Journal of Chromatography B, 881–882 (2012) 83–89

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A liquid chromatography tandem mass spectrometry method for simultaneous determination of acid/alkaline phytohormones in grapes

Zheng Han [a], Gang Liu [a], Qinxiong Rao [a], Bing Bai [a], Zhihui Zhao [a], Hong Liu [b,*], Aibo Wu [a,*]

[a] Institute for Agri-Food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences, 1018 Jinqi Road, Shanghai 201403, PR China
[b] Shanghai Municipal Center for Disease Control and Prevention, 1380 Zhongshan West Road, Shanghai 200336, PR China

ARTICLE INFO

Article history:
Received 12 October 2011
Accepted 2 December 2011
Available online 10 December 2011

Keywords:
Phytohormone
Multiresidue analysis
Liquid chromatography tandem mass spectrometry

ABSTRACT

A high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for simultaneous determination of five acid/alkaline phytohormones, i.e., indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthylacetic acid (NAA), gibberellic acid (GA_3) and isopentenyladenine (2IP), in grapes was developed. After optimization, the samples were extracted with methanol containing 1% formic acid and purified by Oasis HLB SPE cartridges. The analytes were separated on a Thermo Hypersil Gold column (100 mm × 2.1 mm, 3.0 μm) with water and acetonitrile, then determined with Thermo tandem quadrupole mass spectrometer operating in negative electro-spray ionization using selected reaction monitoring (SRM) mode. The established method was further validated by determining the linearity ($R^2 \geq 0.9990$), average recovery (82.5–105.4%), sensitivity (0.05–1.00 ng mL^{-1}), precision (RSD ≤ 13.0%) and stability (RSD ≥ 82.0%). Finally, the application of the approach proposed to thirty grape samples convinced its desirable performance for rapid analysis of multiclass phytohormones, supporting its sufficient capability for multiresidue analyses or other analytical system targeting phytohormones in agriculture field.

1. Introduction

Phytohormones are structurally diverse compounds that play an important role in a variety of processes related to plant growth and development including cell division, enlargement and differentiation, organ formation, seed dormancy and germination, leaf and organ senescence and abscission. Phytohormones are usually grouped into four major classes of auxines, gibberellines, cytokinines and inhibitors [1]. Typically, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthylacetic acid (NAA) are chief representatives of auxines, while gibberellic acid (GA_3) and isopentenyladenine (2IP) represent the groups of gibberellins and cytokinines, respectively [2]. In most cases, multiclass phytohormones existed in plants either by endogenous secretion or exogenous treatment to achieve various enhanced agricultural characteristics during some critical growth stages. However, abusing of the phytohormones as regulators would cause either no significant effects or adverse effects on the target plants [3,4], not calling attention to the effects of these compounds on public health. Thus development and validation of a simple and sensitive method is substantially crucial for the application stage and optimum concentration of the used phytohormones. Moreover, monitoring the phytohormones residues is also controversially related to food safety issues [3]. Regarding the potential risks, the European Union (EU) has set up a maximum residue limit (MRL) of 5 mg kg^{-1} for GA_3 in grapes [5], which signifies the requirements of the sensitive and accurate quantification methods for routine analyses of phytohormones residues in a number of food matrices.

The increasing need for multiple analysis targeting multiclass phytohormones has promoted related research on the methods with the employment of adequate extraction and clean-up procedures. Unfortunately, the development of such a sample pretreatment method is impeded by the chemical diversity of the analytes. For example, IAA, IBA, NAA and GA_3 are acidic while 2IP is basic [2], as a consequence, it required that the extraction and purification procedures must be highly efficient and can accommodate the wide range of chemical properties consisting of different target compounds. In previous studies, several sample pretreatment methods have been developed. These methods generally involved liquid extraction with different acid or alkaline solvents and further purifications via solid phase extraction (SPE) with a wide variety of sorbents, i.e., reversed-phase, immunoaffinity or polymeric materials [6–9]. However, most of SPE cartridges mainly targeted several compounds with similar chemical properties for multiresidue analysis [10,11]. And the phytohormones were extracted for more than 12 h under low temperature to keep

* Corresponding authors. Tel.: +86 21 62202875; fax: +86 21 62203612.
E-mail addresses: hliu@scdc.sh.cn (H. Liu), wuaibo@saas.sh.cn (A. Wu).

1570-0232/$ – see front matter.
doi:10.1016/j.jchromb.2011.12.002

the stability of the compounds [1,2]. Therefore, development and validation of a fast and generic sample pretreatment for different classes of phytohormones is still in urgent need for practical uses.

It is also difficult to provide accurate quantification of multiclass phytohormones in a given plant in a single analysis owing to their presence in trace amounts and the complicated background of a wide range of more abundant primary and secondary metabolites. Several analytical methods based on capillary electrophoresis (CE) [11–13], gas chromatography (GC) [14,15] and high performance liquid chromatography (HPLC) [16,17] have been well established. In general, CE offers attractive features for the analysis of phytohormones as only minute amounts of samples are needed and the analysis time can be relatively short, but the potential reproducibility problem might occur. Whereas GC with electron capture detector or mass spectrometry (MS) detection is limited to the analysis of phytohormones due to the critical and time-consuming derivatization steps prior to analysis. Practically, the most frequently used method for phytohormones analysis is liquid chromatography combined with different detectors since it combines high resolution with increasing sophisticated automation. However, HPLC analysis might suffer from interfering of the target HPLC-UV signals by matrix co-extractives, which render the separation time longer or the sample clean-up procedure more complex [1,18].

In recent years, the availability of ionization sources, i.e., atmospheric pressure chemical ionization (APCI) and electro-spray ionization (ESI), has significantly improved the possibilities of employing HPLC/MS in the multiclass phytohormones analysis, owing to simple sample preparation, high sensitivity and the compatibility with almost the whole range of compound polarities [3,19–23]. No other techniques in the area of instrumental analysis has developed so rapidly as HPLC–MS/MS during the past 10 years [24,25] though the reliability of quantitative assays may not be absolute on some minor occasions. The molecules originating from the sample matrix that co-elute with the compounds of interest can interfere with the ionization process in the mass spectrometer, causing ionization suppression/enhancement, which might adversely affect the quantification results. Hitherto, there is no uniform HPLC–MS/MS method validated for simultaneous determination of IAA, IBA, NAA, GA_3 and 2IP in agricultural products.

The objectives of the work are well defined: (i) to simplify and validate the procedures of extraction and purification of multiclass phytohormones in grapes; (ii) to establish a fast and accurate HPLC–MS/MS method in order to determine five phytohormones; (iii) to test the method and to investigate the actual situations of phytohormone residues in grapes.

2. Experiments

2.1. Chemicals

The standards including IAA, IBA, NAA, GA_3 and 2IP were purchased from Sigma–Aldrich (St. Louis, MO, USA). The chemical structures of the five phytohormones are shown in Fig. 1. Acetonitrile and methanol of HPLC grade were obtained from Merck (Darmstadt, Germany). Other chemicals and solvents were of HPLC or analytical grade. Deionized water was purified using a Milli-Q Gradient A 10 System (Millipore, Billerica, MA, USA).

MCX SPE cartridges (61 mg, 3 cm^3) and Oasis HLB SPE cartridges (60 mg, 3 cm^3) were purchased from Waters (Milford, MA, USA). PCX SPE cartridges (60 mg, 3 cm^3), Diol SPE cartridges (500 mg, 3 cm^3), ODS-C18 SPE cartridges (200 mg, 3 cm^3), PEP SPE cartridges (30 mg, 1 cm^3), PSA SPE cartridges (500 mg, 3 cm^3) and SAX SPE cartridges (500 mg, 6 cm^3) were obtained from Bonna-Agela Technologies Inc. (Wilmington, DE, USA). All the involved SPE cartridges should be pre-conditioned with methanol and water before loading.

2.2. Apparatus

The filtrate was analyzed by direct injection into an HPLC–MS/MS (TSQ QUANTUM ULTRA, Thermo Scientific, USA) using selected reaction monitoring (SRM) mode. Separation was performed on a Thermo Hypersil Gold column (100 mm × 2.1 mm, 3.0 μm) at 35 °C, with a mobile phase flow rate of 0.3 mL min^{-1}. The mobile phase consisted of: (A) water and (B) acetonitrile. A linear gradient elution program was applied as follows: initial 60% B, 4 min 60% B, 6 min 80% B, 6.5 min 95% B, 7 min 95% B, 7.2 min 60% B and hold on for a further 2.8 min for re-equilibration, giving a total run time of 10 min. The injection volume was 5.0 μL (full loop). The following settings were used for MS/MS conditions: spray voltage, 3.5 kV; vaporizer temperature, 300 °C; sheath gas pressure, 30 psi; aux valve flow, 30 arb; and capillary temperature, 350 °C. Data acquisition and processing were performed using Xcalibur software (Thermo Scientific, USA).

2.3. Preparation of standard solutions

Accurately weighed solid portions of IAA, IBA, NAA, GA_3 and 2IP were dissolved in methanol to prepare 0.1 mg mL^{-1} of stock solutions. A mixed stock solution containing 10 μg mL^{-1} of IAA, NAA, 2IP, GA_3 and 5 μg mL^{-1} of IBA was prepared in methanol. All solutions were stored under darkness at −20 °C and the working solutions were prepared from these stock solutions and were serially diluted with the combined solution of methanol/water (50/50, v/v) immediately before use.

2.4. Samples

A total of thirty grape samples were randomly collected from local markets. The related information about the geographic origin of samples was required and registered as follows: samples 1–5 were collected from Xinjiang province; samples 6 and 7 were from Shandong province; sample 8 from Shanxi province; sample 9 from Hebei province; samples 10–13 from Zhejiang province; sample 14 from Liaoning province; the other samples were all from Shanghai. All samples were cut into pieces, and then homogenized with IKA T25 high speed homogenizer (Ika-Werke Gmbh, Staufen, Germany). The homogenate was preserved at −20 °C until analysis.

2.5. Sample pretreatment

The homogenized grape samples (2.0 g) were further homogenized for 2 min with methanol containing 1% formic acid (20 mL), then ultrasonic for 30 min. The mixture was centrifuged (3000 × *g*, 15 °C) for 10 min with Beckman Coulter Allegra 64R centrifuge (Brea, CA, USA). The supernatant was diluted with 180 mL of water to obtain the mixed solution with 10% of methanol. The solution was passed through the reconditioned Oasis HLB SPE cartridges at a rate of about 1–2 drops/s, and then 5 mL of water was passed through the cartridges at a rate of about 1–2 drops/s. All targets were eluted with 6 mL of methanol containing 1% formic acid at a rate of about 1–2 drops/s, and the elute was evaporated to dryness under a stream of nitrogen gas at 40 °C. The residue was re-dissolved by 1 mL combined solution of methanol/water (50/50, v/v) passed through a 0.22 μm filter and ready for injection.

2.6. Evaluation of matrix effects

The stock solutions were diluted with the blank matrix prepared with the analyte-free grape through the whole sample preparation

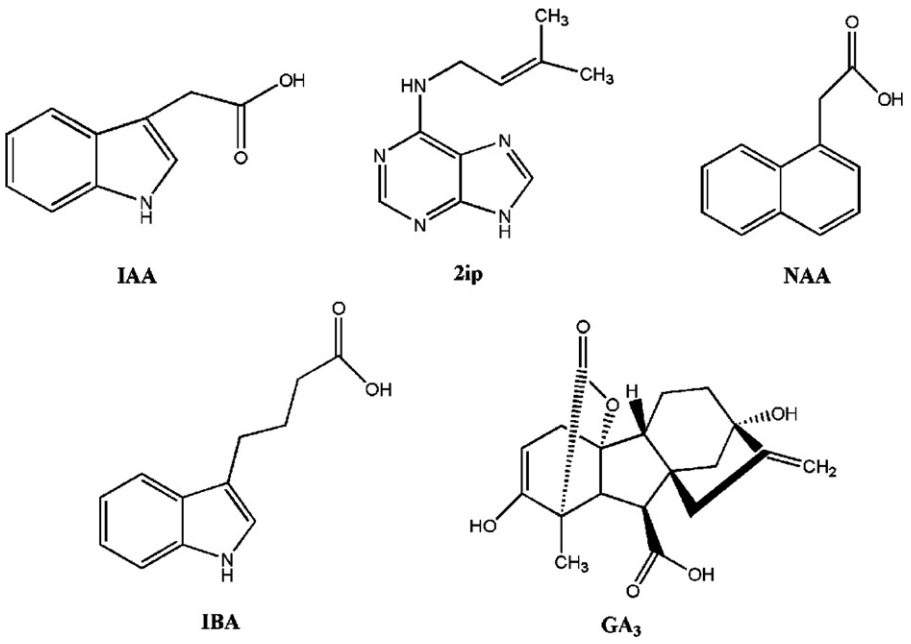

Fig. 1. Chemical structures of IAA, IBA, NAA, GA_3 and 2IP as representative acid/alkaline phytohormones.

and the combined solution of methanol/water (50/50, v/v) to yield a serial of analyte concentrations (5, 10, 25, 50, 100, 250, 500, 1000 ng mL^{-1}), respectively. The slope of the standard addition plot was compared with the slope of standard calibration plot to calculate the signal suppression/enhancement (SSE), which could be commonly used to estimate the matrix effects [26].

3. Results and discussion

3.1. Optimization of sample pretreatment

3.1.1. Selection of extraction method

In the present study, a variety of extraction solvents and their mixtures were tested: (1) methanol–water–formic acid (80/19/1, v/v/v), (2) acetonitrile–water–formic acid (80/19/1, v/v/v), (3) methanol–formic acid (99/1, v/v), (4) acetonitrile–formic acid (99/1, v/v). Twelve portions of the blank sample were spiked with the intermediate levels of each standard solution (50 μg kg^{-1}) while three additional portions were selected as the controls. The samples were macerated with 20 mL of the candidate solutions and pretreated as described in Section 2.5. The results are shown in Table 2. Satisfactory recoveries were obtained ranging from 88.9% to 114.3% when solvent 3 was selected.

After comparison of the previously described method [2] and the currently established one, it could be obviously seen that the recoveries of IAA, NAA, GA_3 and 2IP were almost the same for both methods (Table 2), while the recovery of IBA extracted by the former was lower than that by the latter (75.9% vs. 88.9%). Since the present extraction method only cost no more than 1 h and its extraction efficiencies were almost the same or even higher on some cases, it could be an appropriate approach for the subsequent phytohormones extraction.

3.1.2. Optimization of purification method

In order to remove the interferences and minimize the matrix effects, eight commercially available SPE cartridges were thoroughly compared with their purification efficiencies. Firstly, we evaluated the recovery performance of all candidates by passing mixed standard solutions through the cartridges. The mixed solution (10 μg mL^{-1}) was diluted with the water to yield a concentration of 50 ng mL^{-1}. After purification by the candidate SPE cartridges, the solutions were determined by HPLC–MS/MS. The results showed that the recoveries of most phytohormones were substantially improved on HLB cartridges which were thus selected for further optimization, despite that the recovery of GA_3 as exception was only 16.9% (Fig. 3). Therefore, HLB cartridges were selected, since HLB cartridges could reduce the color intensity of the extracts, result in better peak shapes, and reduce the noise level [3].

After careful investigation of each procedure, i.e., loading, washing and eluting, the results showed that HLB cartridges could not adsorb GA_3 in the methanol–water solution. When 1% of formic acid was added into the loading solution, the recovery of GA_3 was remarkably improved (>90.6%). It might be due to the case that adding some acid into loading solution could lead to the existence of molecular state of GA_3, as a consequence, it could be adsorbed in the reversed HLB cartridges more firmly. Then 20 mL of the spiked sample solutions (50 ng mL^{-1}) were purified with the HLB cartridges. In order to achieve the satisfactory recoveries (>80%), the sample solution was diluted with water to obtain the final loading solution with 10% of methanol. As a result, a standard purification procedure described as Section 2.5 was established.

Table 1
The MS/MS parameters for the five phytohormones.

Phytohormones	Precusor ion (*m*/*z*)	Primary product ion (*m*/*z*)	Collision energy (eV)	Secondary product ion (*m*/*z*)	Collision energy (eV)
IAA	173.9	130.0	14	–	–
IBA	201.8	158.0	17	116.0	18
NAA	185.0	141.0	14	–	–
GA_3	345.0	239.0	18	221.0	25
2IP	201.9	134.0	19	66.0	50

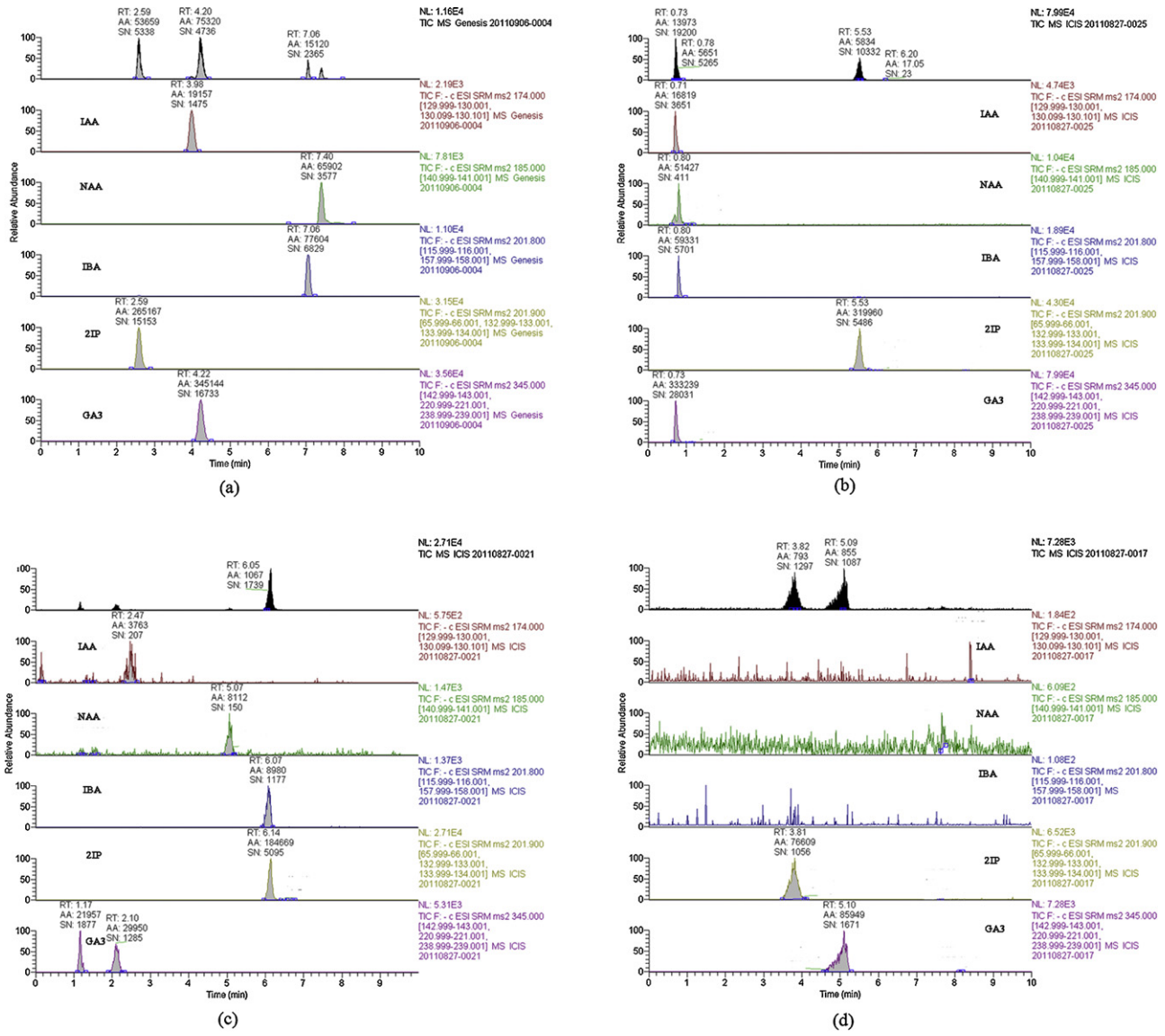

Fig. 2. Comparison of separation and ionization efficiencies of the five phytohormones among four candidate mobile phases.

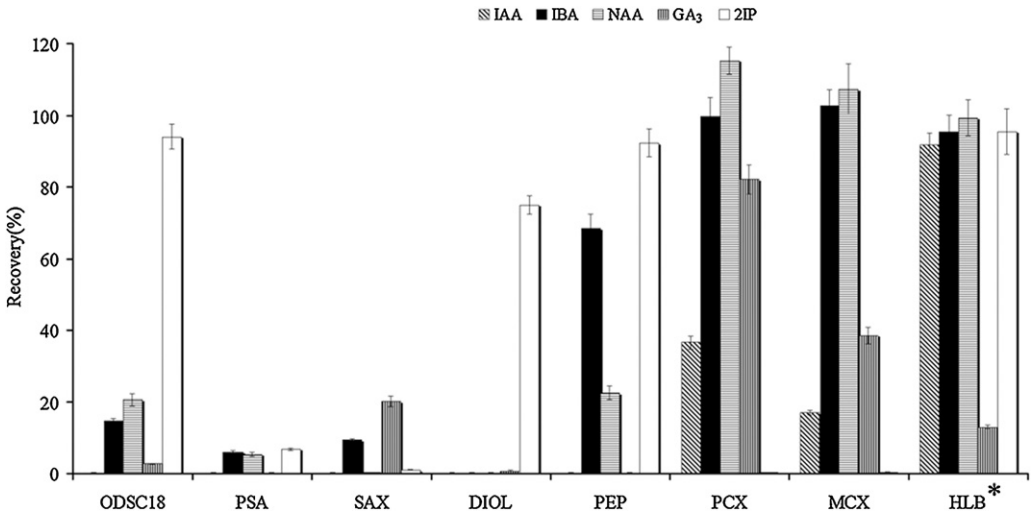

Fig. 3. Comparison of the purification recoveries of the five phytohormones on eight candidate SPE cartridges. *Represents that the HLB cartridges was selected for further optimization.

Table 2
Comparison of the absolute extraction recoveries of the four candidate extraction solvents ($n = 3$).

Solvents	IAA	IBA	NAA	GA_3	2IP
	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$
Methanol–water–formic acid (80/19/1, v/v/v)	103.9 ± 7.6	62.1 ± 5.6	69.7 ± 6.2	76.4 ± 5.8	30.8 ± 2.6
Acetonitrile –water–formic acid (80/19/1, v/v/v)	120.5 ± 8.9	46.1 ± 3.6	99.3 ± 7.2	76.3 ± 6.2	20.6 ± 2.1
Methanol–formic acid (99/1, v/v)	114.3 ± 9.0	88.9 ± 6.2	92.7 ± 8.2	112.8 ± 10.2	105.3 ± 8.3
Acetonitrile–formic acid (99/1, v/v)	99.0 ± 7.0	65.3 ± 5.1	96.1 ± 7.6	119.3 ± 3.5	59.9 ± 4.1

3.2. Optimization of HPLC–MS/MS conditions

3.2.1. Optimization of the mobile phase composition

The composition of the mobile phase was concerned for the ionization efficiency which is correlated to high sensitivity of analysis. In the present study, water, water containing 10 mmol L^{-1} ammonium acetate, water containing 0.2% formic acid and water containing 0.2% aqueous ammonia were compared. Results of various injections indicated that the responses of the five phytohormones were greatly improved and higher sensitivity was obtained when water or water containing 0.2% aqueous ammonia was used (Fig. 2). Alternatively, water was selected as it constituted a more stable, economic and ecological procedure. Under such situation, desirable peak shape and satisfactory separation efficiency were also achieved.

3.2.2. MS/MS parameters

The MS/MS conditions were optimized for each phytohormone by direct injection of each standard solution (500 ng mL^{-1}). Identification of precursor ions was performed in the full scan mode by recording from 100 to 500 (m/z) in both ESI^+ and ESI^- mode. The results showed that the responses of $[M-H]^-$ ions generated from IAA, IBA, NAA and GA_3 under the ESI^- mode were obviously higher than their $[M+H]^+$ ions generated under ESI^+ mode. 2IP could generate ions with high responses under either ESI^- or ESI^+ mode. In order to simplify the analysis, the $[M-H]^-$ ion generated from 2IP under ESI^- mode was selected for compromise of the other compounds. Based on the confirmation of precursor ions, two product ions for each precursor ion were intended to be selected according to the highest sensitivity and optimal selectivity for the target compounds. However, on the nature, only one product ion was selected for IAA and NAA due to their stable parent nucleus. Collision energies were chosen to give the maximum intensity of the fragment ions. The final MS/MS parameters for the five phytohormones are shown in Table 1. Finally, the selected reaction monitoring (SRM) mode was developed for quantification. The transition with the highest signal intensity was preferred for quantitation, while the other one with less intensity plus the ratio of abundances of two transitions was alternatively used for confirmation.

3.2.3. Selection of sample solvent medium before injection

The composition of sample solvent medium before injection directly affected the separation behavior of the analyte in HPLC system and their ionization efficiency during MS/MS determination. To select the suitable sample solvent medium, methanol, acetonitrile, methanol–water (50/50, v/v), acetonitrile–water (50/50, v/v), methanol–water containing 10 mmol L^{-1} ammonium acetate (50/50, v/v), acetonitrile–water containing 10 mmol L^{-1} ammonium acetate (50/50, v/v), methanol–water containing 0.2% of formic acid (50/50, v/v) and acetonitrile–water containing 0.2% formic acid (50/50, v/v) were compared in the pilot test. The analyte-free grape homogenate was spiked with each standard (50 μg kg^{-1}), and then pretreated as described in Section 2.5 until evaporated under a stream of nitrogen gas at 40 °C. Then, the residues were re-dissolved with the eight candidate solvents. Surprisingly, the peak shapes of the analytes were quite terrible if the acetonitrile was included in the solution. When methanol–water containing 10 mmol L^{-1} ammonium acetate was selected, the ionization was significantly mitigated under ESI^- mode so that the abundance and sensitivity were thus accordingly reduced. Although the ionization efficiencies were almost identical when methanol, methanol–water (50/50, v/v) or methanol–water containing 0.2% formic acid was used, methanol–water (50/50, v/v) was selected due to the better peak shapes for all five analytes.

3.3. Evaluation of the matrix effects

The extent of SSE was quite different for the five phytohormones. The signals were significantly suppressed by the sample matrix for IAA, NAA, GA_3 and 2IP with the SSE 66.2%, 58.6%, 56.3% and 38.5%, respectively, while the responses of IBA were obviously enhanced with the SSE of 129.2%. Therefore, it could be concluded that the matrix effects of the grapes were seriously existed for the five phytohormones, which would evidently interfere the accuracy of the established method. Therefore, the external matrix calibration was further used to eliminate the matrix effects.

3.4. Method validation

The analytical method was validated according to the following criteria: linearity, sensitivity, recovery, precision (within- and between-day variability) and stability.

3.4.1. Linearity

The standard solutions, with the concentration sequence of 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 ng mL^{-1}, were prepared in the blank matrix. The calibration curves were created by plotting peak areas of each phytohormone vs. respective concentration. Nice linear relationships and good coefficients of determination ($R^2 \geq 0.9990$) were obtained in both means (Table 3).

3.4.2. Sensitivity

The limit of detection (LOD) was determined by successive analyses of spiked matrices with decreasing amounts of each phytohormone standard until a signal-to-noise ratio 3:1 was reached and the limit of quantitation (LOQ) was adopted as the concentration of a phytohormone giving S/N = 10:1. As shown in Table 5, the LOQs were in the range of 0.05–1.00 ng mL^{-1} and LODs were of 0.02–0.30 ng mL^{-1} (Table 3), which were obviously lower than the values reported in the previous studies [1,3]. Since 2 g of each sample was pretreated as described in Section 2.5 and metered volume to 1 mL, the sensitivity in the sample solution was twice higher than that in the accordingly real sample. In another word, in the real grape sample, the LOQs were in the range of 0.10–2.00 μg kg^{-1} and LODs were of 0.04–0.60 μg kg^{-1}.

3.4.3. Recovery

Recovery was performed in the phytohormone-free grapes employing the method of standard addition. Eighteen portions of the selected sample were spiked with the high, intermediate and low levels of the mixed standards (400, 50, 5 μg kg^{-1} for IAA, NAA, GA_3, 2IP and 200, 25, 2.5 μg kg^{-1} for IBA) while three additional

Table 3
The calibration curves and sensitivities of the five phytohormones in sample matrix.

Phytohormones	Slope	Intercept	R^2	Range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
IAA	90.4	−425.2	0.9991	5–1000	0.3	1
IBA	184.4	577.7	0.9993	2.5–500	0.02	0.05
NAA	316.6	19,918.3	0.9991	5–1000	0.03	0.6
GA_3	324.1	−774.7	0.9999	5–1000	0.3	1
2IP	405.8	−1675.0	0.9991	5–1000	0.1	0.3

Table 4
Recoveries of the five phytohormones in grape matrix (n = 6).

Phytohormones	High level[a] (%)		Intermediate level[b] (%)		Low level[c] (%)	
	$\bar{X} \pm$ SD	RSD	$\bar{X} \pm$ SD	RSD	$\bar{X} \pm$ SD	RSD
IAA	90.5 ± 1.7	1.9	96.5 ± 4.3	4.5	89.2 ± 8.3	9.3
IBA	101.6 ± 2.1	2.0	82.5 ± 2.0	2.4	86.0 ± 6.8	7.9
NAA	91.0 ± 4.0	4.4	91.6 ± 4.6	5.0	90.7 ± 7.9	8.7
GA_3	97.1 ± 5.0	5.2	92.8 ± 3.0	3.2	95.3 ± 8.9	9.4
2IP	105.4 ± 4.2	4.0	90.3 ± 8.9	9.8	95.9 ± 9.2	9.6

[a] High level: IAA, NAA, GA_3, 2IP was designed as 400 μg kg^{-1}; IBA was designed as 200 μg kg^{-1}.
[b] Intermediate level: IAA, NAA, GA_3, 2IP was designed as 50 μg kg^{-1}; IBA was designed as 25 μg kg^{-1}.
[c] Low level: IAA, NAA, GA_3, 2IP was designed as 5 μg kg^{-1}. IBA was designed as 2.5 μg kg^{-1}.

portions were selected as the controls. Samples were pretreated as described in Section 2.5, and the concentrations were calculated using the external matrix calibration. The recoveries were in the range of 82.5–105.4% (Table 4).

3.4.4. Intra- and inter-day precision

Intra- and inter-day precision was determined by assaying the analyte-free samples spiked with high, intermediate and low levels of the individual phytohormone on five consecutive days with six replicates each day. The intra-day precision was in the range of 2.1–11.0%, and inter-day was in the range of 3.5–13.0% (Table 5).

3.4.5. Stability

To assess the stability of IAA, IBA, NAA, GA_3 and 2IP, the blank matrix was spiked with the involved phytohormones at 50 ng mL^{-1}, and the concentrations left at room temperature, −20 °C and 4 °C for 24 h were determined, respectively. The results showed that the mean percentages of calculated concentration vs. theoretical concentration were ≥82.0% for all phytohormones for 24 h even under room temperature, indicating that the analytes were stable through the whole sample preparation procedure (Fig. 4).

In total, all the experimental data indicated that the established method is rapid, robust, sensitive and could be used for simultaneous determination of the five phytohormones in grapes.

Table 5
Intra- and Inter-day precision of the established HPLC–MS/MS method (n = 6, μg kg^{-1}).

Phytohormones	Intra-day precision		Inter-day precision	
	$\bar{X} \pm$ SD	RSD	$\bar{X} \pm$ SD	RSD
IAA	729.4 ± 16.0	2.2	754.9 ± 44.1	5.8
	96.3 ± 4.6	4.8	93.4 ± 8.1	8.7
	9.0 ± 0.7	8.1	8.7 ± 0.8	9.6
IBA	416.3 ± 8.7	2.1	479.3 ± 26.8	5.6
	43.9 ± 2.6	6.0	43.5 ± 4.1	9.5
	4.1 ± 0.2	4.9	4.1 ± 0.2	4.9
NAA	729.2 ± 30.4	4.2	735.8 ± 31.4	4.3
	90.3 ± 3.9	4.4	88.4 ± 4.4	5.0
	9.8 ± 0.7	7.5	9.1 ± 0.9	10.0
GA_3	776.5 ± 40.1	5.2	785.7 ± 34.7	4.4
	85.7 ± 3.0	3.5	84.7 ± 3.7	4.4
	9.8 ± 0.7	6.8	9.1 ± 1.0	10.8
2IP	853.4 ± 32.1	3.8	849.4 ± 30.0	3.5
	91.4 ± 8.9	9.7	92.3 ± 12.0	13.0
	10.3 ± 1.1	11.0	10.2 ± 1.3	12.4

3.5. Method application in real samples

The evaluated method was finally applied to determine the natural occurrence of IAA, IBA, NAA, GA_3 and 2IP in grapes in China. The samples were prepared as described in Section 2.5. The concentrations of the analytes were calculated using the external matrix calibration.

As shown in Table 6, among the collected thirty samples, twenty two contained phytohormones (73.3% of incidence), ranging from 1.0 to 25.8 μg kg^{-1}, which was consistent to the previous values reported by Dasharath et al. (GA_3: 0.05 (±4%) mg kg^{-1}) in grapes [3], but higher than that in Chinese cabbage (no positive sample found) [18]. The three samples with the highest concentration levels of phytohormones (25.4 μg kg^{-1}, 25.8 μg kg^{-1} and 25.4 μg kg^{-1}) were all green grapes collected from Xinjiang province, China. Fortunately, the concentrations of all samples were less than the European Union MRLs [5].

Moreover, the phytohormones demonstrated great variability in type and relative proportions in the twenty two positive samples (Table 6). A total of sixteen samples contained 2IP, the most prevalent phytohormones, in the range of 1.0–10.0 μg kg^{-1}. The mean levels (occurrence) of IAA, GA_3 and NAA in the positive

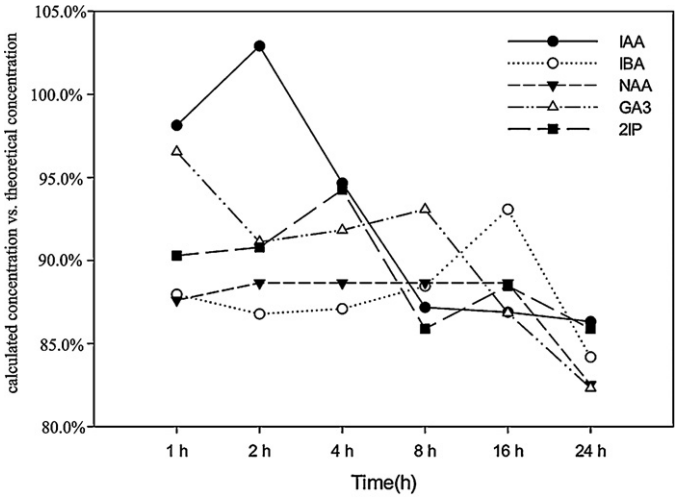

Fig. 4. The stability of IAA, IBA, NAA, GA_3 and 2IP in the blank grape matrix at room temperature for 24 h.

Table 6
The contents of the five phytohormones in the grapes samples collected from local markets ($\mu g\,kg^{-1}$).

Sample code	Origin	IAA	IBA	NAA	GA_3	2IP	SUM
1	Xinjiang province	–	–	1.3	24.1	–	25.4
2	Xinjiang province	–	–	–	21.7	4.1	25.8
3	Xinjiang province	–	–	1.3	7.4	5.4	14.1
4	Xinjiang province	2.8	–	–	–	4.0	6.8
5	Xinjiang province	–	–	–	–	1.5	1.5
6	Shandong province	–	–	–	–	–	–
7	Shandong province	–	–	–	–	–	–
8	Shanxi province	7.2	–	–	–	6.3	13.5
9	Hebei province	10.8	–	–	–	–	10.8
10	Zhejiang province	–	–	–	–	–	–
11	Zhejiang province	–	–	–	–	–	–
12	Zhejiang province	–	–	8.4	2.8	–	11.2
13	Zhejiang province	–	–	–	–	–	–
14	Liaoning province	–	–	–	–	–	–
15	Shanghai	–	–	–	–	3.7	3.7
16	Shanghai	–	–	1.4	–	–	1.4
17	Shanghai	3.3	–	–	–	1.6	4.9
18	Shanghai	–	–	1.9	–	10.0	11.9
19	Shanghai	–	–	–	–	1.8	1.8
20	Shanghai	–	–	1.9	–	1.1	2.9
21	Shanghai	–	1.6	–	–	1.9	3.4
22	Shanghai	–	–	5.4	–	–	5.4
23	Shanghai	–	–	–	–	–	–
24	Shanghai	–	–	–	–	–	–
25	Shanghai	–	–	–	–	1.0	1.0
26	Shanghai	–	–	–	3.2	–	3.2
27	Shanghai	–	–	–	1.9	2.5	4.4
28	Shanghai	–	–	2.0	–	3.6	5.7
29	Shanghai	–	–	–	–	2.0	2.0
30	Shanghai	3.3	–	–	–	5.2	8.5

–: not detected.

samples were 5.5 $\mu g\,kg^{-1}$ (22.7%), 10.2 $\mu g\,kg^{-1}$ (27.3%) and 3.0 $\mu g\,kg^{-1}$ (36.4%), respectively. Trace amount of IBA (1.6 $\mu g\,kg^{-1}$) was detected only in one single sample. Hence, the results demonstrate that the developed HPLC–MS/MS is a sensitive, stable and robust method for rapid analyses of multiclass phytohormones, which will be applicable in rountie monitoring, fundamental or applied research of phytohormones.

4. Conclusions

The phytohormones have aroused increasing attention in the system of food safety management. In this study, we have developed a reliable HPLC–MS/MS method for simultaneous quantification of IAA, IBA, NAA, GA_3 and 2IP in native forms without derivatization in grapes and also simplified the procedures for extraction and purification. The phytohormones analyzed represent structurally diverse compounds with different chemical properties (acid/alkaline). After careful validation by determining the sensitivity, linearity, precision, stability and matrix effects, the established analytical method was successfully applied to determine the five phytohormones in grape samples collected from different origins in China. The achieved satisfactory results are sufficient to prove that this method was suitable for rapid multiresidue analyses of multiclass phytohormones in agriculture field.

Acknowledgements

The authors acknowledge the financial support by Shanghai Agriculture Commission Project (2009NO 6-1; 2011NO.4-3) and Shanghai Municipal Center for Disease Control and Prevention.

References

[1] S. Hou, J. Zhu, M. Ding, G. Lv, Talanta 76 (2008) 798.
[2] S.D. Chiwocha, S.R. Abrams, S.J. Ambrose, A.J. Cutler, M. Loewen, A.R. Ross, A.R. Kermode, Plant J. 35 (2003) 405.
[3] D.P. Oulkar, K. Banerjee, M.S. Ghaste, S.D. Ramteke, D.G. Naik, S.B. Patil, M.R. Jadhav, P.G. Adsule, J. AOAC Int. 94 (2011) 968.
[4] X. Pan, R. Welti, X. Wang, Phytochemistry 69 (2008) 1773.
[5] EC Regulation No. 149/2008 (29 January 2008) Off. J. Eur. Union 1.3, L58, 1–398.
[6] W. Chen, Y. Gai, S. Liu, R. Wang, X. Jiang, J. Integr. Plant Biol. 52 (2010) 925.
[7] L. Ge, C.Y. Peh, J.W. Yong, S.N. Tan, L. Hua, E.S. Ong, J. Chromatogr. A 1159 (2007) 242.
[8] P. Tansupo, P. Suwannasom, D.L. Luthria, S. Chanthai, C. Ruangviriyachai, Phytochem. Anal. 21 (2010) 157.
[9] T. Henriksen, R.K. Juhler, G. Brandt, J. Kjaer, J. Chromatogr. A 1216 (2009) 2504.
[10] A. Rodriguez, R.S. Sanchez Tames, Anal. Biochem. 146 (1985) 184.
[11] M.L. Chen, Y.Q. Huang, J.Q. Liu, B.F. Yuan, Y.Q. Feng, J. Chromatogr. B 879 (2011) 938.
[12] S. Wang, L. Jia, D. Xing, D. Chen, J. Zhao, J. Sep. Sci. 31 (2008) 859.
[13] L. Ge, J.W. Yong, S.N. Tan, E.S. Ong, Electrophoresis 27 (2006) 2171.
[14] X.G. Chu, W. Yong, H.X. Cai, J.W. Pan, Se Pu 19 (2001) 286.
[15] A. Royer, F. Laporte, S. Bouchonnet, P.Y. Communal, J. Chromatogr. A 1108 (2006) 129.
[16] Y. Wu, B. Hu, J. Chromatogr. A 1216 (2009) 7657.
[17] G.L. Gambino, P. Pagano, M. Scordino, L. Sabatino, E. Scollo, P. Traulo, G. Gagliano, J. AOAC Int. 91 (2008) 1245.
[18] Q. Lu, L. Zhang, T. Chen, M. Lu, T. Ping, G. Chen, Rapid Commun. Mass Spectrom. 22 (2008) 2565.
[19] L. Ge, J.W. Yong, N.K. Goh, L.S. Chia, S.N. Tan, E.S. Ong, J. Chromatogr. B 829 (2005) 26.
[20] V. Tureckova, O. Novak, M. Strnad, Talanta 80 (2009) 390.
[21] Z. Ma, L. Ge, A.S. Lee, J.W. Yong, S.N. Tan, E.S. Ong, Anal. Chim. Acta 610 (2008) 274.
[22] S. Fan, X. Wang, P. Li, Q. Zhang, W. Zhang, J. Sep. Sci. 34 (2011) 640.
[23] Y. Izumi, A. Okazawa, T. Bamba, A. Kobayashi, E. Fukusaki, Anal. Chim. Acta 648 (2009) 215.
[24] X. Pan, X. Wang, J. Chromatogr. B 877 (2009) 2806.
[25] C. Susawaengsup, M. Rayanakorn, S. Wongpornchai, S. Wangkarn, Talanta 85 (2011) 897.
[26] Z. Han, Y. Ren, H. Zhou, Z. Cai, L. Luan, Y. Wu, J. Chromatogr. B 879 (2011) 411.