Simultaneous analysis of thirteen phytohormones in fruits and vegetables by SPE-HPLC–DAD

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Abstract Determination of phytohormones have attracted increasing attentions in food safety field. In this study, an efficient and quantitative method was developed which can simultaneously determinate thirteen phytohormones in fruits and vegetables using solid phase extraction (SPE) combined with high performance liquid chromatography–diode array detection (HPLC–DAD). The samples were extracted with 80% methanol containing 0.5% (V/V) formic acid, and the extracts were then concentrated and purified using primary secondary amine (PSA) and C18 tandem dual SPE cartridges. The analytes were separated on a Waters XBridge™ C18 column and eluted utilizing a gradient elution program of water and methanol. Mean recoveries of the thirteen analytes varied from 74.69 to 92.40%, with relative standard deviations < 3.57%. The limits of detection and quantitation were 0.005–0.018 mg/kg and 0.02–0.10 mg/kg, respectively. The phytohormones in kiwi fruit, strawberry, bean sprout, and green pepper were detected using the above method, respectively. Only the IAA content of 0.14 mg/kg was detected for the strawberry from a supermarket, which was lower than the prescribed limit in food safety standards (0.2 mg/kg).

Keywords Phytohormone · High performance liquid chromatography–diode array detection · Simultaneous analysis · Fruit and vegetable

Introduction

Phytohormones are nonnutritious chemicals that artificial synthesized or extracted from microorganisms and applied externally in plants to regulate the growth of plants. The use of lower concentrations of phytohormones can promote or inhibit the growth and development of plants (Hou et al., 2008; Santner et al., 2009). The fruits and vegetables with phytohormone residues may have little effect on human body in the short term, but after long-term consumption, it will cause endocrine disorder and affect the metabolic balance in the body (Cho et al., 2013; Oulkar et al., 2011). The abuse or improper use of phytohormones has resulted in increasing food safety problems. In recent years, the government and legislative setup have stipulated maximum residue limits of phytohormones to mitigate its potential risks for the people's health (Li et al., 2015). Therefore, it is imperative and urgent to formulate and validate a simple and generic pretreatment method which is applicable to different classes of phytohormones.

The methods used to analyze phytohormones are varied, owing to the significant differences in the chemical structures of the phytohormone varieties. The determination methods including spectral analysis (Li et al., 2009; Zhang et al., 2010), enzyme-linked immune sorbent assay (ELISA) (Wang et al., 2001), capillary electrophoresis (CE) (Chen et al., 2011), gas chromatography (GC) (Royer et al., 2006), high-performance liquid chromatography (HPLC) (Aral et al., 2013), and liquid chromatography–mass spectrometry (LC–MS) (Cai et al., 2015; Luo et al.,

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2017) have been used to detect phytohormones. In general, the accuracy of spectral analysis techniques is relatively low, and ELISA methods require further improvement to reduce the cross-reaction and improve accuracy. The derivatization methods for GC analysis are different for different phytohormones. HPLC can overcome the defects that need to be derived using GC analysis, and moreover, it can be used to detect the multiple residues of different phytohormones. The LC–MS has used for the identification of residues in excess of established limits. Unfortunately, the determination of multiple phytohormones is still a challenging issue. In contrast, a potential alternative for the determination and confirmation of phytohormone residues is the use of HPLC–DAD methods, which are cost-effective and can be used for the simultaneous detection of many analytes.

Sample pretreatment is an important step in chromatography. It is a time-consuming procedure, and also greatly affects the accuracy and precision of the analysis. There has been progress in sample preparation technology for the analysis of phytohormones, including the use of liquid–liquid extraction (LLE) (Wells et al., 2013), solid-phase extraction (SPE) (Dobrev et al., 2005; Ma et al., 2008; Pan et al., 2008), magnetic solid-phase extraction (MSPE) (Cai et al., 2014), vapor-phase extraction (VPE) (Engelberth et al., 2003), solid-phase microextraction (SPME) (Liu et al., 2010), and molecularly imprinted solid-phase extraction (MISPE) (Yan et al., 2012). Compared with other techniques, SPE has emerged as an eminent tool for sample pretreatment with less organic solvents involved, and matrix interferences can be removed and target analytes can be pre-concentrated simultaneously. However, single SPE process is usually inadequate to remove lipids and lipophilic matrix substances. Hence, tandem dual SPE processes have been adopted to purify various classes of phytohormones in complex plant matrices, but some of them suffer from low recovery and reproducibility (Dobrev and Kamínek, 2002; Susawaengsup et al., 2011). The challenges in the simultaneously determination of multi-class phytohormones originate are not only from their low abundance and the complex matrix of plants, but also from the difficulties of simultaneous extraction and purification of multi-class phytohormones owing to their chemical diversity.

This study developed a method for simultaneously determination of thirteen phytohormones: Gibberellic acid (GA), zeatin (Z), paclobutrazol (PBZ), 4-fluorophenoxyacetic acid (4-FPA), 4-chlorophenoxyacetic acid (4-CPA), 3-indolyl-acetic acid (IAA), 3-indolyl-butyric acid (IBA), 6-benzylaminopurine (6-BA), abscisic acid (ABA), 1-naphthyl acetic acid (NAA), forchlorfenuron (CPPU), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in fruits and

vegetables. The method involved sample pretreatment with multiple SPE and analytical determination with HPLC–DAD, which was an uncomplicated instrumentation system available in most laboratories. The objectives of this study were to optimize and compare, in terms of efficiency, the sample pretreatment involved in the extraction and purification of multiple phytohormones and develop an efficient analytical method for their simultaneous determination in fruits and vegetables. This method could be applied for the rapid detection of phytohormones at a limit of detection (LOD) low enough for quantitative determination below established limits.

Materials and methods

Reagents and chemicals

GA (98.0%), Z (98.5%), PBZ (98.5%), 4-FPA (98.5%), 4-CPA (98.0%), IAA (99.5%), IBA (99.9%), 6-BA (99.0%), ABA (99.5%), NAA (98.5%), CPPU (98.7%), 2,4-D (99.0%), and 2,4,5-T (99.0%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The chemical structures of the phytohormones are shown in Fig. 1. HPLC-grade methanol was obtained from Burdick & Jackson (Muskegon, MI, USA) and HPLC-grade acetic acid from E. Merck (Darmstadt, Germany). Throughout the study, water from a Milli-Q system from Millipore (Billerica, MA, USA) was used.

Oasis HLB (60 mg, 3 mL) and PSA (60 mg, 3 mL) SPE cartridges were purchased from Waters (Milford, MA, USA). C18 (60 mg, 3 mL) SPE cartridges were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Kiwi fruit, strawberry, bean sprout, and green pepper were purchased local supermarket.

Standard solutions

Using 50% methanol as solvent, standard stock solutions of GA, Z, PBZ, 4-FPA, 4-CPA, IAA, IBA, 6-BA, ABA, NAA, CPPU, 2,4-D, and 2,4,5-T were prepared to a final concentration of 1.0 mg/mL. These solutions were stored under refrigeration (− 20 °C). Diluted aliquots of the standard stock solution with 50% methanol and prepared the working solution daily.

Sample extraction

The plant hormones were extracted from plant samples according to the method of Dobrev et al. (2005) with modifications. All plant samples were cut into pieces, and then homogenized with high speed homogenizer. The homogenized plant samples (5 g) were added to a 50 mL

Fig. 1 Chemical structures of the thirteen phytohormone compounds

centrifuge tube and extracted twice with 40 mL of the extractants, followed by mixing after ultrasonic extraction for 15 min. The mixture was centrifuged ($2960\times g$) for 20 min, and the supernatant were combined. The combined extracts were evaporated and diluted with methanol/water/formic acid (10/95/0.1, v/v/v) to 5 mL for further analysis.

Purification using C18 cartridge

The extracted supernatants were dissolved in 5.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v) and purified by C18 cartridges. The cartridges were activated and conditioned with 6.0 mL of methanol and equilibrated with 3.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v). Each sample was loaded on a cartridge and washed with 3.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v). The phytohormones were eluted with 2×3.0 mL of methanol/water/formic acid (80/20/0.1, v/v/v). The obtained eluates were dried using nitrogen evaporators and dissolved with 1 mL 80% methanol, and then filtered using a $0.45\ \mu\text{m}$ microfiltration membrane prior to HPLC analysis.

Purification using Oasis HLB cartridge

The purification method was carried out according to Urbanová et al. (2013) with minor modifications. The extracted supernatants were dissolved in 5.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v), and then loaded on Oasis HLB cartridges pre-conditioned with 6.0 mL of MeOH and allowed to equilibrate with 3.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v). After washing the cartridges with 3.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v), the target phytohormones were eluted with 3.0 mL of methanol/water/formic acid (80/20/0.1, v/v/v) twice. The obtained eluates were dried using nitrogen evaporators and dissolved with 1 mL 80% methanol, and then filtered using a $0.45\ \mu\text{m}$ microfiltration membrane prior to HPLC analysis.

Purification using PSA and C18 cartridges

The extracted supernatants were dissolved in 5.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v) and purified using PSA and C18 tandem dual cartridges. The SPE procedure was as follows: The PSA SPE column was

connected in series above C18 SPE column with a string joint. The cartridges were activated and conditioned with 12.0 mL of methanol and equilibrated with 6.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v). Each sample was loaded on tandem dual cartridge and washed with 6.0 mL of methanol/water/formic acid (10/95/0.1, v/v/v). The target phytohormones were eluted with 3.0 mL of methanol/water/formic acid (80/20/0.1, v/v/v) twice. The obtained eluates were dried using nitrogen evaporators and dissolved with 1 mL 80% methanol, and then filtered using a 0.45 μm microfiltration membrane prior to HPLC analysis.

HPLC–DAD analysis

HPLC was performed with a Waters 1525 (Waters, Milford, MA, USA) system, including Waters 1525 secondary pump, Waters 2998 DAD, and Waters 2707 autosampler. The Waters Empower3 software controlled the HPLC parameters and processed the data. The XBridgeTM C18 column (250 mm $\times$ 4.6 mm, 5 μm) (Waters, Milford, MA, USA) was used for separation. Eluent A (water/acetic acid = 99.9/0.1) and eluent B (methanol) were combined in a linear gradient as follows: 0–40 min, 20–70% B; 40–50 min, 70–95% B; 50–54 min, 95% B. The flow rate was 0.9 mL/min, and the column heater was set at 30 °C. The DAD absorption spectrum of the compounds were recorded between 210 nm and 500 nm.

Method validation

The analytical method validation was conducted according to AOAC guidelines (Han et al., 2017). The following validation parameters were established: linearity range, LOD, limit of quantitation (LOQ), repeatability, reproducibility, recovery and stability. Relative standard deviation (RSD) of repeated experiment was used to evaluate the precision. The recoveries of the phytohormones in the samples were assessed by spiking a standard mixture at three concentrations (0.1 mg/kg, 0.3 mg/kg and 0.5 mg/kg). The intra- and inter-day accuracies and precisions were determined by samples spiked with high, medium and low levels of phytohormones on five consecutive days, and on each day six replicates. Standard calibration was performed by plotting analyte concentrations versus peak areas. LOD and LOQ were determined by signal-to-noise ratios of 3 and 10, respectively.

Statistical analysis

All data were analyzed using the Statistical Package for Social Science 23 (SPSS 23) and all charts were produced using Origin 9.0. Significant differences were evaluated

using Statistix 9 ($p < 0.05$). All values are expressed as the mean $\pm$ standard deviation (SD).

Results and discussion

Evaluation of chromatographic conditions

The chromatographic conditions for simultaneous separation and quantification of thirteen phytohormones were optimized by evaluating the variables that affect the chromatographic process. According to the literature, phytohormones are usually separated through acid mobile phases on reverse phase column (Kantiani et al., 2009). Different systems were evaluated in which various types of column and mobile phases were employed. Once the column and mobile phase were selected, the separation of the analytes was optimized with different gradient programs.

In this study, various reverse-phase C18 columns were tested, including XBridgeTM C18, 250 mm $\times$ 4.6 mm, 5 μm (Waters, Milford, MA, USA), Zorbax Eclipse XDB-C18, 250 mm $\times$ 4.6 mm, 5 μm (Agilent Technologies, Santa Clara, CA, USA), and GraceSmart RP18, 250 mm $\times$ 4.6 mm, 5 μm (Grace, Columbia, MD, USA). It was found that the peaks of the thirteen phytohormones were well separated in 50 min on the XBridgeTM C18 columns.

The effect of mobile phase composition on chromatographic separation was investigated using acetonitrile-0.2% acetic acid, acetonitrile-0.05% phosphoric acid, and methanol-0.05% phosphoric acid. Several elution gradient procedures using acetic acid (0.2%) as eluent A and acetonitrile with acetic acid (0.2%) as eluent B were studied. The chromatograms obtained cannot resolve all the signals satisfactorily. It was observed from Fig. 2, the mobile phase of methanol-0.1% acetic acid (v/v) with gradient program yielded the most effective separation.

According to the above results, the optimal chromatographic conditions were XBridgeTM C18 column kept at 30 °C, eluent A (methanol/water/acetic acid = 10/89.9/0.1) and eluent B (methanol/water/acetic acid = 90/9.9/0.1) were selected. Further improvement in separation was obtained by mobile phase gradient. The gradient condition is as follows: 0–40 min, 20–70% B; 40–50 min, 70–95% B; 50–54 min, 95% B. As a result, resolution improved considerably, and narrow and symmetrical shape peaks were achieved.

The wavelengths of the target analytes were selected by a DAD full-wavelength scan (190–500 nm). As the thirteen phytohormones showed different UV absorption properties, different detection wavelengths were simultaneously set to monitor these compounds in a single run: 220 nm (for 4-FPA, IBA, and 2,4,5-T), 240 nm (for Z, GA, PBZ, IAA,

Fig. 2 HPLC chromatograms of standard mixtures of phytohormones. Peak identities: 1, Z; 2, GA; 3, PBZ; 4, 4-FPA; 5, 4-CPA; 6, IAA; 7, IBA; 8, 6-BA; 9, ABA; 10, NAA; 11, CPPU; 12, 2,4-D; 13, 2,4,5-T

6-BA, CPPU and 2,4-D), and 290 nm (for 4-CPA, ABA and NAA). The obtained results are presented in the chromatogram in Fig. 2.

Study on extraction methods

According to the physicochemical properties of the phytohormones and previous reports (Aral et al., 2013; Cai et al., 2015), acetonitrile–formic acid (99.5/0.5, v/v), acetonitrile–water–formic acid (80/19.5/0.5, v/v/v), acetonitrile–water (80/20, v/v/v), and methanol–water–formic acid (80/19.5/0.5, v/v/v) for extracting phytohormones from fruits or vegetables were investigated, using the samples

with the added target phytohormones of 0.5 mg/kg. Non-spiked fresh fruits or vegetables samples were also processed in parallel as sample blanks.

The results showed that the most efficient extractions of the target phytohormones with the least amount of interfering substances including pigments, lipids, and other lipophilic matrix substances were obtained using the methanol systems (Table 1). Whereas 4-FPA, IAA, and 2,4,5-T showed slightly lower extraction efficiencies than acetonitrile. This may be due to the good solubilities of 4-FPA, IAA, and 2,4,5-T in acetonitrile. The comparison between acidified and non-acidified extraction solvents showed that slightly higher recoveries of the

Table 1 Comparison of the recoveries (%) of the extraction solvents (n = 3)

Analyte	Methanol–formic acid (99.5/0.5, v/v)	Methanol–water–formic acid (80/19.5/0.5, v/v/v)	Methanol–water (80/20, v/v/v)	Acetonitrile–formic acid (99.5/0.5, v/v)	Acetonitrile–water–formic acid (80/19.5/0.5, v/v/v)
Z	79.35 ± 7.52 ^{abc}	89.35 ± 7.35 ^c	86.38 ± 6.27 ^{bc}	72.35 ± 4.28 ^a	75.34 ± 4.58 ^{ab}
GA	68.37 ± 4.57 ^a	90.24 ± 6.74 ^{cd}	92.37 ± 4.53 ^d	81.27 ± 5.37 ^{bc}	79.58 ± 5.27 ^b
PBZ	86.42 ± 5.34 ^a	103.47 ± 5.37 ^b	89.57 ± 5.27 ^a	86.37 ± 5.46 ^a	90.27 ± 4.20 ^a
4-FPA	91.57 ± 6.31 ^a	91.27 ± 6.58 ^a	90.25 ± 4.39 ^a	102.57 ± 5.38 ^b	95.31 ± 5.21 ^{ab}
4-CPA	75.67 ± 5.28 ^a	86.37 ± 5.41 ^b	81.27 ± 5.69 ^{ab}	75.38 ± 4.39 ^a	89.37 ± 4.38 ^b
IAA	101.24 ± 6.37 ^{bc}	95.37 ± 4.37 ^b	106.37 ± 5.64 ^c	69.87 ± 5.68 ^a	101.24 ± 5.38 ^{bc}
IBA	74.28 ± 7.27 ^a	105.37 ± 4.27 ^b	76.37 ± 8.67 ^a	73.68 ± 7.21 ^a	75.21 ± 4.38 ^a
6-BA	68.27 ± 6.51 ^a	110.57 ± 6.87 ^c	81.29 ± 5.61 ^b	85.31 ± 4.65 ^b	85.37 ± 5.21 ^b
ABA	84.37 ± 5.64 ^a	87.64 ± 5.34 ^a	102.37 ± 7.14 ^b	89.68 ± 7.27 ^a	85.37 ± 5.27 ^a
NAA	84.27 ± 4.27 ^{bc}	90.38 ± 6.18 ^{cd}	75.29 ± 4.29 ^{ab}	95.07 ± 7.08 ^d	72.38 ± 5.74 ^a
CPPU	68.31 ± 5.64 ^a	103.57 ± 5.87 ^c	80.37 ± 6.28 ^b	86.17 ± 4.50 ^b	106.37 ± 5.21 ^c
2,4-D	75.14 ± 6.38 ^a	92.74 ± 7.27 ^b	81.67 ± 5.79 ^a	76.38 ± 4.89 ^a	81.14 ± 4.35 ^a
2,4,5-T	77.38 ± 3.28 ^a	89.27 ± 4.16 ^b	83.10 ± 6.47 ^{ab}	86.47 ± 4.27 ^{ab}	98.24 ± 5.20 ^c

Values in the same line with different letters were significantly different at $p < 0.05$

phytohormones were achieved using 80% methanol with 0.5% formic acid. In terms of molecular structure of the phytohormones, 4-FPA, ABA, 4-CPA, IBA, NAA, IAA, 2,4-D, and 2,4,5-T all possess carboxyl groups. The ionic form of the carboxyl groups will be inhibited in an acidic environment. The addition of 0.5% formic acid to the extracting solution can hence improve the recovery. In general, methanol–water–formic acid (80/19.5/0.5, v/v/v) was more suitable for the extraction of the target phytohormones. The recovery of phytohormones was in the range of 86.37–110.57%.

Optimization of SPE conditions

Several SPE cartridges with various sorbents have been widely applied for the removal of the co-extracted interferences in the analysis of phytohormones due to their convenience and simple method. The commonly used solid phase extraction columns are C18 and hydrophilic-lipophilic balance column (Oasis HLB) according to the polarity of weak acidic phytohormones (Hou et al., 2008; Kojima et al., 2009). The nonpolar substance like chlorophyll can be removed by C18, and some strongly polar components can also be washed out by adjusting eluent. Ma et al. (2008) purified coconut juice using C18 column, and IBA, ABA, GA can be detected using HPLC combined with mass spectrometer.

Figure 3 shows the recoveries of the 0.5 mg/kg addition standards with extracts from strawberry obtained using the different purification methods. When the sample was purified using C18 SPE alone, it was found that the

recovery rate of the phytohormones was not satisfied yet. The separation ability of the C18 SPE was limited when the plants containing numerous polar and medium polar substances, as many interfering peaks appeared. Oasis HLB is a hydrophilic-lipophilic balance column. Oasis HLB cartridges produced slightly improved recoveries compared to the C18 cartridges. For IAA, NAA, CPPU and 2,4,5-T, improved recoveries were observed on Oasis HLB cartridges, but this cartridge showed poor recoveries (< 70%) for the other phytohormones (Z, 4-CPA, IBA, 6-BA, ABA and 2,4-D). Although the polarity of this column is high, the separation is unsatisfied using a single column, as it cannot purify complex plant extracts. This result is consistent with previous study (Ge et al., 2007).

PSA is a polar adsorbent, which has good adsorption effect on polar compounds. At present, it is widely used in the analysis of fruits and vegetables residue matrix dispersion solid phase pretreatment (Huang et al., 2015). After adsorption on PSA SPE, the recovery rate of the target compound was obviously increased (Fig. 3). During the process of experiment, it was found that PSA SPE effectively adsorbed pigments in fruits and vegetables extracts, but the adsorption of target compounds was less. After the purification process using PSA and C18 tandem dual SPE cartridges, almost all the standard recoveries reached > 80.37% and higher than those of the samples purified using C18 SPE (or Oasis HLB SPE) alone. Considering the recoveries of the target phytohormones and their separation efficiencies from the plant samples, a two-step cleanup method using PSA and C18 SPE was developed and used for the analysis of multiple phytohormones in fruits and vegetables.

Fig. 3 Recovery rates of addition standards with extracts from strawberry by different purification procedures ($n = 3$). Bars by different purification procedures with different letters were significantly different at $p < 0.05$

Validation of the method

The standard solutions containing thirteen analytes were prepared and diluted to appropriate concentrations to plot the calibration curves. The curves between peak area and concentration of each analyte were plotted at six concentrations of the thirteen analytes. The linear relationships and good coefficients of determination ($R^2 \geq 0.9924$) were obtained. This showed good linearity comparable to that reported in the study of different tissues of model plants (Cai et al., 2015). As shown in Table 2, the LOQs and LODs were in the range of 0.005–0.018 mg/kg and 0.02–0.10 mg/kg, respectively, which were lower than previously reported value (Aral et al., 2013).

The intra-day and inter-day precisions were determined by analyzing known concentrations of the thirteen phytohormones in six replicates every day for three consecutive days. To verify the repeatability of the method, six different concentrations of thirteen phytohormones prepared from the same sample were analyzed. Table 3 shows that

the intra- and inter-day precisions ranged from 1.25 to 4.07% and 0.57 to 2.37%, respectively. The repeatability RSD values of thirteen phytohormones were all $< 1.68\%$. These results indicated that the developed method had high reproducibility.

The accuracy of the method was further investigated by the recovery test using three concentration levels of the mixed standard solutions. The standards were added into the samples for extraction and purification, and then analyzed. The average recoveries were calculated as the ratio of the detected amount to the added amount. As shown in Table 3, the recovery of the method was in the range of 74.69–92.40%, and the RSD was $< 3.57\%$, indicating that the established method was accurate for the determination of thirteen phytohormones in fruits and vegetables. Thirteen phytohormones were added into strawberry samples at 0.1 mg/kg. There was no interference in the retention time of the phytohormones, indicating this method has good selectivity.

Table 2 Linear regression data, LODs, and LOQs of the thirteen analytes

Analyte	Regression equation ^a	Linear range (μg/mL)	R ²	LOD (mg/kg)	LOQ (mg/kg)
Z	$Y = 5203.1X + 124.6$	0.50–80	0.9987	0.018	0.06
GA	$Y = 7025.8X - 257.6$	0.05–90	0.9958	0.009	0.05
PBZ	$Y = 1147.2X + 102.8$	0.50–80	0.9924	0.015	0.07
4-FPA	$Y = 3352.9X + 121.7$	0.30–90	0.9951	0.014	0.06
4-CPA	$Y = 7620.7X + 68.9$	0.05–90	0.9959	0.013	0.07
IAA	$Y = 8627.9X - 25.9$	0.05–90	0.9984	0.009	0.02
IBA	$Y = 9863.8X + 152.9$	0.07–90	0.9980	0.009	0.03
6-BA	$Y = 7104.9X + 42.7$	0.07–90	0.9985	0.007	0.02
ABA	$Y = 5012.7X - 40.9$	0.05–90	0.9996	0.012	0.10
NAA	$Y = 7453.9X + 78.6$	0.05–90	0.9957	0.010	0.07
CPPU	$Y = 5809.7X - 42.9$	0.04–90	0.9968	0.005	0.02
2,4-D	$Y = 8573.9X + 135.0$	0.07–90	0.9984	0.007	0.03
2,4,5-T	$Y = 22,478.9X - 59.3$	0.20–90	0.9958	0.010	0.07

^aY is the peak area, X is the concentration injected

Table 3 Precision, repeatability, stability, and recovery of the thirteen analytes

Analyte	Precision (RSD, %)		Repeatability (RSD, %, n = 6)	Stability (RSD, %, n = 6)	Recovery (%, n = 3)	
	Intra-day (n = 6)	Inter-day (n = 5)			Mean	RSD, %
Z	2.37	0.57	0.58	2.28	74.69	3.54
GA	1.98	1.58	1.27	3.57	88.52	2.89
PBZ	2.27	1.57	1.68	2.27	82.78	3.12
4-FPA	3.27	1.17	0.58	1.89	83.45	2.57
4-CPA	2.89	2.37	0.68	1.57	81.64	2.68
IAA	2.57	1.27	0.98	2.57	79.44	1.57
IBA	1.89	1.98	0.47	3.51	89.84	1.74
6-BA	3.6	0.57	0.59	2.54	78.58	2.57
ABA	4.07	2.37	0.59	1.82	84.64	2.15
NAA	3.87	2.14	0.68	1.58	92.40	3.57
CPPU	1.25	1.29	1.59	3.27	84.76	2.58
2,4-D	2.47	2.34	0.97	0.98	80.04	2.91
2,4,5-T	1.57	1.57	1.27	2.51	83.40	3.17

Sample analysis

Kiwi fruit, strawberry, bean sprout, and green pepper were detected using the above method, with the samples of 5 g each. The IAA content was 0.14 mg/kg for the strawberry sample from a supermarket, which was lower than the prescribed limit in food safety standards (0.2 mg/kg). None of the thirteen phytohormones were detected from other samples. In conclusion, the proposed method is suitable for trace phytohormones determination and analysis in fruits and vegetables with the merits of simplicity, sensitivity, reliability and cost-effective.

The detection of phytohormones has become increasingly imperative for food safety. This study established the extraction and purification methods for different phytohormones in fruits and vegetables, and optimized a sensitive HPLC–DAD method to simultaneously determine these phytohormones. Through methodology investigation, the established analysis method was successfully applied for the determination of thirteen phytohormones in fruits and vegetables. This method was proved suitable for the rapid simultaneous analysis of different classes of phytohormones in agriculture.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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