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Determination of Ten Plant Growth Regulators in Bean Sprouts by Mixed Solid Phase Extraction Columns Separation and LC-MS/MS

Chenggang Cai [1], Feng Yao [1], Chuanpeng Li [1], Yannan Xiang [1], Pinggu Wu [2,*], Zhengyan Hu [2], Junlin Wang [2] and Ruiyu Zhu [1,*]

[1] School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou 310023, China; ccg0516@zust.edu.cn (C.C.); 222103855017@zust.edu.cn (F.Y.); 222103855022@zust.edu.cn (C.L.); 212103817019@zust.edu.cn (Y.X.)

[2] Zhejiang Province Center for Disease Control and Prevention, Hangzhou 310051, China; zhyhu@cdc.zj.cn (Z.H.); lywang@cdc.zj.cn (J.W.)

* Correspondence: pgwu@cdc.zj.cn (P.W.); zhuruiyu7@126.com (R.Z.); Tel.: +86-0571-87115263 (P.W.); +86-0571-85070375 (R.Z.)

Abstract: (1) Background: Plant growth regulators (PGRs) can accelerate growth or improve the quality and quantity of bean sprouts but are forbidden to use in bean sprout cultivation, as the sprouting process's increased chemicals will disturb the PGRs analysis. This article aimed to increase the accuracy and level of sensitivity of the LC-MS/MS method for the simultaneous analysis of 10 PGRs after mixed solid phase extraction (SPE) purification. (2) Methods: An LC-MS/MS detection method for 10 kinds of PGRs was established based on ESI ionization in the positive ion mode for 6-furfurylaminopurine (6-KT), paclobutrazol (PBZ), indole-2-acetic acid (IAA), and indole-3-butyric acid (IBA) and in the negative ion mode for gibberellin A3 (GA_3), 2,4-dichlorophenoxy acetic acid (2,4-D), 4-chlorophenoxyacetic acid (4-CPA), forchlorfenuron (FCF), thidiazuron (TDZ), and 6-benzyl adenine (6-BA). (3) Results: The 10 PGR compounds were detected within a concentration range of 1.0–50 ng/mL. The average recovery was 68.3–97.3% with relative standard deviations (RSD) of 4.6–15.2% (n = 6); the limit of detection (LOD) and limit of quantification (LOQ) were found to be 2 and 5 ng/g, respectively. PGRs were surveyed in 36 soybean sprouts and 33 mungbean sprouts; the results showed that 4-CPA and IAA were detected in 10 soybean sprouts and 10 mungbean sprouts, respectively. Five samples contained both 4-CPA and IAA. (4) Conclusions: The established method is simple, rapid, accurate, and highly sensitive for the detection of PGR residues in bean sprout products.

Keywords: bean sprout; food safety; liquid chromatography/mass spectrometry/mass spectrometry; plant growth regulator

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1. Introduction

Sprouting is one of the processing methods used to enhance the bioavailability of some nutrients of agricultural products. Bean sprouts are a nutritious, delicious, economical, and popular food. Soybean and the mungbeans are two of the most important edible beans and have been consumed for long time, and germinated soybean and mungbean sprouts are popular fresh vegetables at all times of the year [1,2]. Plant growth regulators (PGRs) represent one of the most important pesticides, regulating several processes of plants such as growth, sprouting, and maturity, as well as protective responses [3].

PGRs are often added during the bean sprout cultivation process to accelerate growth or improve the quality and quantity of the products. However, PGRs such as gibberellin A3 (GA_3), 2,4-dichlorophenoxy acetic acid (2,4-D), 4-chlorophenoxyacetic acid (4-CPA), indole-2-acetic acid (IAA), indole-3-butyric acid (IBA), forchlorfenuron (FCF), thidiazuron

(TDZ), 6-benzyl adenine (6-BA), paclobutrazol (PBZ), and 6-furfurylaminopurine (6-KT) are forbidden for use in bean sprout production [2,4,5]. Nevertheless, reports of illegal PGR addition, such as through the application of "AB powder" (A contains 6-BA, and B contains GA_3), appear frequently enough to cause concern [6]. The overuse of PGRs has become a major issue in the current field of food safety, as these banned substances are known to be causative agents for cancer, osteoporosis, and precocious puberty [7–9].

Several methods have been developed for analyzing the presence of PGRs in food samples, including GC [10,11], HPLC [12–18], GC-MS [5,19,20], electrochemical sensing method [21], and HPLC-MS [22–27]. The GC, HPLC, and GC-MS methods are often utilized, but some of these methods showed the complexity of samples pre-treatment and the low sensitivity of the detection results. Recently, Wang et al. [28] and Feng et al. [29] described rapid, accurate, and highly sensitive LC- and HPLC-MS/MS methods for the detection of PGR residues in food/bean sprout products.

Some pretreatments for sample preparation included the development of graphene-oxide-functionalized cotton-fiber-based solid phase extraction [18], and a hollow core/shell-structured $CuO@SiO_2$ microspheres [21] and a novel magnetic β-cyclodextrin-modified graphene oxide adsorbent [30] were used for PGRs purification before analysis. Although there are different analysis methods with some novel pretreatments for PGR collection and purification, solvent extraction and solid phase extraction methods are commonly used in this stage [28–30]. HPLC with tandem mass spectrometry (LC-MS/MS) offers high sensitivity and high specificity and has become the preferred detection method for the analysis of PGRs. Combining it with the Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) method as pretreatment and the LC-MS/MS method, Pu et al. [24] analyzed 24 plant growth regulators in grapes. The determination coefficients were higher than 0.995, with the limit of quantification ranging from 0.1 to 5 ng/mL. Chlormequat chloride and FCF were analyzed in the 50 tested grape samples. Different from the previous studies, this study aimed to develop an LC-MS/MS method for the simultaneous analysis of 10 PGRs that are likely to be used in bean sprouts. The sprout products contain more complex chemical ingredients, and there are no standard operation procedures for surveying PGRs in sprout products in the local market, so it is necessary to establish a relatively easily operatable method for PGR analysis in bean sprout products. And because of the complicated medium in the sprout production system, the utilization of mixed SPE matrixes was also studied for a fraction of the PGRs. This study aimed to increase the accuracy and sensitivity of an LC-MS/MS method and a standard procedure for the simultaneous analysis of 10 PGRs in bean sprouts.

2. Results and Discussion

2.1. LC-MS/MS Method Development

In this study, a method for 10 PGRs analysis was developed. At present, there is no suitable and inexpensive internal standard for the detection of these compounds, including the isotope internal standard. Standard solutions of 1, 2, 5, 10, 20, and 50 ng/mL of FCF, TDZ, 6-BA, PBZ, and 6-KT were prepared via the addition of 0.01 mL, 0.02 mL, 0.05 mL, 0.1 mL, 0.2 mL, and 0.5 mL of 100.0 ng/mL PGR mixed stock solutions and 10% MeOH/water solution to 1 mL. Then, the standard curve was drawn after LC-MS/MS analysis based on the peak areas and concentrations of standards; the linear range of the standards was 1–50 ng/mL, with correlation coefficients (R^2) ranging from 0.997 to 1.000. The retention times for the 10 compounds are listed in Table 1 and Figure 1. The retention time of the tested PGRs in Table 1 ranged from 2.52 min (IAA) to 4.10 min (PBZ), indicating a rapid analysis of the compounds. The MRM chromatograms of the 50 ng/mL mixed standard solution are listed in Figure 2.

Table 1. Retention time, quantitative ions, qualitative ions, and other mass parameters for the detection of PGRs.

Chemicals	Retention Time (min)	Quantitative Ions *	Qualitative Ions *	Ratio of Quantitative to Qualitative Ions
IAA	2.52	176.3 > 130.1 (10, 24)	176.3 > 103.0 (10, 28)	0.659
GA_3	2.64	345.1 > 143.1 (15, 35)	345.1 > 239.1.1 (15, 20)	0.695
4-CPA	2.83	185.0 > 127.0 (15, 15)	187.0 > 129.0 (15, 10)	0.193
IBA	2.94	204.0 > 130.1 (24, 40)	204.0 > 186.0 (24, 25)	0.194
6-KT	3.01	216.0 > 81.0 (2, 12)	216.0 > 148.0 (2, 16)	0.243
2,4-D	3.02	218.9 > 160.9 (10, 18)	218.9 > 124.9 (10, 35)	0.028
6-BA	3.24	224.0 > 133.1 (15, 36)	224.0 > 106.2 (15, 21)	0.056
TDZ	3.54	219.1 > 100.1 (8, 13)	219.1 > 71.2 (8, 35)	0.095
FCF	3.81	246.0 > 127.0 (10, 15)	246.0 > 91.0 (10, 35)	0.065
PBZ	4.10	294.0 > 70.0 (10, 25)	294.0 > 124.9 (10, 40)	0.142

* (cone voltage, V; collision energy, eV).

The total ion chromatogram of the 10 PGR standard solutions after the addition of standard bean sprout elution I is shown in Figure 3, and the total ion chromatogram of the 5 PGRs (GA_3, 2,4-D, 4-CPA, IBA, IAA) present in elution I from one sample after the addition of 5 ng/g standard solutions is shown in Figure 4. The other PGRs, FCF, 6-BA, SBL, DXZ, and 6-KT, were present in elution II from the same sample, and the total ion chromatogram after the addition of 5 ng/g standard solutions is shown in Figure 5.

Several studies utilized HPLC [17–20] or LC-MS [24–27,31] to analysis the PGR concentration in agricultural or food products. Since PGRs are found in very low levels in sprouts and require a short analysis time and a low detection limit, a new method using LC-MS/MS [28] was developed to analyze the four PGRs of GA_3, 2,4-D, 4-CPA, and 1-naphthalene acetic acid (NAA). For the linear ranges of 0.3–50 ng/mL for GA_3 and 2,4-D, 0.2–50 ng/mL for 4-CPA, and 0.5–50 ng/mL for NAA ($r \geq 0.998$), the detection limits (S/N = 3) ranged from 1.0 to 2.5 ng/g, and the recoveries for spiked soybean sprout samples were in the range of 75.0–93.3%. The change in the recoveries was due to the matrix effects that influenced the analysis results. Due to the types and combinations of the different PGRs, several studies focused on the simultaneous detection of their presence in the sample; for example, Liu et al. [6] analyzed 6 PGRs with the LC-MS/MS method in bean sprouts, Wu et al. [5] detected 10 kinds of PGRs with the GC-MS method in bean sprouts, and there were 5 [26], 7 [27], and 21 [25] kinds of PGRs analyzed with the LC-MS method.

Due to the presence of many complex substrates and interfering substances in the bean sprout matrix, the purified eluent I substrate still had an inhibitory effect on the quantitative analysis of GA_3, 2,4-D, 4-CPA, IAA, and IBA. Quantification required the addition of a standard within the bland matrix. Eluent II demonstrated no obvious effect on the quantification of FCF, TBT, 6-BA, PBZ, and 6-KT, so it was not necessary to carry out supplemental quantification. In order to qualitatively analyze the PGRs, one parent ion and more than two daughter ions were selected for each component. Under the same experimental conditions, the deviation of retention time between the sample and the standard solution should be less than 5%. The relative abundance of the qualitative ions of each component in the sample chromatogram was compared with that of the corresponding qualitative ions in the standard solution chromatogram at a similar concentration. If the deviation did not exceed the range specified in Table 1, it was determined that the corresponding analyte was detected in the sample.

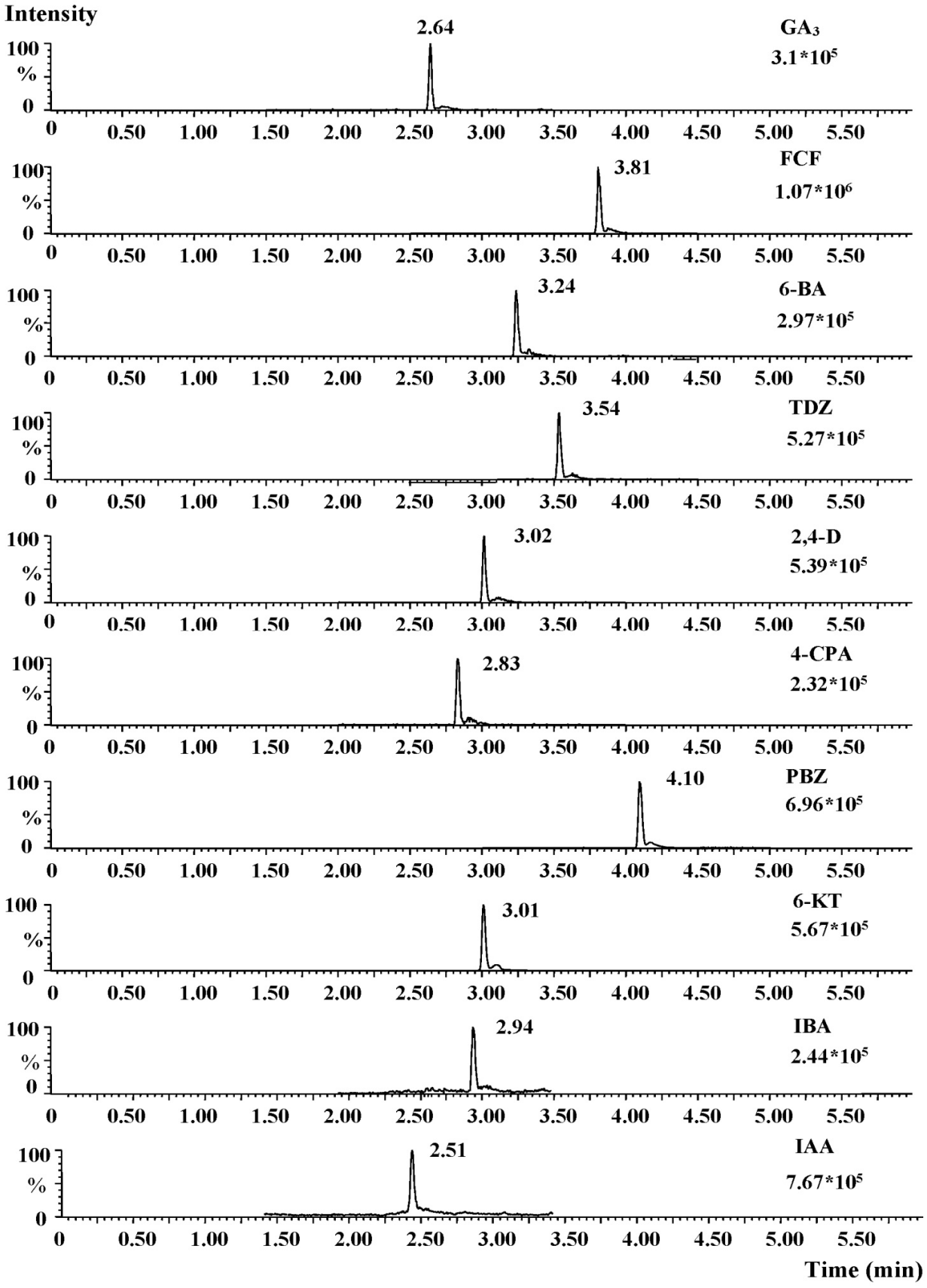
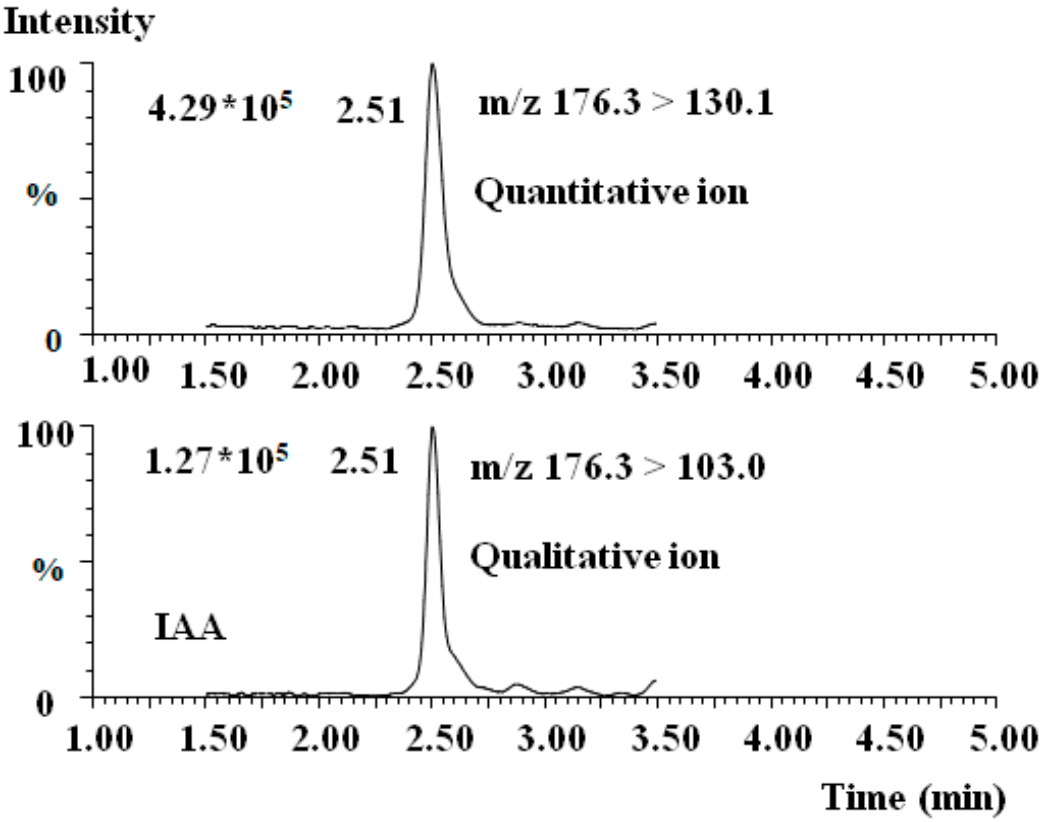
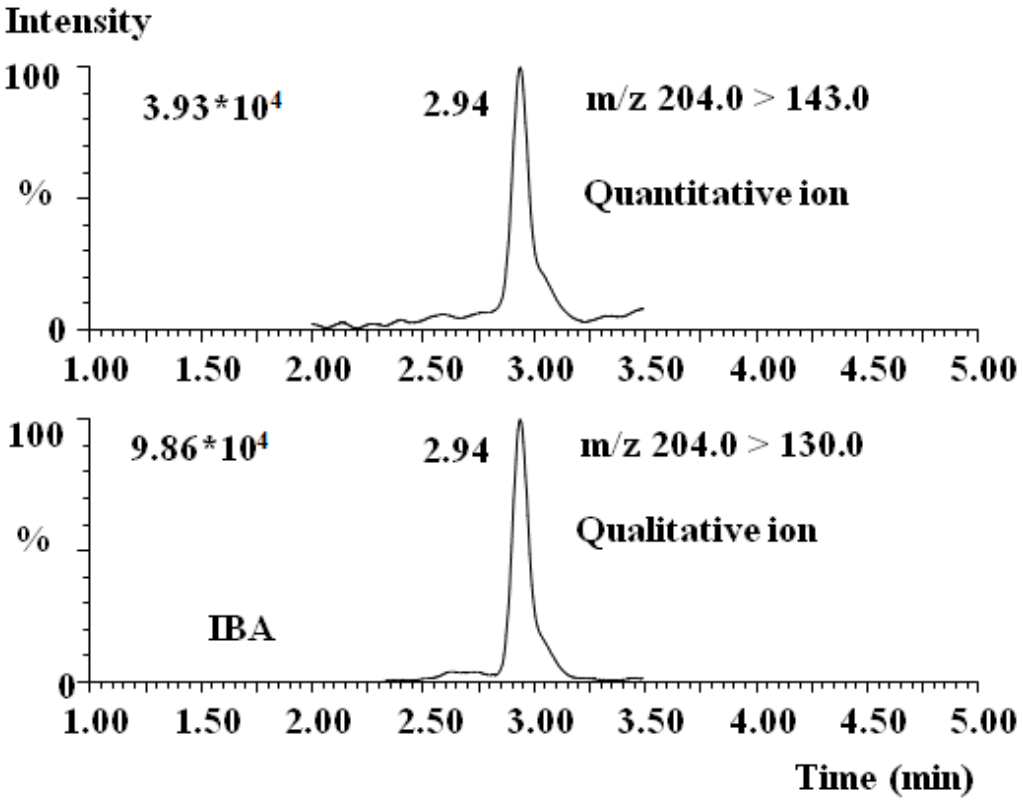


Figure 1. Total ion chromatogram of the 10 PGR standard solutions. Numbers under the PGRs indicate the signal intensity.

Numbers under the PGRs indicates the signal intensity.

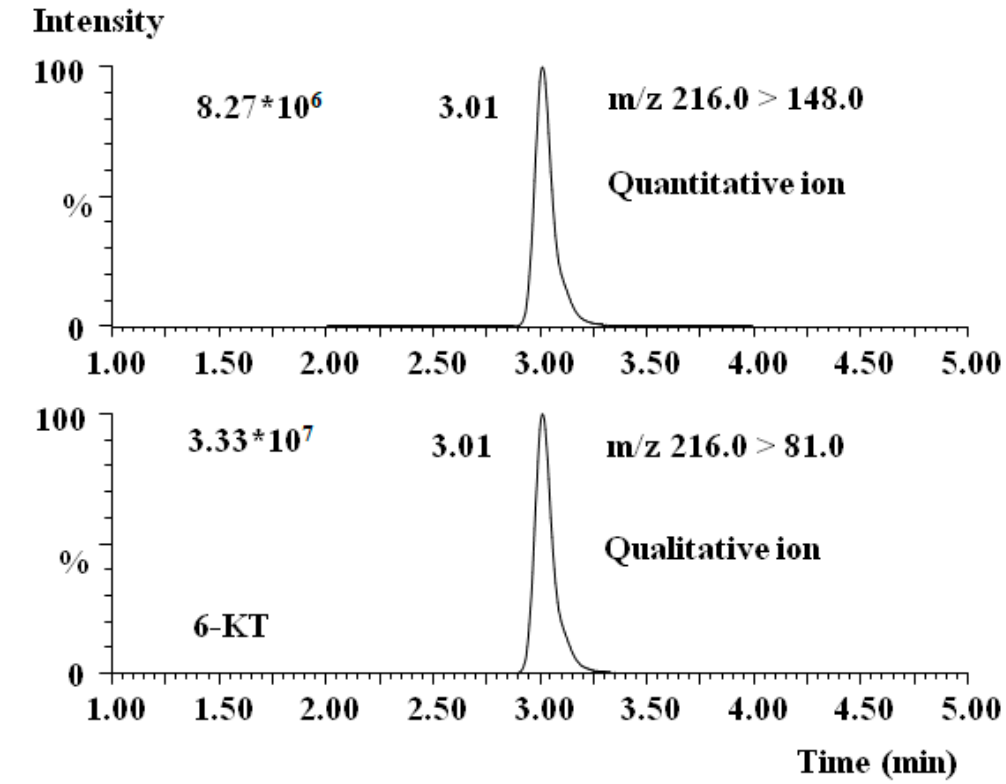


(**a**)

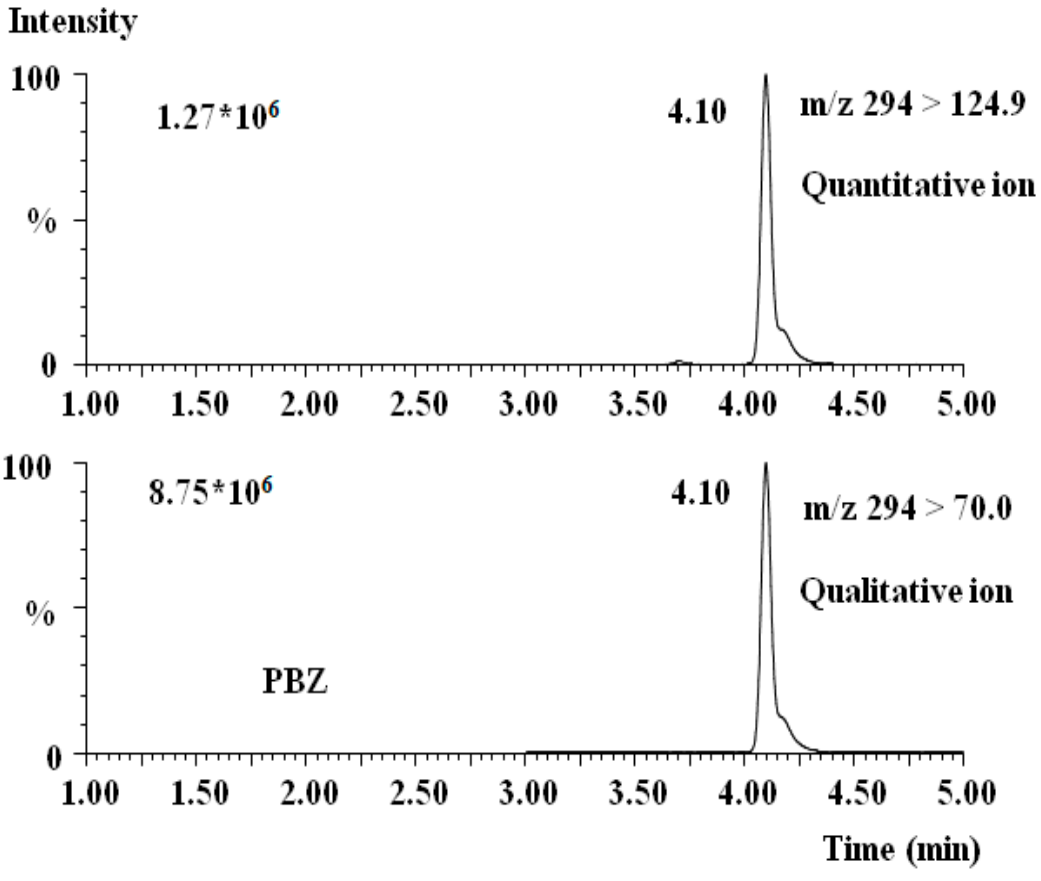


(**b**)

Figure 2. *Cont.*

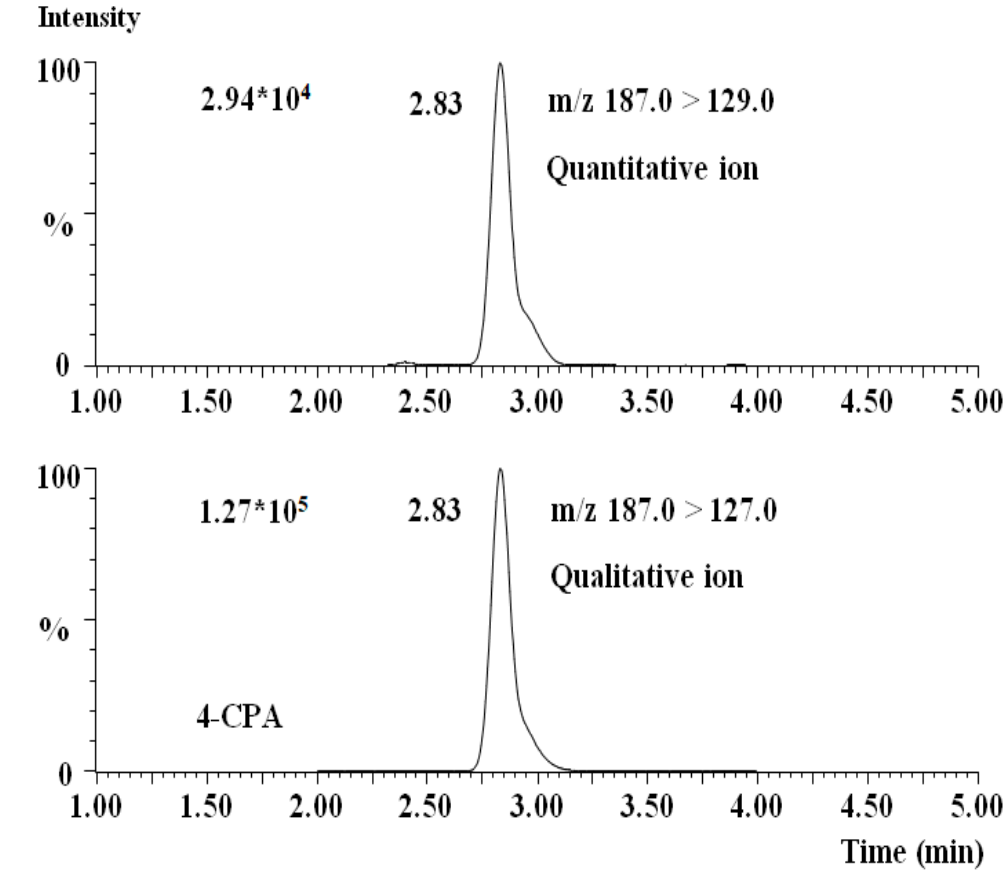


(**c**)

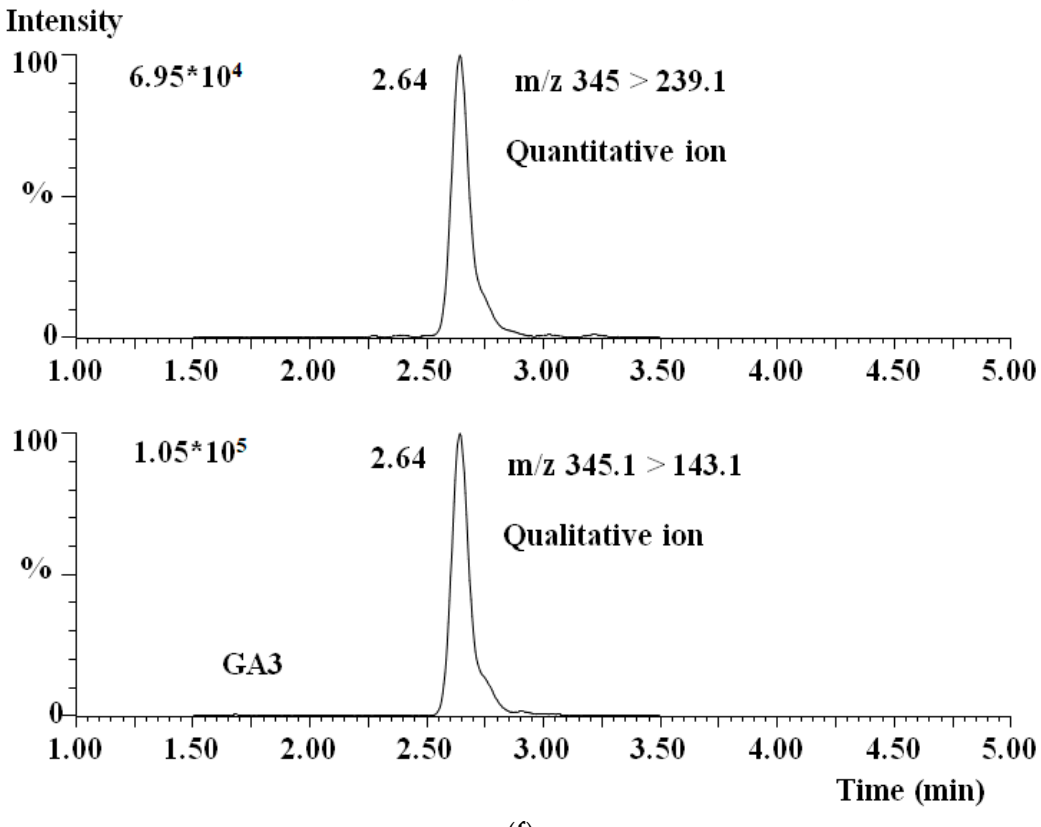


(**d**)

Figure 2. *Cont.*

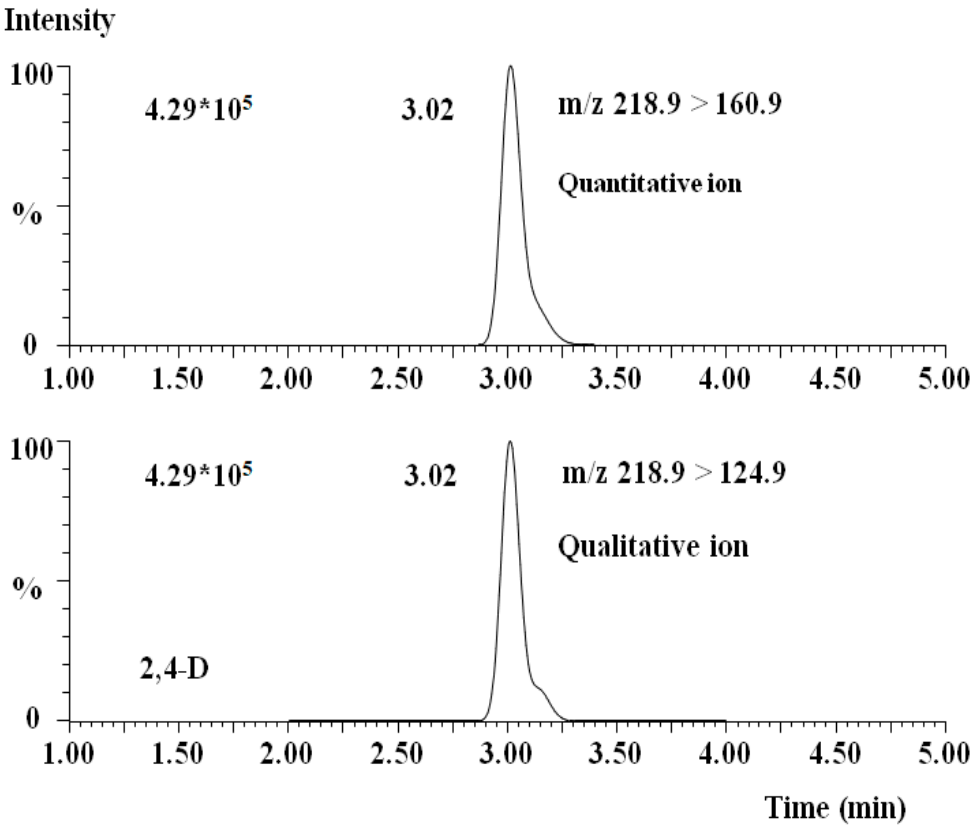


(**e**)

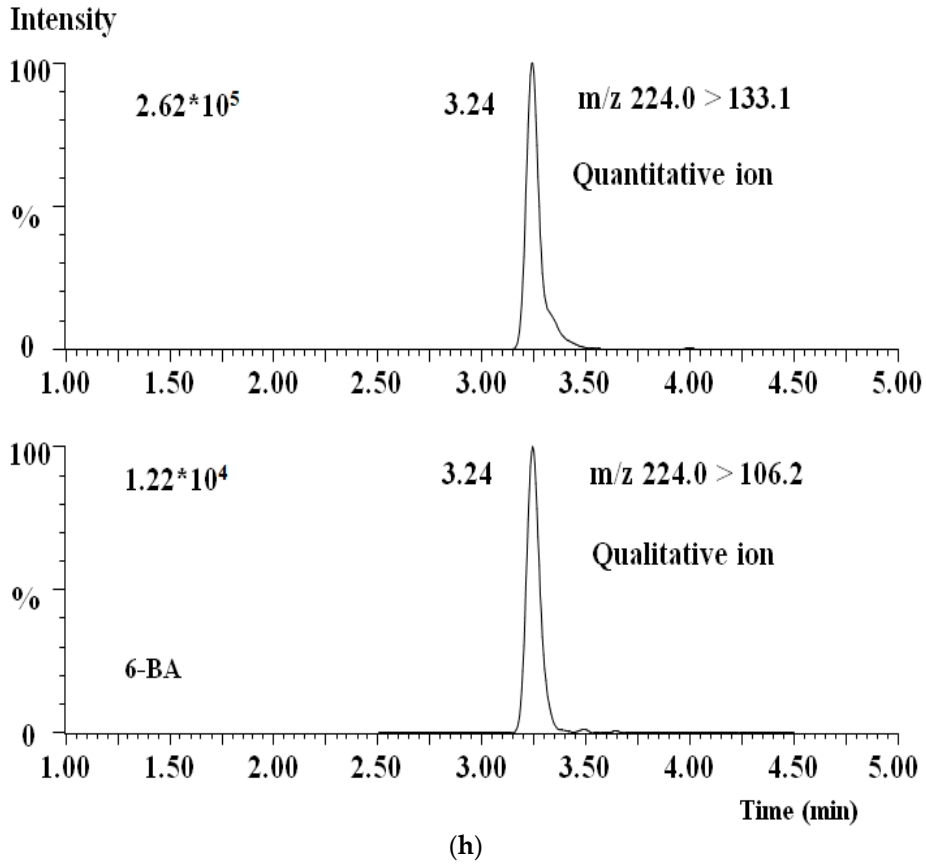


(**f**)

Figure 2. *Cont.*

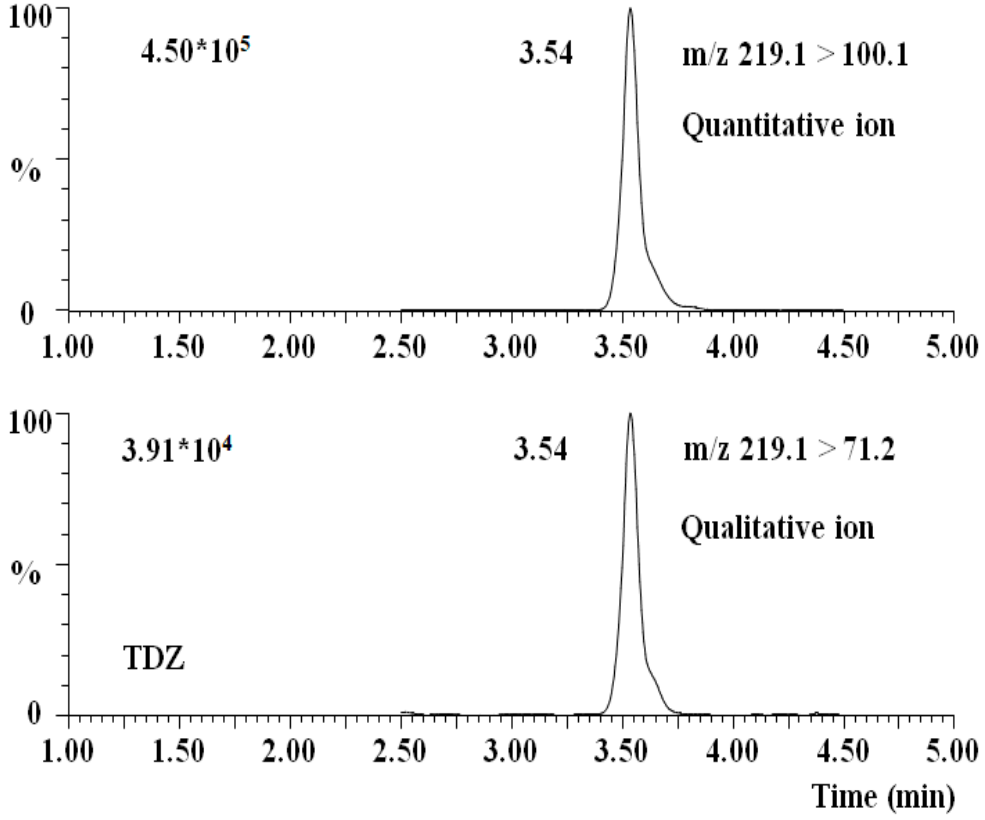


(**g**)

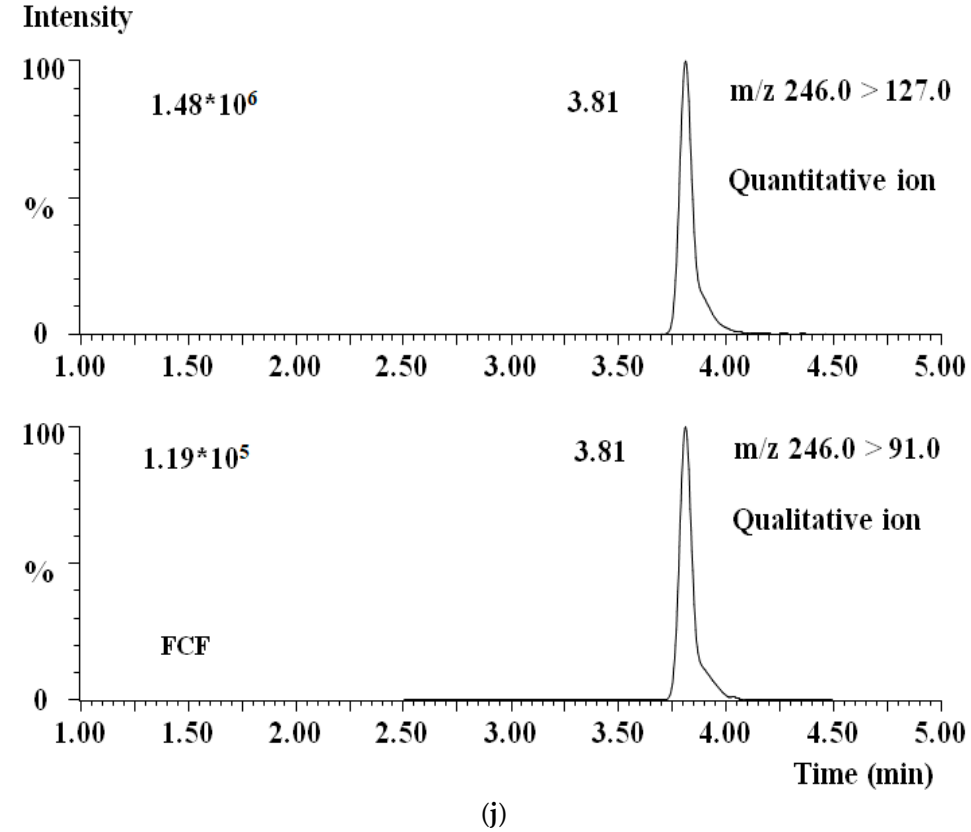


(**h**)

Figure 2. *Cont.*



(**i**)



(**j**)

Figure 2. MRM chromatograms of 50 ng/mL mixed standard solution: (**a**) to (**j**) are IAA, IBA, 6-KT, PBZ, 4-CPA, GA_3, 2,4-D, 6-BA, TDZ, and FCF, respectively.

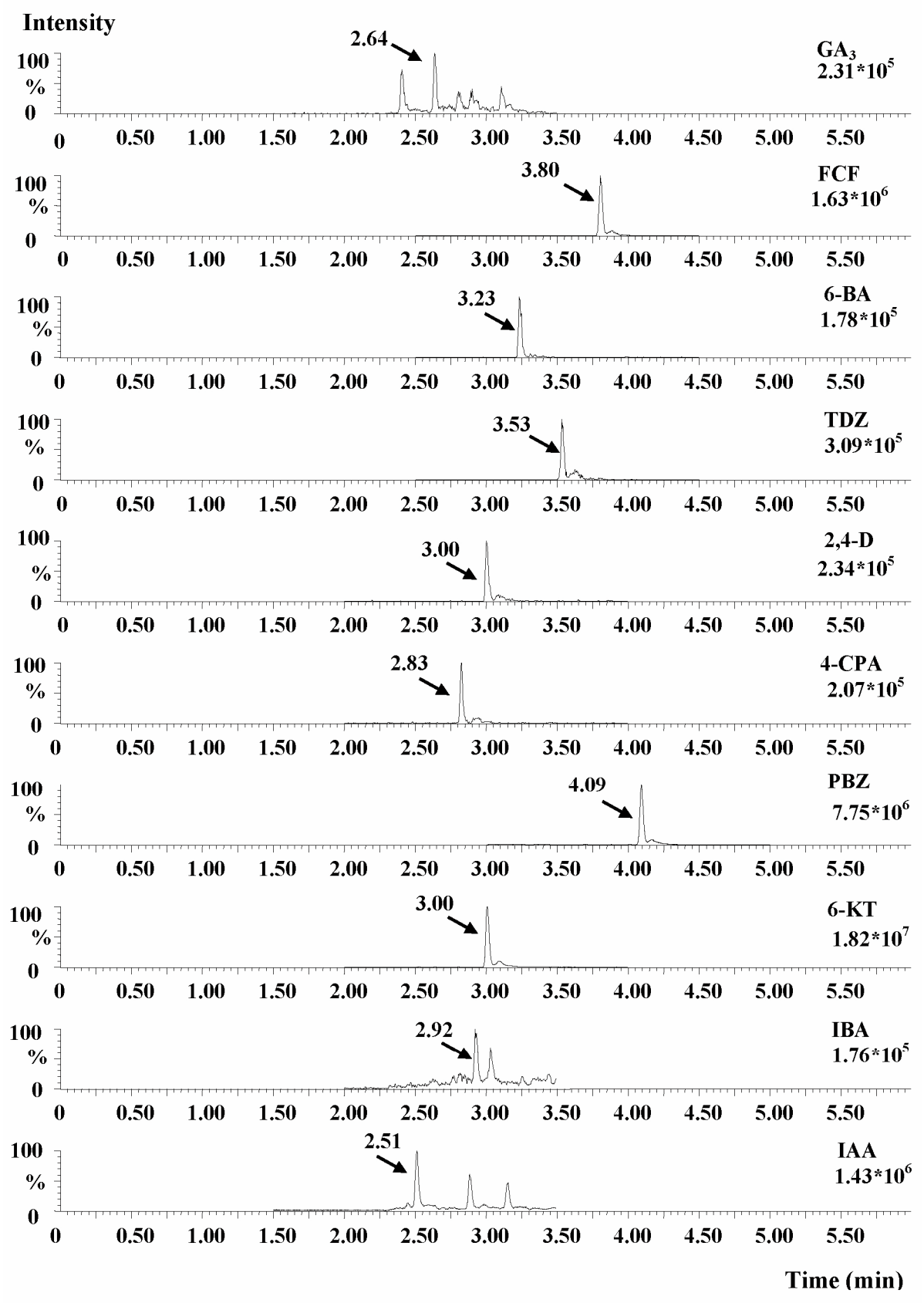


Figure 3. EICs of the 10 PGR standard solutions after the addition of standard (bean sprout elution I).

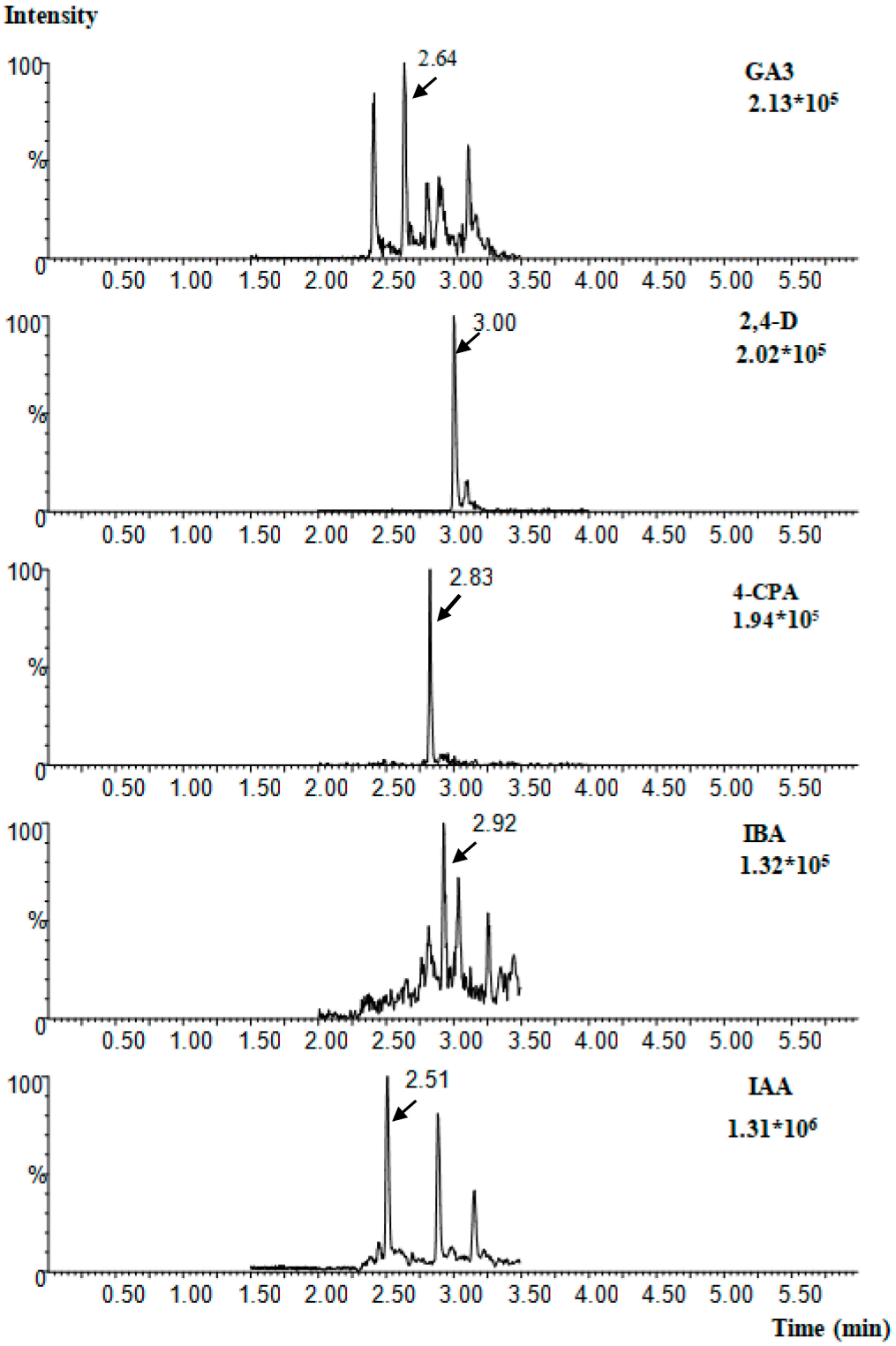


Figure 4. EICs of the five PGRs present in elution I from sample 1 after the addition of 5 ng/g standard solutions.

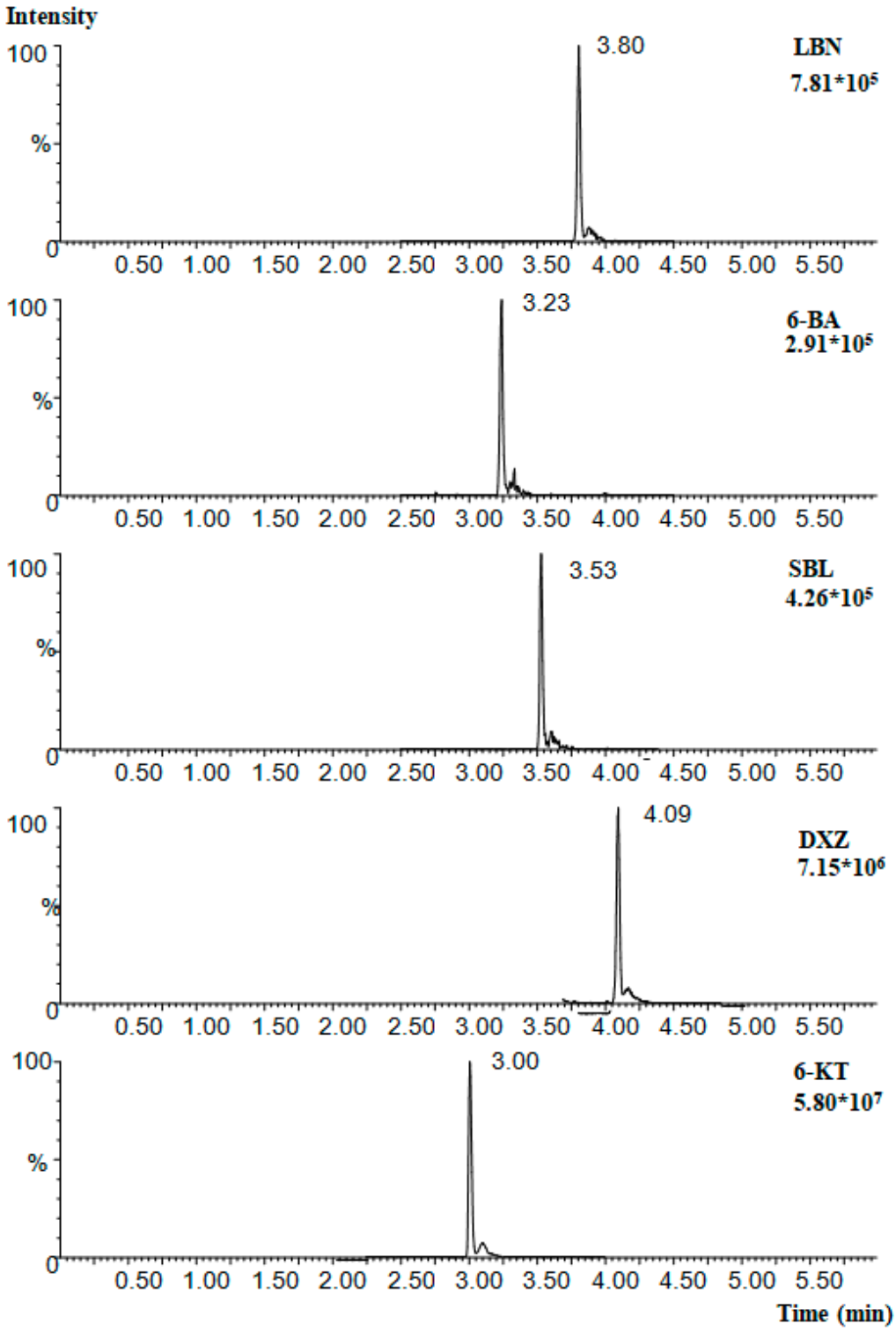


Figure 5. EICs of the five PGRs present in elution II from sample 1 after the addition of 5 ng/g standard solutions.

Some other studies have been performed for PGR analysis [26,32,33]. Compared with this study, there were some differences, including the analytical methods, the involvement of the apparatus, PGRs, the sample pretreatments, and the analysis results. For example, new, cauliflower-like phloroglucinol-glyoxylic acid resin microspheres (PGRMs) with controllable diameters and tunable surface roughness were prepared and used for the efficient extraction and determination of PGRs in cucumbers. This method was successfully

used for KT and 6-BA in cucumbers [34], and a label-free opto-fluidic ring resonator (OFRR) biosensor was used to identify the plant growth regulator of 6-benzylaminopurine [30]. Wang et al. [26] developed a triple quadrupole tandem mass (QqQ LC/MS) using liquid chromatography for the simultaneous determination of nine plant growth regulators in navel oranges. The extraction was performed by acetone and ethyl acetate (v/v, 1:2) with the microwave-assisted extraction method, and the cleanup of extracts was performed with dispersive-solid phase extraction (d-SPE) using active carbon as the sorbent. Compared with the other methods reported, the established method of this study is a standard procedure for rapid, accurate, and highly sensitive PGR analysis. There have been few reports on the simultaneous analysis of these 10 chemicals, and this developed method can be easily adapted for the detection and analysis of these 10 PRG residues in fruit and vegetable products. Some other studies focusing on PGRs reported the sample pretreatment, the simultaneous analysis of different PGRs, or a survey of the PGRs in sprout samples.

2.2. Accuracy, Precision, and Quality Control of the Developed Method

A sequential clean-up method was developed for the quantification of 10 plant growth regulators in bean sprouts by the GC/MS method in our previous study [5]. The analytes were extracted by the acidic acetonitrile after the concentration and extract were re-dissolved in methanol. After purification by a QuECHERS cartridge and MCS (C8+ strong cation exchange resin (SCX)) solid phase extraction column, two different parts of the ten PGRs were analyzed. Acetonitrile is a commonly used extraction solvent for pesticide residue detection, and the addition of sodium chloride can effectively separate water-soluble compounds from fat-soluble compounds, so methanol was not selected for PGR extraction in this study. After analysis, the results showed that after the spiking of 0.01–0.1 mg/kg selected plant growth regulators, the average recovery ranged from 70.0% to 93.2%, the relative standard deviations were 5.2%–12.3%, and the LOQ ($S/N \geq 10$) and LOD ($S/N \geq 3$) were 0.01–0.025 mg/kg and 0.003–0.008 mg/kg, respectively [5].

Three concentrations (5, 50, and 500 ng/g) of PGRs were spiked into one soybean sprout sample (n = 6 for each group) for a recovery test. The accuracy and precision of the method are shown in Table 2. The results show that the average recovery was 68.3%–97.3%, and the relative standard deviations (RSD) were 4.6–15.2%.

Table 2. Recovery of PGRs in one bean sprout sample (n = 6).

Chemicals	Standard Spiked (ng/g)	Average Recovery (%)	RSD%	LOQ (ng/g)	LOD (ng/g)
GA_3	5	68.3	15.2	5	2
	50	81.2	9.7	5	2
	500	86.4	6.4	5	2
2,4-D	5	68.3	15.2	5	2
	50	74.6	11.7	5	2
	500	90.5	7.2	5	2
4-CPA	5	75.7	10.6	5	2
	50	88.6	9.1	5	2
	500	95.3	5.8	5	2
IBA	5	73.1	14.2	5	2
	50	78.8	10.8	5	2
	500	84.2	8.2	5	2
IAA	5	74.3	13.4	5	2
	50	80.2	8.4	5	2
	500	83.7	6.3	5	2
FCF	5	80.3	9.2	5	2
	50	83.4	7.6	5	2
	500	89.3	5.2	5	2

Table 2. *Cont.*

Chemicals	Standard Spiked (ng/g)	Average Recovery (%)	RSD%	LOQ (ng/g)	LOD (ng/g)
TDZ	5	76.3	11.3	5	2
	50	79.3	9.4	5	2
	500	83.4	6.1	5	2
6-BA	5	78.6	9.3	5	2
	50	82.5	8.4	5	2
	500	91.3	5.6	5	2
PBZ	5	83.4	10.3	5	2
	50	86.4	7.8	5	2
	500	97.3	4.6	5	2
6-KT	5	77.8	12.4	5	2
	50	81.2	9.1	5	2
	500	89.1	6.7	5	2

When the standard in 5 ng/g was added, the quantitative ion signal noise ratio (SNR) of each compound was greater than 10, so the limit of quantification (LOQ) of the developed method was 5 ng/g, and the limit of detection (LOD) was 2 ng/g according to 1/3 of the LOD. At this time, the quantitative ion SNR of each compound was greater than 3.

2.3. Sample Analysis

The PGRs were different in varied samples. Mou et al. [27] developed a LC-MS/MS method with solid phase extraction to determine seven PGRs, including 2,4-D, GA_3, IBA, 4-CPA, 4-fluorophenoxyacetic acid, IBA, and α-naphthalene acetic in fruits. Only GA_3 was detected in one grape and one cucumber sample at 6.32 and 7.87 μg/kg, respectively. Liu et al. [34] reported the simultaneous detection of seven plant growth regulator residues in bean sprouts by a quick, easy, cheap, effective, rugged, and safe (QuEChERS)-HPLC-MS/MS method and detected 4-CPA between 30 μg/kg to 650 μg/kg and 6-BA between 10 μg/kg–39 μg/kg in 21 samples and 4 samples (52 samples total), respectively.

Besides samples of vegetables or sprouts, the PGRs in Chinese herbal medicine were also analyzed by QuEChERS and solid extraction methods. The results showed that compared to the liquid method, the solid method required a smaller amount of sample, which was critical for the PGR analysis of rare valuable herbal medicines. The two methods successfully were applied for the determination of 20 PGRs in different Chinese herbal medicines [35].

After the method was developed in this study, 69 sprout samples, including 36 soybean sprouts and 33 mungbean sprouts, were surveyed for PGRs. The results show that 4-CPA was detected in 7 soybean sprouts and 5 mungbean sprouts and IAA was detected in 4 soybean sprouts and 9 mungbean sprouts, while only 5 samples contained both 4-CPA and IAA simultaneously (Table 3). Because the consumption of plant sprouts as part of human day-to-day diets is gradually increasing, and their health benefit is attracting interest across multiple disciplines [36], consumers are concerned about the safety of these food materials, and due to the low level of the PGRs, a reliable analysis method is very important to ensure the sprouts' food safety.

Table 3. PGRs in bean samples.

Chemicals	Content Range (mg/kg)	Soybean Sprout Detection Rate (%)	Mungbean Sprouts Detection Rate (%)	Total Detection Rate (%)
Gibberellin	<LOD	/	/	/
2,4-D	<LOD	/	/	/
4-CPA	0.02–0.26 *	19.4 (7/36)	15.2 (5/33)	17.4 (12/69)
IBA	<LOD	/	/	/
IAA	0.04–2.5 *	11.1 (4/36)	27.3 (9/33)	18.8 (13/69)

Table 3. *Cont.*

Chemicals	Content Range (mg/kg)	Soybean Sprout Detection Rate (%)	Mungbean Sprouts Detection Rate (%)	Total Detection Rate (%)
FCF	<LOD	/	/	/
TDZ	<LOD	/	/	/
6-BA	<LOD	/	/	/
PBZ	<LOD	/	/	/
6-KT	<LOD	/	/	/

* calculated value. The PGR 4-CPA was detected in seven soybean sprouts and five mungbean sprouts, while IAA was detected in four soybean sprouts and nine mungbean sprouts.

3. Materials and Methods

3.1. Reagents and Instruments

Formic acid, ammonia, hydrochloric acid, ammonium acetate, sodium chloride in analytical purity and methanol (MeOH), and acetonitrile (MeCN) in chromatographic purity were from Hangzhou Huipu Chemical Corp. Ltd. (Hangzhou, China). Ultra-pure water was obtained using a Milli-Q reverse osmosis water polishing system (Millipore, Milford, MA, USA). MCS (C8+ strong cation exchange resin) solid phase extraction columns (500 mg/6 mL) were obtained from Hangzhou Fuyu Technology Corp. Ltd. (Hangzhou, China). Analytical standards (99.0% purity) of GA_3, 2,4-D, 4-CPA, IAA, IBA, FCF, TDZ, 6-BA, PBZ, and 6-KT were obtained from Dr Ehrenstorfer GmbH. Corp., Augsburg, Germany. High-purity nitrogen (99.9999% purity) was from Hangzhou JinGong Special Gas Corp., Ltd. (Hangzhou, China). Filter (0.22 μm, nylon membrane) was from Lepei Experimental Equipment Corp. Ltd. (Haimen, Jiangsu, China).

3.2. Preparation of Standard Solutions and Reagents

Stock solutions of each standard plant growth regulator (1.0 mg/mL) were prepared by dissolving 0.0250 g of material in a 25 mL volumetric flask with MeCN. For 6-KT, MeOH was utilized as the solvent. The resulting standard solutions were stored at 4 °C.

Mixed stock solutions of PGR compounds (10.0 μg/mL) were made by mixing 1 mL of each 1.0 mg/mL standard stock solution and diluting to 100 mL with MeCN. Mixed stock solutions of PGR compounds (100.0 ng/mL) were made by diluting 1 mL of 10.0 μg/mL mixed stock solutions in 100 mL MeCN. All mixed solutions were stored at 4 °C.

3.3. Sample Preparation

Thirty-six soybean sprout samples and thirty-three mungbean sprout samples were purchased from a local supermarket in Hangzhou, Zhejiang Province, China. A flow chart of the extraction and purification process of PGRs in samples is shown in Figure 6.

3.3.1. Sample Extraction

Bean sprout samples (5.0 g), formic acid (20 μL), and MeCN (20 mL) were added to a 50 mL centrifuge tube. The capped tube was vortexed for 1 min and treated in an ultrasonic water bath (Kunshan Ultrasonic Instruments Corp. Ltd., Suzhou, China) for 5 min at 25 °C. The resulting suspension was centrifuged at 9400× *g* for 5 min, and the top layer was decanted to another 50 mL centrifuge tube. NaCl (2.0 g) was added to this solution, and the capped centrifuge tube was vortexed for 2 min (vortex, Scientific Industries, Bohemia, New York, NY, USA). The centrifuge tube containing MeCN and NaCl was centrifuged at 9400× *g* for 2 min (centrifuge, Eppendorf China, Shanghai, China); then, 2 mL of the upper MeCN layer was transferred to a 15 mL centrifuge tube and concentrated to dryness by flowing nitrogen, and the residue was reconstituted with 0.2 mL MeOH and vortex mixing. Then, 40 mM aqueous HCl (3 mL) was added, and the mixture was transferred to another 15 mL centrifuge tube, capped, and subjected to centrifugation at 14,000× *g* for 5 min. The top layer was loaded on the activated MCS SPE column for further purification.

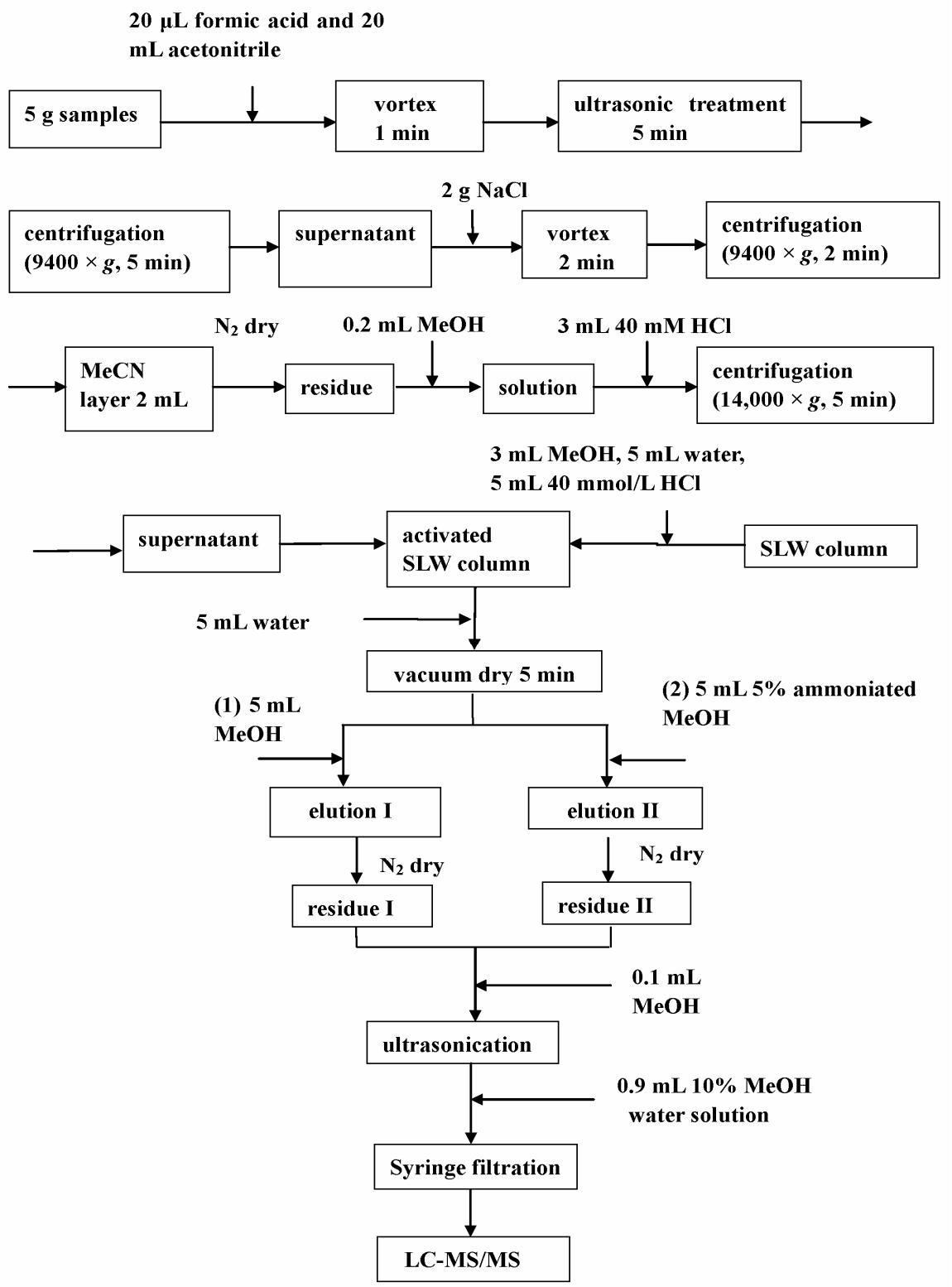


Figure 6. Flow chart of the extraction and purification processes of PGRs in samples.

3.3.2. Purification

The MCS column was activated by the sequential addition of MeOH (5 mL), water (5 mL), and 40 mM aqueous HCl (5 mL). The supernatant prepared in Section 3.3.1 was then loaded to the activated MCS column and washed with 5 mL water. Thereafter, the column was vacuum-dried for 5 min. To elute the target analytes, MeOH (5 mL) was added

to the column and collected as elution I. Next, 5% ammoniated MeOH (5 mL) was added to the column and collected as elution II. Both fractions were concentrated by flowing nitrogen and reconstituted in MeOH (0.1 mL) ultrasonically. After the addition of another 0.9 mL 10% MeOH in water solution, the two fractions were individually filtered through a 0.22 μm filter membrane (for organic reagent) prior to injection into the LC-MS/MS. Elution I was found to contain GA_3, 2,4-D, 4-CPA, IAA, and IBA. Elution II was found to contain FCF, TDZ, 6-BA, PBZ, and 6-KT.

3.4. LC-MS/MS Conditions

Liquid chromatography was performed using an ACQUITY UPLC I-Class/Xevo TQ-S System (Waters, Milford, MA, USA). Chromatographic separation was carried out on a Waters BEH C_{18} column (1.7 μm, 2.1 mm × 100 mm) (Waters, Milford, MA, USA) at 40 °C. Mobile phases consisted of 5 mM ammonium acetate water solution (A) and MeCN (B). The elution gradient (flow rate 0.3 mL/min) was set as follows: 0–1 min, 2%B; 1–2 min, 30% B; 2–4 min, 95% B; 4–5 min, 95% B; 5–6 min, 2% B; 6–8 min, 2% B. The injection sample volume was 2 μL.

Mass spectrometric detection was performed on an ACQUITY UPLC I-Class/Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters, Milford, MA, USA). ESI ionization was performed in the positive ion mode for 6-KT, PBZ, IAA, and IBA and in the negative ion mode for GA_3, 2, 4-D, 4-CPA, FCF, TDZ, and 6-BA. The tandem mass spectrometer was operated at unit resolution in the multi-reactions monitoring mode (MRM).

The capillary voltage was 0.5 kv, the ion source temperature was maintained at 150 °C, and the dissolvent temperature was 400 °C. The curtain gas as well as auxiliary heat gas were of high-purity nitrogen, and the collision gas was of high-purity argon.

The mass spectrometric conditions were optimized by continuous infusion of the standard solution at a rate of 10 μL/min using a Harvard infusion pump. Quantification was performed via peak area ratio. All data were processed using MassLynx software (Version 4.1). The quantitative and qualitative ions, as well as cone voltage and collision energy of the mass, are listed in Table 1.

3.5. Method Validation

3.5.1. Standard Curve Preparation

Standard solutions of 1, 2, 5, 10, 20, and 50 ng/mL FCF, TDZ, 6-BA, PBZ, and 6-KT were prepared by addition of 0.01 mL, 0.02 mL, 0.05 mL, 0.1 mL, 0.2 mL, 0.5 mL of 100.0 ng/mL PGR mixed stock solutions and 10% MeOH/water solution to 1 mL. Then, the standard curve was drawn after LC-MS/MS analysis based on the peak areas and concentrations of the standards, and data fitting was performed using a linear regression method.

Eluent I was used to analyze 5 PGRs, including GA_3, 2, 4-D, 4-CPA, IAA, and IBA, which needed to be quantitatively analyzed with blank matrix. After extraction and purification of six blank bean sprout samples according to Section 3.3, six MeOH eluents were obtained, then added to 0.01 mL, 0.02 mL, 0.05 mL, 0.1 mL, 0.2 mL, and 0.5 mL of 100 ng/mL mixed PGR stock solutions in 100.0 ng/mL, respectively. After that, 10% MeOH/water solution was added to the six eluents to 1.0 mL and mixed thoroughly, and the eluents were then filtered through 0.22 μm nylon membrane to prepare standard solutions of GA_3, 2, 4-D, 4-CPA, IAA, and IBA in concentrations of 1, 2, 5, 10, 20, and 50 ng/mL, respectively. The standard curves were drawn according to the results after LC-MS/MS analysis.

3.5.2. Accuracy, Precision, and Recovery

The precision and accuracy of the LC-MS/MS method were assessed by the following criteria: at the limit of quantification (LOQ), the RSD value should not exceed 20%, and the relative standard deviation (RSD) value determined at each concentration level should not exceed 15%. The percentage deviation between the measured and nominal concentration

was defined as the accuracy of the method. The precision and accuracy of the method were assessed by the following criteria: at the limit of quantification (LOQ), the RSD value should not exceed 20%, and the RSD value determined at each concentration level should not exceed 15%. The recovery value should be within 70% to 120%.

Three concentrations (5, 50, and 500 ng/g) of PGRs were spiked into one soybean sprout sample (n = 6 for each group) for a recovery test. The values of recovery were calculated by comparing the PGRs concentrations added to the soybean sprout sample with the sample concentrations analyzed from the calibration curve.

3.5.3. Limit of Detection and Limit of Quantification

The limit of detection (LOD) was set to the lowest concentration where the signal of the compound was threefold higher than background noise (S/N > 3). The LOQ was chosen experimentally as the minimal concentration in samples that could be determined, where the ratio of signal to noise exceeds 10.

3.6. Calculation

The concentration of PGRs in the bean sprout samples were calculated according to the following equation: (methods for PGRs analysis) (1)

$$X = \frac{C \times 2}{m \times 20 \times 1000} \quad (1)$$

where

X = concentration of PRGs in the sample (mg/kg);
C = concentration of PRGs in the prepared sample solutions (ng/mL);
m = mass of bean sprout samples subject to extraction (g);
2 = volume of MeCN extract taken for purification (mL);
20 = volume of MeCN used to extract the bean sprout samples (mL);
1000 = the dilution factor
All the data were average values from three trials.

3.7. Statistical Analysis

Significant differences in mean levels of PGRs in samples were identified by one-way analysis of variance (ANOVA) and least-significant-difference (LSD) test at $p < 0.05$. Analysis was conducted using the SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA).

4. Conclusions

Bean sprouts are commonly consumed in China and Asia for their nutrition and special taste. PGRs can stimulate the sprouts' growth and promote the product quantity. While some PGRs are harmful to the health of consumers, it is necessary to develop analytical methods to survey the PGRs in bean sprouts. A simple, rapid, sensitive, and accurate enhancement of the accuracy and sensitivity of the LC-MS/MS method for the detection of PGR residues in bean sprout products was developed for the analysis of 10 plant growth regulators in bean sprouts using a serial purification procedure and the addition of an external standard. This method can effectively remove interfering compounds from the bean sprout matrix. Ten PGR compounds were detected within a concentration range of 1.0–50 ng/mL. The average recovery was 68.3.4–97.3%, with relative standard deviations (RSD) of 4.6–15.2% (n = 6). The LOQ and LOD were found to be 0.005 and 0.002 mg/kg, respectively. This newly established method can be used for the analysis of GA_3, 2,4-D, 4-CPA, IAA, IBA, FCF, TDZ, 6-BA, PBZ, and 6-KT in fruit and vegetable products. Sixty-nine sprout samples, including thirty-six soybean sprouts and thirty-three mungbean sprouts, were surveyed for PGRs. The results show that PGRs were detected in 10 soybean sprouts and 10 mungbean sprouts. The detected PGRs were 4-CPA and IAA, respectively, and both 4-CPA and IAA were detected in five samples. This method

of analysis could be used for other plant materials, and by standardizing the method, the procedure could be improved.

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