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Analysis of Plant Growth Substances (*Auxins, Gibberelins and Cytokinins*) in Vegetables Using High-Performance Liquid Chromatography Coupled to Electrospray Ionization Tandem Mass Spectrometry

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Abstract:

This study was applied to extract and analyze major plant growth substances in vegetables. Plant tissues were washed by tap water before frozen in liquid nitrogen and kept under 4°C in the freeze until to be analyzed. After grinding into powder, plant hormones in the samples were extracted by 2-propanol/H₂O/concentrated HCl (2:1:0.002, vol/vol/vol) and Dichloromethane (CH₂Cl₂), respectively. The extract was concentrated by nitrogen flow during an hour and analytes were dissolved in MeOH and then directly injected into column and analyzed by HPLC-ESI-MS/MS. 9 types of plant growth substances belong to auxin, gibberellin and cytokinin, including: Gibberelin A₃ (GA₃), Gibberelin A₄ (GA₄), Gibberelin A₇ (GA₇), Indole-3-butyric acid (IBA), Indole-3-carboxylic acid (ICA), Trans-zeatin (tZ), N6-Benzyladenine (BA), Kinetin (K) and N6-Isopentenyladenine (iP) had identified and quantified in the plant tissues. The method showed good linearity for all 9 analytes with regression coefficients $R^2 > 0.996$. Limit of detection ranged from 0.19 ng.g⁻¹ to 1.20 ng.g⁻¹ for cytokinins (BA, tZ, K and iP), from 2.06 ng.g⁻¹ to 3.22 ng.g⁻¹ for auxins (ICA and IBA), and from 4.14 to 5.16 ng.g⁻¹ for gibberellins (GA₃, GA₄ and GA₇). The mean recoveries for all of the analytes were from 83.38 to 104.50% and the repeatability standard deviation of the recovery for hormones in three different concentrations as low, medium and high levels was from 0.22% to 11.07%. To our knowledge, this is a rapid, simple, sensitive, accurate and efficient method for analysis plant growth substances in vegetables as well as the first study analyzing 9 plant hormones: GA₃, GA₄, GA₇, IBA, ICA, tZ, BA, K and iP in plant tissues using high-performance liquid chromatography equipped with an electrospray ionization tandem mass spectrometry. This plays a pivotal role in studying, determining and quantifying residues of plant growth substances that accumulate in agricultural products in general and green vegetables in particular. Since then, helping national managers have appropriate measures for using plant growth regulators in the production of agricultural products as well as recommending to consumers know which products are at risk of many plant growth stimulants and potentially affecting human health.

Key words: Plant growth substance, gibberellin, auxin, cytokinin, HPLC-ESI-MS/MS

1. Introduction

Plant hormones play a pivotal role in most physiological processes during a plant's life cycle, such as growth, differentiation, metabolism and morphogenesis from germination to senescence. On the basis of their structures and physiological functions, plant hormones are classified into several major groups including abscisic acids (ABAs), auxins, cytokinins, gibberellins and ethylene, and other phytohormones counting jasmonates, salicylates, brassinosteroids, polyamines, strigolactones and brassinosteroids [1]. The determination and quantification of phytohormones in plants has been of great interest and essentials for researchers in plant biology and physiology to understand their functions in plant metabolism and ecological interactions [2, 3].

The presence of hormones are at very low concentrations in plant tissues (10⁻⁹ to 10⁻⁶ M), therefore, the development of method for the determination of hormones is challenging [4, 5]. In addition, another challenge is that

different hormones have diverse chemical and structural properties, which makes the simultaneous and equitable evaluation of their quantities difficult [6]. A variety of methods for determination and quantification of phytohormones has been reported. Bioassays were considered as a classical method for hormone analysis and were first used in the determination of auxins in 1928 [7]. However, this method was tedious in sample preparation, low in specificity and poor in repeatability; therefore the application of bioassays has attracted little attention in recent years [6]. After that, immunoassays were used as the first highly sensitive methods for quantitative phytohormones [8, 9]. Nevertheless, the presence of cross reactions and the long period of antibody preparation limit their further application. Furthermore, the specific antibody is not suitable for the simultaneous detection of multiple plant hormones in different classes. In the previous studies, gas chromatography – mass spectrometry (GC-MS) was a well recognized technique in the field of phytohormone analysis [10-12]. GC-MS was acknowledged as an efficient method for the structural identification and accurate quantification in multiple phytohormone analysis, but the requirement for sample volatility limits its application for all classes of phytohormones. Moreover, some thermally labile components are likely to break down at the high temperature of the GC injector and column, which limits the range of plant hormones fit for GC analysis. According to Qingfen et al. (2014), high-performance liquid chromatography (HPLC) with electrochemical detection performs poorly in the quantitative analysis of endogenous plant hormones at nanomolar levels in plant tissue samples, because the purification process is complicated and the limit of quantitation (LOQ) that meets the determination requirements is high and difficult to achieve with small amounts of sample [13]. In recent years, mass analyzers (MS) coupled to liquid chromatography has been developed and implemented for phytochemical analysis allowing an increase in sensitivity and selectivity and, in addition, enabling simultaneous determination and quantification of multiple compounds in a single run [3, 14, 15]. However, different approaches might be adopted depending on the separation method using liquid chromatography and the spectrometers as triple-quadrupole, iontrap, time of flight (TOF), orbitrap, etc. applied during the quantification. Therefore, development of a rapid, simple, sensitive, accurate and efficient method for the analysis of phytohormones from complex biological samples is necessary and significant for botanical research.

The main goal of this study was to develop a simple, fast and robust method to simultaneous quantification of 9 plant growth substances in vegetables including Gibberellin A₃ (GA₃), Gibberellin A₄ (GA₄), Gibberellin A₇ (GA₇), Indole-3-butyric acid (IBA), Indole-3-carboxylic acid (ICA), trans-zeatin (tZ), N6-Benzyladenine (BA), Kinetin (K) and N6-Isopentenyladenine (iP) using HPLC/Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS).

2. Materials and Methods

2.1. Chemicals

Auxins (IBA, ICA), Cytokinins (tZ, BA, K, iP) and Gibberelins (GA₃, GA₄ and GA₇) were purchased from OlChemim Ltd. (Olomouc, Czech Republic). Methanol (MeOH) for HPLC with 99.80% of purify and formic acid (FA) were products of Sigma Aldrich Company (Singapore) whereas deionized water was produced by Milli-Q Integral 3 (Merck Millipore, France). Structures of the plant growth substances were shown on figure 1.

Figure 1: Structure of the Plant Growth Substances

2.2. Reversed-Phase High-Performance Liquid Chromatography

The plant growth regulators (PGR) were analyzed using an Ultimate 3000HPLC system (Thermo Fisher Scientific, Bremen, Germany), equipped with (3 μ m, 150 x 2.1 mm) Hypersil GOLD aQ C18 column (Thermo Fisher Scientific, Bremen, Germany). The samples were maintained at 8°C in the autosampler tray and the column temperature was kept at 40°C. Deionized water involving 0.1% FA and MeOH containing 0.1% FA were used as A and B mobile phase solvents in analysis of plant growth promoters at a constant flow rate of 0.1 mL/min. A gradient elution program was established as following: the initial value of methanol was kept at 30% for 3 min, and from that point methanol concentration was linearly increased to 90% in 6 min. Afterwards, this conditions were maintained for 3 min and then the solvent composition was restored to foremost conditions for 1 min and maintained for 2 min to allow column re-equilibration, giving a gradient program of 15-min duration. HPLC instrument was controlled using Chromelonesoftware (Thermo Fisher Scientific, Bremen, Germany).

2.3. Mass Spectrometry

The quantification and confirmation of plant growth promoters and their major metabolites were performed by LCQ Fleet MS (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization (ESI) source. Standard solutions of plant growth promoter compounds were prepared in MeOH/water/FA (50/50/0.1%, v/v/v). For the selection of diagnostic precursor-to-product ion in negative and positive ionization modes, mixture of standard solutions (1 ng. μ L⁻¹) for plant hormones was infused into ion-trap mass spectrometer, fitted with the ESI ion source using a 500 μ L Hamilton syringe (USA) at a flow rate of 5 μ L/min. The optimized electrospray ion source (ESI) operating conditions were as follows: heater temperature 100°C, Sheath gas flow rate 25 (arb), auxiliary gas flow rate 5 (arb), sweep gas flow rate 0 (arb), capillary temperature 200°C; the spray voltage was 3.6 and -3.0 (kV), capillary voltage was 4 and -31 (V), and tube lens -125 and 70 (V) for positive and negative ions, respectively. All of analytes were monitored using the selected reaction monitoring (SRM) mode. In order to enhance the sensitivity of the system, PGRs were implemented on the basis of retention time stability so that only ions eluted during the specified retention time were monitored. MS systems and data acquisition, processing and evaluation were performed by Xcalibur software (Version 2.2, Thermo Fisher Scientific).

2.4. Extraction and Purification of Plant Materials

After cutting, fresh vegetable samples were washed with tap water to remove dirt and soil from the leaves before frozen in liquid nitrogen and kept under 4°C in the freezer until to be analyzed. The tissues were ground into powder with a mortar and pestle, and approximately 300 mg of each sample were weight and transferred to 15 mL screw-cap tubes. About 3 mL of 2-propanol/H₂O/concentrated HCl (2:1:0.002, vol/vol/vol) were added as extraction solvent, then were shaken at 4°C for 30 min in the Starlab shaker. Subsequently, 6 mL of dichloromethane (CH₂Cl₂) was added, followed by agitation for another 30 min at 4°C and then centrifugation at 4,000 rpm for 10 min. After centrifugation, two phases were formed and plant debris was in the middle of two layers. Around 1 mL of the lower layer was concentrated using a nitrogen evaporator with nitrogen flow for an hour and then redissolved in 1 mL of MeOH. After vortexing and centrifuge at 13,000 g and 4°C for 10 min, the supernatant was directly injected to column and analyzed by HPLC-ESI-MS/MS.

2.5. Calibration Curves and Validation of the Method

For the creation of calibration curves, 1 mg.mL⁻¹ stock solution of each PGR were prepared in methanol and stored at -20°C until used. Working solutions of original plant growth regulator standards were prepared diluting stock solution in 50% MeOH containing 0.1% FA and 50% of deionized water containing 0.1% FA at the same concentration of 100 μ g.mL⁻¹ for each PGR. The standard mixtures containing 1 mL of each plant growth substance including GA₃, GA₄, GA₇, IBA, ICA, tZ, BA, K, iP and 1 mL of MeOH and deionized water (1:1), such that the final concentration for each of 9 hormones was 10 μ g.mL⁻¹. The calibration curves were prepared in matrix using 10 μ g. mL⁻¹ of the intermediate standard mixture solution at different concentration levels of 1, 5, 10, 20, 50, 100, 200, 500 and 1000 μ g. mL⁻¹ to check the selectivity of the method. The calibration curves of plant hormones were obtained by plotting the peak area, which were used for validation and quantification.

A linear equation was established as $y = ax + b$, wherein a and b were slope and intercept, respectively. The limit of detection (LOD) ($3 \times SD/a$) and the limit of quantification (LOQ) ($10 \times SD/a$) were determined by using the residual standard deviation of the regression line (SD) and the slope of the calibration line (a) [16].

Recovery of the method was calculated by comparing the amount of each plant hormone present in the spiked/extracted and extracted/spiked quality controls. Quality controls were used to assess the method's accuracy and precision. Quality controls were prepared spiking 300 mg of fresh vegetable tissues with 300 μ L of three different levels of each plant growth hormone including 10, 100 and 1000 μ g.mL⁻¹ as low, medium and high levels, respectively. The spiked samples were extracted as described in Extraction and Purification of plant samples. The dry residues were dissolved in MeOH containing the final concentration of each plant hormone, which corresponds to 0.91 μ g. mL⁻¹, 9.09 μ g. mL⁻¹ and 90.09 μ g. mL⁻¹, respectively. All quality controls were prepared in triplicate. The results are presented as the mean $\pm$ standard deviation (SD).

3. Results and Discussion

3.1. Optimization of the Mass Spectrometry's Conditions

Figure 2: Precursor and Product Ions Specific for Each Plant Growth Substance

GA₃, GA₄, GA₇, IBA, ICA were analyzed in negative scan mode as $[M-H]^-$ ions; while tZ, BA, K and iP were analyzed in positive scan mode $[M+H]^+$ ions using ESI-MS/MS. Precursor and product ions specific for each hormone were identified via authentic compounds (Figure 2) and appropriate precursor-to-product ion transitions representing a major fragmentation path and unique for each plant hormone were chosen (Table 1). The MS/MS conditions for analytes were optimized to produce maximal signal (Table 1). Upon the collision energy from 22 to 28 (eV), precursor ion of plant growth substances were fragmented into many product ions, wherein the product ion appearing in highest signal was selected for quantification whereas the two product ions with the next high signal were used as confirmation ions (Table 1). It is interesting to note that, Gibberelin compounds (GA₃, GA₄, GA₇) were measured via the quantitative ions with the mass of 239, 313, 223 (m/z), respectively; while cytokinin such as tZ, BA, K and iP were detected in order of the mass of 202, 91, 148 and 136 (m/z), respectively; and the mass of 134 and 116 (m/z) were quantitative fragment ions of Auxins (IBA, ICA), respectively. In the previous study of Sheila et al. (2003), the mass of 223 and 136 (m/z) were selected as the main fragment ions in the transition of precursor to product ion of GA₇ and iP, respectively [17]. Terezia et al.

(2013) indicated that the parent mass of 345 (m/z) of GA₃ in plant tissues was changed into the mass of 239 (m/z) under analytical conditions using liquid chromatography coupled to electrospray ionization tandem mass spectrometry [16]. In the other researches of Xiangqing et al. (2008 and 2010), the mass of 116 (m/z) was used as the quantitative ion for ICA whereas the authors selected the mass of 158 (m/z) as the main product ion for IBA [18, 19]. The HPLC system was coupled to a tandem quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface (ESI), which was used by Ondrej et al. (2008) to analyze cytokinins in plant tissues such as tZ, iP and BA via the daughter ions including 136, 136 and 91 (m/z), respectively [20]. These results suggest that our investigation was similar to the previous studies in analysis of plant hormones using liquid chromatography – electrospray ionization tandem mass spectrometry; however, there are no studies focusing on 9 types of plant growth substances in vegetables such as GA₃, GA₄, GA₇, IBA, ICA, tZ, BA, K and iP.

Analytes	Scan mode	Retention time (Rt)	Precursor ion	Product ions		Collision energy (eV)
				Quantitative ion	Confirmation ions	
GA ₃	-	4.63	345	239	143 283	22
GA ₄	-	7.10	331	313	287 269	22
GA ₇	-	6.74	329	223	267 311	23
IBA	-	6.01	202	134	116 158	23
ICA	-	5.06	160	116	62 144	26
tZ	+	4.83	220	202	136 148	24
BA	+	6.10	226	91	148 209	28
K	+	5.31	216	148	188 173	26
iP	+	6.44	204	136	148 186	23

Table 1: Optimized MS/MS Conditions for Plant Growth Substance Analysis

3.2. Separation and Identification of Plant Growth Substances in Vegetables Using HPLC-ESI-MS/MS

To separate individual plant hormones in a mixture, each of the analytes were subjected to chromatography on a (3µm, 150 x 2.1 mm) Hypersil GOLD aQC18 reversed-phase column (Thermo Fisher Scientific, Bremen, Germany), followed by a analysis via ESI-MS/MS. Eluates were monitored by the selected reaction monitoring (SRM) mode. Typical LC-ESI-MS/MS chromatograms of 9 phytohormones including 5 compounds such as GA₃, GA₄, GA₇, IBA, ICA were analyzed in negative ion scan mode and 4 compounds obtaining tZ, BA, K and iP were analyzed in positive ion scan mode are shown in Figure 3 and Figure 4, respectively.

Figure 3: Representative LC Chromatograms of 5 Plant Growth Substances Analyzed in Negative Ion Scan Mode (TIC: Total Ion Current of All Authentic Phytohormones in Negative Scan)

Generally speaking, all of the compounds were eluted before 10 min of retention time. In particular, as can be seen from Figure 3, among the substances detected as negative ions, GA₃ was the earliest eluted ion at 4.63 min, followed by ICA ion at 5.15min. It is interesting to note that, IBA and GA₇ ions were the third and the fourth eluted ion positions at 6.01 min and 6.83 min, respectively. Finally, 7.10 min was reported as the retention time of GA₄ negative ion in the analytical processing.

It can be observed from the Figure 4 that, there are four different retention times of four plant hormones detected in positive ion mode including tZ, BA, K and iP. Initially, tZ was eluted out of the C18 reversed-phase column at 4.83 min of retention time. Subsequently, K was washed at 5.31 min by a gradient elution program of the mobile phases of MeOH and deionized water as described in the reversed-phase high-performance liquid chromatography above. After that, the next compound was removed as BA at 6.10 min, and the last positive ion was rinsed at 6.44 min namely iP. The different precursor-to-product ion (SRM) transitions allowed specific detection of each compound in the mixtures of plant hormones (Figure 2). In addition, monitoring of precursor-to-product ion transition typically resulted in the identification of several peaks including one at the retention time of the target phytohormone (Figure 3 and 4). These data indicate that a combination of the retention time in the liquid chromatography and the diagnostic precursor ion to fragment ions transition in ESI-MS/MS are required together. This is the specificity necessary for the quantification of the plant hormones in vegetable samples.

Figure 4: Representative LC Chromatograms of 4 Plant Growth Substances Analyzed in Positive Ion Scan Mode (TIC: Total Ion Current of All Authentic Phytohormones in Positive Scan)

3.3. Calibration Curve and Linearity of Plant Growth Substances

Figure 5: Calibration Curves of Plant Growth Substances in Vegetables

Method validation was accomplished by calculation of LOD and LOQ. The calibration curves including different concentrations of plant hormones at levels of 1, 5, 10, 20, 50, 100, 200, 500 and 1000 $\mu\text{g.mL}^{-1}$ were analyzed in triplicate and shown in Table 2. The correlation coefficients (R^2) for the calibration curves were fluctuated between 0.9961 and 0.9999, wherein 0.9997 for GA₃, 0.9990 for GA₄, 0.9961 for GA₇, 0.9987 for IBA, 0.9985 for ICA, 0.9999 for tZ, 0.9989 for BA, 0.9982 and 0.9995 for K and iP, respectively (Table 2).

LOD is the lowest analyte concentration, which can be distinguished from the noise in blank samples and it is defined as a concentration with signal/noise (S/N) of 3. As it can be seen from Table 2, gibberelin compound group (GA₃, GA₄ and GA₇) had at highest level of LOD ranging from 4.14 to 5.16 ng.g^{-1} . This is followed by the LOD of auxins as IBA and ICA at 3.22 ng.g^{-1} and 2.06 ng.g^{-1} , respectively. In particular, the lowest analyte concentration was reported at levels of 0.19 ng.g^{-1} , 0.31 ng.g^{-1} , 0.46 ng.g^{-1} and 1.20 ng.g^{-1} in cytokinins including tZ, BA, iP and K, respectively (Table 2).

LOQ is defined as the lowest analyte concentration, which can be quantified precisely and accurately, and yield a peak with S/N of at least 10. It is interesting to note that from Table 2 there are differences in the quantitative limit of plant hormones analyzed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. It can be highlighted that the lowest figure of LOQ was tZ, at the vicinity of a negligible 0.63 ng.g^{-1} . BA and iP ranked second and third positions in the limit of quantification at approximately of 1.02 ng.g^{-1} and 1.54 ng.g^{-1} , respectively. The fourth and fifth positions belonged to K and ICA, at 3.99 ng.g^{-1} and 6.86 ng.g^{-1} , respectively. GA₇ and IBA shared the same position of level of LOQ, at roughly 10.75 ng.g^{-1} while the limit of quantification of GA₄ was 13.82 ng.g^{-1} . To specific, GA₇ was quantified at highest concentration of 17.19 ng.g^{-1} (Table 2).

This suggests that at the same analytical conditions plant growth hormones in vegetables are detected and quantified at different concentrations, in which the lowest concentration limits of determination and quantification belonged to cytokinin group, followed by auxin group and the highest is the gibberelin group.

Compound	Curves	R^2	LOD (ng.g^{-1})	LOQ (ng.g^{-1})
GA ₃	$y = 1.4636x + 14.33$	0.9997	5.16	17.19
GA ₄	$y = 4.4224x + 175.56$	0.9990	4.14	13.82
GA ₇	$y = 2.12x + 95.658$	0.9961	4.32	10.75
IBA	$y = 2.4612x + 20.595$	0.9987	3.22	10.75
ICA	$y = 4.4533x + 127.66$	0.9985	2.06	6.86
tZ	$y = 554.43x - 783.52$	0.9999	0.19	0.63
BA	$y = 487.79x + 3857.9$	0.9989	0.31	1.02
K	$y = 418.45x + 2251.5$	0.9982	1.20	3.99
iP	$y = 2084x - 10030$	0.9995	0.46	1.54

Table 2: Parameters of Calibration Curve for Each Plant Growth Substance

3.4. Recovery of the Extraction Procedure of Plant Growth Substances in Vegetables

Recovery was determined by the ratio between the amount of each plant growth substance present in spiked/extracted and extracted/spiked samples as described in Calibration curves and validation of the method. As can be seen from the Table 3, the values of recovery for PGRs were shown in different concentrations of 10 $\mu\text{g.mL}^{-1}$, 100 $\mu\text{g.mL}^{-1}$ and 1000 $\mu\text{g.mL}^{-1}$, respectively; and the repeatability standard deviation (RSD) of each level for each hormone. In addition, the overall recovery corresponds to the mean of recovery in different levels. It is interesting to note that the recovery for all of plant growth substances ranged from 83.38-104.50%. Particularly, the highest recoveries were reported in the gibberellin group (GA₃, GA₄, GA₇) with a figure higher than 100%. Subsequently, the overall average recovery of auxins was approximately at 93%, whereas those of the cytokinins was fluctuated in the range of 83.38% and 92.49%. On the other hand, the repeatability standard deviation of the recovery for plant growth regulators in three different concentrations as low, medium and high levels was fluctuated between 0.22% and 11.07% (Table 3). These results proved that the matrix affects the recovery distinctly depending on the analyte and on the concentration level. It also shows the significance of performing the calibration curve in the matrix and of validating the analytical method in plant growth substances in vegetable samples.

PGR	Spiked Concentration	Repeatability (RSD, %)	Recovery Mean (%)	Overall Average of Recovery (%) *
GA ₃	10	11.07	119.47	104.50 ± 13.42
	100	5.52	101.87	
	1000	3.68	92.58	
GA ₄	10	1.92	105.35	101.60 ± 4.31
	100	1.42	96.90	
	1000	0.63	102.56	
GA ₇	10	4.54	108.60	102.35 ± 5.80
	100	0.44	97.14	
	1000	1.13	101.31	
IBA	10	6.19	92.31	93.32 ± 3.10
	100	10.71	90.86	
	1000	3.74	96.80	

PGR	Spiked concentration	Repeatability (RSD, %)	Recovery mean (%)	Overall average of recovery (%) *
ICA	10	2.99	85.71	92.49 ± 5.92
	100	0.61	96.68	
	1000	3.13	95.07	
tZ	10	0.67	87.26	88.40 ± 1.40
	100	1.25	87.97	
	1000	1.60	89.96	
BA	10	0.56	92.47	83.38 ± 6.87
	100	0.06	82.48	
	1000	0.02	75.20	
K	10	1.46	90.81	92.30 ± 3.90
	100	1.91	89.36	
	1000	0.28	96.72	
iP	10	1.22	97.51	90.95 ± 7.29
	100	0.58	92.23	
	1000	0.22	83.09	

Table 3: Percentage of Recovery during the Extraction of Plant Growth Substances in Vegetables

(*) The Data Was Presented as the Mean ± Standard Deviation, N = 3

4. Conclusion

In summary, 9 types of plant growth substances in vegetables such as Gibberelin A₃ (GA₃), Gibberelin A₄ (GA₄), Gibberelin A₇ (GA₇), Indole-3-butyric acid (IBA), Indole-3-carboxylic acid (ICA), trans-zeatin (tZ), N6-Benzyladenine (BA), Kinetin (K) and N6-Isopentenyladenine (iP) were determined and quantified by HPLC system linked a (3μm, 150 x 2.1 mm) Hypersil GOLD aQ C18 reversed-phase column and coupled to a tandem quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface (ESI) based on a combination of the retention time in the liquid chromatography and the diagnostic precursor ion to fragment ions transition in ESI-MS/MS. The method showed good linearity for all 9 analytes with regression coefficients R² > 0.996. Limit of detection ranged from 0.19 ng.g⁻¹ to 1.20 ng.g⁻¹ for cytokinins (BA, tZ, K and iP), from 2.06 ng.g⁻¹ to 3.22 ng.g⁻¹ for auxins (ICA and IBA), and from 4.14 to 5.16 ng.g⁻¹ for gibberellins (GA₃, GA₄ and GA₇). The mean recoveries for all of the analytes were from 83.38 to 104.50% and the repeatability standard deviation of the recovery for hormones in three different concentrations as low, medium and high levels was from 0.22% to 11.07%. To our knowledge, this is a rapid, simple, sensitive, accurate and efficient method for analysis plant growth substances in vegetables as well as the first study analyzing 9 plant hormones: GA₃, GA₄, GA₇, IBA, ICA, tZ, BA, K and iP in plant tissues using high-performance liquid chromatography equipped with an electrospray ionization tandem mass spectrometry. This plays a pivotal role in studying, determining and quantifying residues of plant growth substances that accumulate in agricultural products in general and green vegetables in particular. Since then, helping national managers have appropriate measures for using plant growth regulators in the production of agricultural products as well as recommending to consumers know which products are at risk of many plant growth stimulants and potentially affecting human health.

5. Acknowledgement

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6. Conflict of Interest

The authors declare that they have no conflict of interest.

7. Abbreviations

GA₃ : Gibberelin A₃

GA₄ : Gibberelin A₄

GA₇ : Gibberelin A₇

IBA : Indole-3-butyric acid

ICA Indole-3-carboxylic acid

tZ : trans-zeatin

BA : N6-Benzyladenine

K : Kinetin

iP : N6-Isopentenyladenine

ESI-MS/MS : Electrospray Ionization Tandem Mass Spectrometry

TOF : time of flame

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