9	2.2	7.7
	1	96.3	1.6	3.7	97.3	5.4	11.0
4-CPA	0.05	87.7	2.8	9.3	99.5	5.3	7.4
	0.5	86.5	3.1	4.3	96.2	3.8	10.4
	1	86.4	3.9	10.2	100.9	2.9	5.9
BA	0.05	78.8	2.3	5.5	80.5	4.1	11.1
	0.5	74.9	1.7	7.4	77.3	7.3	6.7
	1	87.7	0.8	13.0	88.8	2.8	5.1

analytical method, expressed as RSD, was lower than 13 % for all compounds tested in all matrixes. The calculated LOQs (0.90–31 μg kg^{-1}) were much lower than the established MRLs. The procedure was validated and the results demonstrate that it is suitable for analyzing residues of these four PGRs in soybean sprout and mung bean sprout samples. Our work is a reference for routine controls in China and lots of market bean sprout samples have been detected.

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References

Fan SF, Wang XP, Li PW, Zhang Q, Zhang W (2011) Simultaneous determination of 13 phytohormones in oilseed rape tissues by liquid chromatography-electrospray tandem mass spectrometry and the evaluation of the matrix effect. J Sep Sci 34:640–650

Flores MI, Romero-González R, Frenich AG, Vidal JL (2011) QuEChERS-based extraction procedure for multifamily analysis of phytohormones in vegetables by UHPLC-MS/MS. J Sep Sci 34:1517–1524

Giannarelli S, Muscatello B, Bogani P, Spiriti MM, Buiatti M, Fuoco R (2010) Comparative determination of some phytohormones in wild-type and genetically modified plants by gas chromatography-mass spectrometry and high-performance liquid chromatography-tandem mass spectrometry. Anal Biochem 398:60–68

Hou S, Zhu J, Ding M, Lv G (2008) 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 76:798–802

Hussain A, Krischke M, Roitsch T, Hasnain S (2010) Rapid determination of cytokinins and auxin in cyanobacteria. Curr Microbiol 61:361–369

López-Carbonell M, Gabasa M, Jáuregui O (2009) Enhanced determination of abscisic acid (ABA) and abscisic acid glucose ester (ABA-GE) in *Cistus albidus* plants by liquid chromatography-mass spectrometry in tandem mode. Plant Physiol Biochem 47:256–261

Perrine F, Rolfe B, Hynes M, Hocart C (2004) Gas chromatography-mass spectrometry analysis of indoleacetic acid and tryptophan following aqueous chloroformate derivatisation of Rhizobium exudates. Plant Physiol Biochem 42:723–729

Wu Y, Hu B (2009) Simultaneous determination of several phytohormones in natural coconut juice by hollow fiber-based liquid-liquid-liquid microextraction-high performance liquid chromatography. J Chromatogr A 1216:7657–7663

Zhang Y, Li Y, Hu Y, Li G, Chen Y (2010) Preparation of magnetic indole-3-acetic acid imprinted polymer beads with 4-vinylpyridine and β-cyclodextrin as binary monomer via microwave heating initiated polymerization and their application to trace analysis of auxins in plant tissues. J Chromatogr A 1217: 7337–7344