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Simultaneous determination of five plant hormones in cotton leaves using QuEChERS combined with HPLC–MS/MS

WANG Wenhao[1,2], SONG Xianpeng[1], WANG Dan[1], MA Yajie[1], SHAN Yongpan[1], REN Xiangliang[1], HU Hongyan[1], WU Changcai[1,3,4*], YANG Jun[2*] and MA Yan[1,3,4*]

Abstract

Background Plant hormones profoundly influence cotton growth, development, and responses to various stresses. Therefore, there is a pressing need for an efficient assay to quantify these hormones in cotton. In this groundbreaking study, we have established QuEChERS-HPLC–MS/MS method, for the simultaneous detection of multiple plant hormones in cotton leaves, allowing the analysis and quantification of five key plant hormones.

Results Sample extraction and purification employed 0.1% acetic acid in methanol and C18 for optimal recovery of plant hormones. The method applied to cotton demonstrated excellent linearity across a concentration range of 0.05–1 mg·L^{-1}, with linear regression coefficients exceeding 0.99. The limits of quantification (LOQs) were 20 μg·kg^{-1} for GA_3 and 5 μg·kg^{-1} for the other four plant hormones. Recovery rates for the five plant hormones matrix spiked at levels of 5, 10, 100, and 1000 μg·kg^{-1} were in the range of 79.07% to 98.97%, with intraday relative standard deviations (RSDs) ranging from 2.11% to 8.47%. The method was successfully employed to analyze and quantify the five analytes in cotton leaves treated with plant growth regulators.

Conclusion The study demonstrates that the method is well-suited for the determination of five plant hormones in cotton. It exhibits excellent selectivity and sensitivity in detecting field samples, thus serving as a robust tool for in-depth research into cotton physiology.

Keywords Cotton, Plant hormones, QuEChERS, HPLC–MS/MS

*Correspondence:
Wu Changcai
ccwu00@163.com
Yang Jun
yang22181@163.com
Ma Yan
aymayan@126.com
[1] National Key Laboratory of Cotton Biobreeding and Integrated Utilization, Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, Henan 455000, China
[2] College of Agriculture, Hebei Agricultural University, Baoding, Hebei 071000, China
[3] Zhengzhou Research Base, National Key Laboratory of Cotton Biobreeding and Integrated Utilization, Zhengzhou University, Zhengzhou, Henan 450001, China
[4] Western Agricultural Research Center, Chinese Academy of Agricultural Sciences, Changji, Xinjiang 831100, China

Background

Plant hormones are vital signaling molecules produced by plant cells in response to specific environmental cues, orchestrating diverse physiological processes throughout plants' life cycle. These functions encompass activities such as cell division, organ development, seed dormancy, germination, organ senescence, and abscission, which are pivotal in plant growth, development, metabolism, and responses to biotic and abiotic stressors (Liu et al., 2019; Jiang et al., 2020). The recognized plant hormones include growth hormone, cytokinin (CK), abscisic acid (ABA), gibberellin (GA), Brassinosteroid (BR), salicylic acid (SA), jasmonic acid

(JA), ethylene (ET), and more recently discovered strigolactones (SL) (Bowman et al., 2019). Plant hormones do not act alone, exhibiting intricate interactions among each other, including synergistic and antagonistic relationships (Wang et al., 2020c). This results in a comprehensive and coordinated regulation of plant growth and development, which is hormone concentration dependent (Li et al., 2019; Wu et al., 2009).

Cotton, as the world's predominant fiber crop, representing nearly 40% of global fiber production, has long been a focal point of plant research (Wu et al., 2020). When cotton is stressed by unfavourable external conditions, phytohormones sense and promptly regulate its response to the external influences (Wang et al., 2022). Previous studies by Guinn et al., (1993) suggested that elevated ABA levels, reduced indole acetic acid (IAA), and decreased stomatal conductance mediated by zeatin-riboside (ZR) in cotton can attenuate plant metabolism, thereby minimizing stress-induced losses. Likewise, Zhang et al., (2017) revealed that numerous gene were deferentially expressed with upregulated hormones such as JA, SA, and BR in response to waterlogging in cotton. Furthermore, Nguyen et al.'s hypothesis posited that JA, SA, and BR acted in a signaling cascade network, aiding plants in adapting to abiotic stresses (Nguyen et al., 2016). Thus, fluctuations in plant hormone concentrations and their intricate interactions are pivotal in cotton's adaptation to the challenging conditions (Zhang et al., 2021).

Detecting plant hormones is a formidable task given their trace amounts. Analyzing these substances necessitates complex pretreatment and precise instrumentation (Wang et al., 2020; Bari et al., 2009). Currently, commonly employed techniques for hormone detection encompass electrochemical analysis, immunoassays, chromatography, and chromatography/mass spectrometry (Cao et al., 2023). Immunoassays are straightforward and suitable for assessing plant hormones across various species. However, cross-contamination issues among different hormones may result in false positives (Tan et al., 2016). In recent years, liquid chromatography–mass spectrometry (LC–MS) has emerged as a favored method for quantitative phytohormone analysis (Lin et al., 2020). Its high sensitivity, specificity, and ability to concurrently analyze multiple hormones offer a powerful tool for both quantitative and qualitative assessment at tissue and cellular levels (Antoniadi et al., 2015; Verslues, 2017). When compared with gas chromatography, LC–MS can bypass complicated derivatization processes, making it the premier choice for phytohormone detection (Jiang et al., 2020). To date, HPLC–MS/MS has been applied for the quantification of various phytohormones such as auxin, ABA, CKs, and SLs simultaneously (Šimura et al., 2018; Jiang et al., 2020; Cao et al., 2016; Xin et al., 2020; Wu et al., 2009).

Regardless of the detection method employed, pretreatment steps are indispensable for purifying plant substrates, eliminating impurities, and enriching target compounds (Wu et al., 2009). Various pretreatment techniques have been adopted, including dispersed liquid–liquid microextraction (DLLME) (Behbahani et al., 2014), solid phase extraction (SPE) (Wang et al., 2007), solid phase microextraction (SPME), liquid extraction (LPE) (Uslu et al., 2016), ultrasonic extraction (UE) (Roknul Azam et al., 2020), microwave-assisted extraction (MAE) (Fang et al., 2012), accelerated solvent extraction (ASE) (Wang et al., 2020a), liquid phase microextraction (LPME) (Jalili et al., 2020), and supercritical fluid extraction (SFE) (Ngowi et al., 2007). Among these, SPE is the most commonly utilized method for extracting plant hormones (Hou et al., 2008; Dobrev et al., 2005; Ivanov Dobrev et al., 2002). Nevertheless, the intricate nature of the pretreatment process translates to longer preparation times, rendering it impractical for large-scale phytohormone assays (Musarurwa et al., 2019; Lee et al., 2018; Rahman et al., 2018b; Nuapia et al., 2016). In this context, the QuEChERS pretreatment method has emerged as a superior alternative. This approach simplifies the process, reducing sample preparation steps to just two, and has gained prominence due to its capability to extract polar analytes, offering improved selectivity, detectability, and direct compatibility with liquid chromatography coupled with mass spectrometry, reduced extraction solvent and sample preparation time requirements, and superior recovery rates (Zhang et al., 2014; Rong et al., 2018; Lehotay et al., 2010).

The quantification of plant hormones in cotton is a challenging endeavor owing to their inherently low concentrations. There exists a pressing need for comprehensive research to develop methods that enable the simultaneous detection of multiple plant hormones in cotton. The primary objective of this study was to devise an analytical approach characterized by its simplicity, convenience, affordability, sensitivity, and remarkable selectivity. This method was subsequently applied to authentic cotton samples, facilitating the precise, in-depth, and quantitative assessment of plant hormones. These findings are expected to significantly contribute to advancing physiological studies in cotton and related areas.

Materials and methods

Chemicals and reagents

Analytical standards for zeatin (HPLC ≥ 96%), zeatin riboside (HPLC ≥ 96%), indole acetic acid (HPLC ≥ 98%), gibberellin A_3 (HPLC ≥ 90%), and abscisic acid (HPLC ≥ 98%) were purchased from Shanghai Yuanye Bio-Technology Co Ltd. (Shanghai, China), 2,6-di-tert-butyl-4-methylphenol (BHT) was purchased from Shanghai Aladdin Bio-Chem Technology Co. (Shanghai, China), ultrapure water was prepared using a Milli-Q water purification system (Millipore, USA), HPLC-grade acetonitrile, methanol, formic acid, and acetic acid were purchased from Merck (Merck KGaA, Darmstadt, Germany). Analytical grades of magnesium sulfate anhydrous ($MgSO_4$) were obtained from Beijing Chemical Company (Beijing, China). Primary secondary amine (PSA, 40 μm), Cleanert C18 (C18, 40 μm), and graphitized carbon black (GCB, 40 μm) were purchased from Bonna-Agela Technologies (Tianjin, China). Syringe filters (0.22 μm, nylon) were purchased from the Youpu Reagent Company (Tianjin, China).

HPLC–MS/MS analysis

Chromatographic separation of zeatin (ZT), zeatin riboside (ZR), indole-3-acetic acid (IAA), gibberellin A_3 (GA_3), and abscisic acid (ABA) was performed on an ExionLC™ AC (AB Sciex) and SCIEX Triple Quad™ 4500 (AB Sciex) equipped with a Shim-pack GIS C18 column (150 mm × 3.0 mm, 3 μm particle size, Kyoto, Japan). The mobile phase consisting of 0.1% acetic acid in methanol (Phase A) and 0.1% formic acid in ultrapure water (Phase B) was pumped at a flow rate of 0.3 mL min^{-1}. The gradient elution program was as follows: 0.0 – 1.0 min, 95% B; 1.0 – 3.0 min, 95% – 5% B; 3.0 – 4.5 min, 5% B; 4.5 – 4.6 min, 5% – 95% B; 4.6 – 6.0 min, 95% B, equilibration of the column. The column oven temperature was maintained at 40 ℃, and the temperature in the autosampler was set at 15 ℃. The sample volume injected was 4 μL.

Mass spectrometry analysis was conducted using an AB SCIEX Triple Quad™ 4500 equipped with an electrospray ionization source (ESI). Multiple reaction monitoring (MRM) was in positive mode (ESI +) for ZR and ZT, and IAA was in negative mode (ESI-) for GA_3 and ABA. The typical MS/MS instrument basic parameter settings are shown in Table S1. Analyst 1.6.3 software (AB Sciex Corp., USA) and SCIEX OS-Q (AB Sciex Corp., USA) were utilized for instrument control, data acquisition, and analysis.

Plant material and overview of the experimental site

Cotton for method optimization and validation (*Gossypium hirsutum* L.) was planted at Dongchang experimental site of Institute of Cotton Research, Chinese Academy of Agricultural Sciences (Anyang, Henan, China). Cotton was planted in equal spacing, 80 cm between rows and 25 cm between plants, with a planting density of 50 000 plants·hm^{-2}. The experiment was carried out from the early to full flowering stage of cotton. The experiment was carried out from the early flowering stage to the full flowering stage of cotton, and no other plant growth regulators other than the formula were sprayed in the experimental plots. Other management measures were the same as those in the field.

The experiment was started on July 5, 2023, and fresh cotton leaves were picked at 0, 14, and 28 days post plant growth regulator treatment and frozen in liquid nitrogen immediately after picking. All samples were harvested within 1 day.

Sample extraction and purification

The QuEChERS pretreatment consists of two main steps: liquid–liquid extraction and dispersive solid-phase extraction cleanup (Musarurwa et al., 2019). Fresh cotton leaves were collected at Dongchang Experimental Station of Institute of Cotton Research Institute, Chinese Academy of Agricultural Sciences (CAAS). They were cold-excited with liquid nitrogen and stored at −80 °C immediately after picking. A cotton leaf sample of 2.5 g (± 0.05 g) was weighed in a 50 mL polytetrafluoroethylene centrifuge tube, ground and pulverised using a grinder. Then, 7.5 mL of methanol solution containing 0.1% acetic acid (containing 1 mmol·L^{-1} BHT) and 0.25 mL of ultrapure water were added. The extract was homogenized using a high-speed homogenizer for 1 min and then centrifuged at RCF (relative centrifugal force) 5 000 × *g* for 5 min. Next, 1.5 mL of supernatant was transferred from the centrifuge tube to a 2 mL tube (containing 150 mg $MgSO_4$ and 30 mg C18) using the vortex mixer vortex for 1 min. Ultimately, after centrifuging for 5 min at RCF 4 000 × *g*, the upper acetonitrile layer was filtered through 0.22 μm nylon syringe filters into autosampler vials for HPLC–MS/MS analysis.

Method validation

The method was evaluated for selectivity, linearity, matrix effect, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, and stability. Untreated cotton leaves were analyzed to verify the absence of interfering peaks around the retention times of the five target compounds to assess their selectivity. The linearity of the method was assessed by analyzing solvent standard solutions and matrix standard solutions (5 ~ 1 000 μg·L^{-1}). The slopes of the matrix standard solutions were calculated as follows:

$$\text{Slope} = \frac{A - B}{X}$$

A is the peak area of the spiked matrix solution, B is the peak area of the blank matrix standard, and X is the spiked concentration.

The following equation determines the matrix effect:

$$\text{Matrix effect}(\%) = \frac{Slope\ A - Slope\ B}{Slope\ B} \times 100\%$$

Slope A is the slope of the matrix standard curve, and *Slope B* is the slope of the solvent standard curve.

The LOQ was the lowest spike level of the validation satisfying the criteria. The LODs of the five compounds were considered to be the concentration that produced a signal-to-noise (S/N) ratio of 3. It was estimated from the chromatogram corresponding to the lowest point used in the matrix-matched calibration (S/N = 3). The accuracy and precision of the method were assessed by validating the recoveries. Five replicates of each spiked cotton leaf sample at four levels (0.005, 0.01, 0.1, and 1 mg·kg $^{-1}$) were prepared on three different days. The precision in these conditions for repeatability, expressed as the RSD, was determined by the intra- and inter-day assays.

The stability of these four compounds was determined in solvent and matrix. The stability of the stock solutions was tested monthly by injection of a newly prepared working solution. Matrix-matched standards of 0.01 mg·mL^{-1} were analysed monthly, and all samples were stored at 20 ℃.

Application to field cotton samples

In order to investigate the impact of various plant growth regulators on the modulation of plant hormones in cotton, an array of treatment combinations was designed. These treatments were labeled as follows, DD: mepiquat + diethyl aminoethyl hexanoate; DB: mepiquat + 24-epibrassinolide; DP: mepiquat + prohexadione calcium; DPC: mepiquat; DA: diethyl aminoethyl hexanoate; BR: 24-epibrassinolide; PC: prohexadione calcium; CK: water treatment. All plant growth regulators were applied at the recommended field application doses, with mepiquat at 45 g·hm^{-2}, diethyl aminoethyl hexanoate at 120 mL·hm^{-2}, 24-epibrassinolide at 120 g·hm^{-2}, and prohexadione calcium at 450 mL·hm^{-2}.

Results

Optimization of HPLC–MS/MS conditions

The negative ions GA_3 and ABA exhibited low response strength in this experiment, making them challenging to detect. As a result, we chose methanol as the organic phase. Our findings indicated that the five target compounds could not be adequately separated using a mobile phase combination of methanol and water. However, we discovered that the addition of formic acid and acetic acid to the water at concentrations of 1% and 0.1%, respectively, allowed for successful preparation (Fig. 1). Despite this, the response values did meet the test requirements. To address this, we experimented with adding formic acid and acetic acid to methanol at concentrations of 1% and 0.1%. A comparison showed that using 0.1% acetic acid in methanol as the organic phase improved the recovery of the five target compounds. In comparison to methanol and pure water, the final choice of a mobile phase combination consisting of 0.1% acetic acid in methanol and 0.1% formic acid in water provided superior peak shapes, greater sensitivity, and a more stable baseline. This enhanced the ability to quantify peak areas in the experiment (Fig. 2). Consequently, we established a comprehensive HPLC–MS/MS analytical method for determination.

The basic mass spectrometer instrument parameters include: Curtain gas (CUG), ion spray voltage (IS), ion source temperature (TEM), collision energy (CE), spray gas (ions source gas, GS1), auxiliary heater (ions source gas, GS2), collision gas (CAD), cell exit potential (CXP), and specific settings of each parameter are shown in Table S1.

In this research, we focused on optimizing instrumental acquisition parameters and MRM ion-pairing channel selection to monitor plant hormones. The detection of the five target compounds involved the use of mixed standard solutions (0.1 mg·L^{-1}) in ESI + ionization mode. We initially conducted a primary mass spectrometry (MS) scan to obtain precise parent ions. Subsequently, a secondary MS scan was performed for the five target compounds to identify the daughter ions of each target component. For qualitative or quantitative purposes, we selected the two ions with the highest response and greatest stability. Optimization of ionization parameters for each compound included adjustments to the declustering potential (DP), collision energy (CE), and ESI source temperature. The optimized mass spectrometry parameters for the compounds are detailed in Table 1.

Extraction optimization

In this experiment, we examined the recoveries of six different methanol-based extraction solutions for ZR, ZT, IAA, GA_3, and ABA. The specific recoveries are presented in Fig. 3, which illustrates that the inclusion of 2,6-di-tert-butyl-4-methylphenol, also known as butylated hydroxytoluene (BHT), led to enhanced recoveries of the three plant hormones by 0.75% to 9.02% when compared with extractions using pure methanol alone. Furthermore, the addition of an acidic solution contributed to the recovery efficiency. In order to determine the

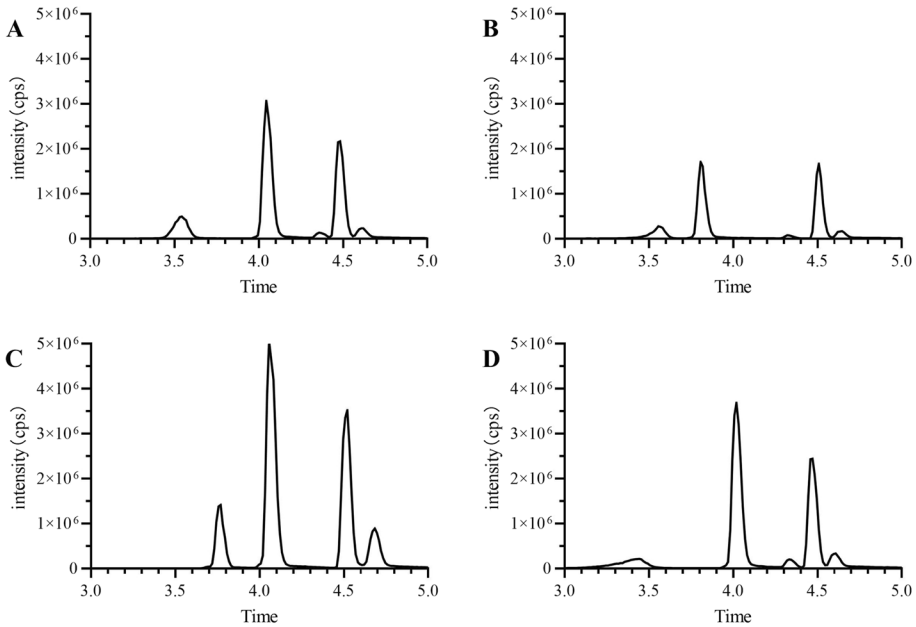

Fig. 1 Effect of different combinations of aqueous phase as mobile phase on the recovery: **A** 0.1% formic acid in water. **B** 1% formic acid in water. **C** 0.1% acetic acid in water. (D) 1% acetic acid in water

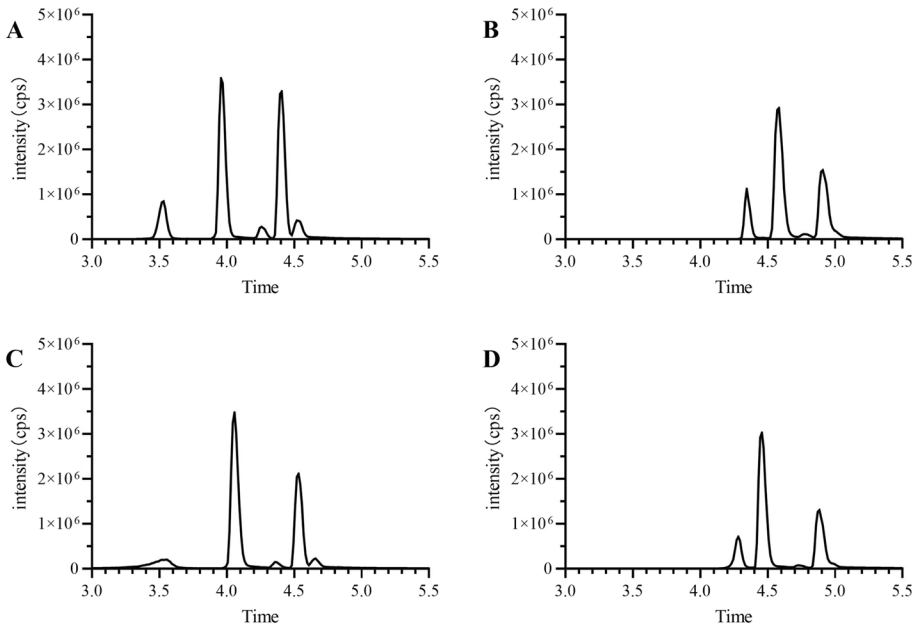

Fig. 2 Effect of methanol of different acid solutions on the recovery: **A** 0.1% acetic acid methanol. **B** 1% acetic acid methanol. **C** 0.1% formic acid methanol. **D** 1% formic acid methanol

most effective extraction protocols for the five plant hormones in cotton, we compared the effects of four different extraction protocols: 0.1% acetic acid in methanol solution + BHT, 1% acetic acid in methanol solution + BHT, 0.1% formic acid in methanol solution + BHT, and 1% formic acid in methanol solution + BHT. The results indicated that the extraction efficiency of 0.1% acetic acid in methanol solution + BHT surpassed that of other three options, resulting in a 2.45% increase in recovery efficiency when compared with the use of methanol + BHT.

Table 1 MS/MS parameters for multiple reaction monitoring (MRM)

Compound	Ion source	Precursor (m/z)	Product (m/z)	DP /V	CE /V
ZR	ESI +	352.2	220.0	100	27
			136.1		42
ZT	ESI +	220.3	136.1	77	23
			202.0		18
IAA	ESI +	176.2	130.0	24	20
			102.9		42
GA_3	ESI-	345.1	143.0	80	46
			221.1		35
ABA	ESI-	263.3	152.8	53	15
			219.0		19

This comprehensive assessment established 0.1% acetic acid in methanol solution + BHT as the optimal extraction solution for this experiment. The chosen solution demonstrated a significant improvement, with recoveries in cotton improved by 2.45% to 25.46% in contrast to methanol alone.

Clean-up optimization

In this investigation, various adsorbents were paired with different amounts, including 30, 50, 80, and 100 mg, alongside 150 mg of water adsorbent $MgSO_4$, and the findings are presented in Fig. 4. Notably, all combinations involving $MgSO_4$ with graphitized carbon black (GCB) and primary secondary amine (PSA) failed to meet the recovery requirements set for this study. The sole exception was the combination of 150 mg $MgSO_4$ and 30 mg of C18, which yielded recoveries exceeding 80% for all five target compounds. As a result, 150 mg $MgSO_4$ and 30 mg C18 was chosen as the preferred purification agent for this test compound.

Method validation

Specificity

To evaluate the specificity of the method, seven blank matrix samples were spiked at a concentration of 100 μg·L^{-1}. The results, as depicted in Fig. 5, revealed the absence of any interfering peaks near the retention time of the target analyte in all samples. Additionally, to assess the method's stability and reliability, matrix-matched standard working solution with a concentration of 100 μg·L^{-1} was subjected to monthly analysis.

Linearity and matrix effects

In this research, we conducted seven-point calibration curves for five different plant hormones across the concentration range of 5 to 1 000 μg·L^{-1}. The coefficient of determination (R^2) values for all five analytes exceeded 0.99, indicating a remarkably strong linear relationship (Table 2).

As a general rule, matrix effects (ME) within the range of −20% to 20% are typically considered negligible.

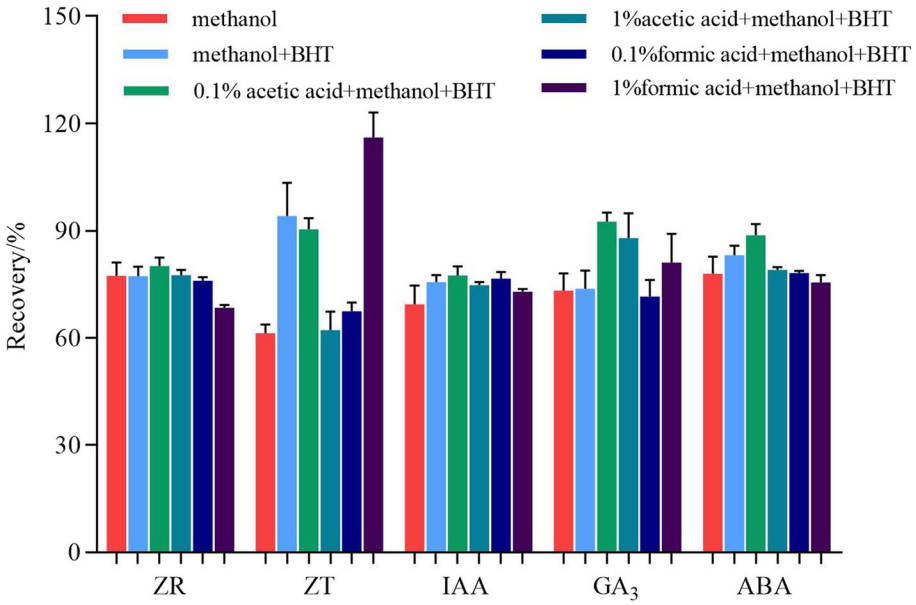

Fig. 3 Recovery of five analytes spiked at 100 μg·kg^{-1} in different extraction solutions (n = 3). The effect of different extraction solutions on the recovery of methanol-based extracts with the addition of antioxidants or the adjustment of acidity was examined

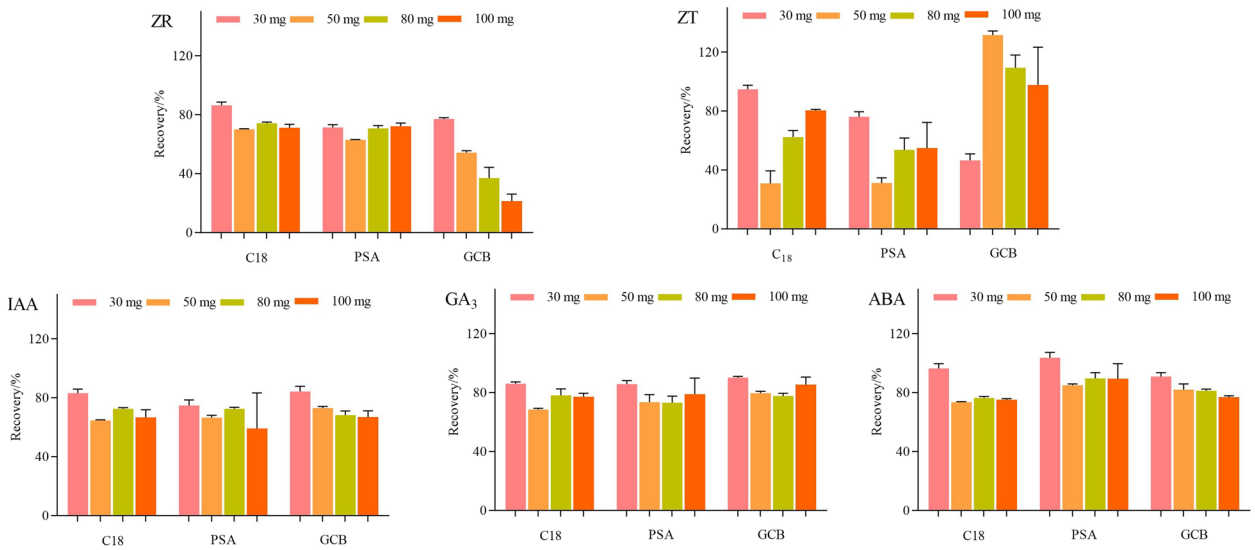

Fig. 4 The recovery of five analytes spiked at 100 μg·kg^{-1} with different adsorbents. 150 mg of $MgSO_4$ was mixed with 30, 50, 80, and 100 mg of C18, PSA and GCB, respectively, as adsorbents to complete the pre-treatment process, and the recovery was compared with determine the optimal adsorbent agent

However, when ME falls between −20% and −50% or between 20% and 50%, it signifies a moderate level of matrix interference.

The detailed results are presented in Table 2. It is noteworthy that, except ABA, all four plant hormones exhibited matrix inhibitory effects. The effects varied in intensity, with milder matrix inhibitory effects observed for ZT and GA_3, more pronounced matrix inhibitory effects for ZR and IAA. On the contrary, ABA shows a strong matrix-enhancing effect.

LOQ and LOD

We determined the limit of detection (LOD) and limit of quantification (LOQ) by examining the lowest spiked cotton leaf samples and calculating signal-to-noise ratios equal to 3 and 10, respectively. Consequently, the LOQs for this method were found to be 5 μg·kg^{-1} for ZR, ZT, IAA, and ABA, while GA_3 had an LOQ of 20 μg·kg^{-1} (Table 2). The LODs for ZR, ZT, IAA, GA_3, and ABA in cotton leaves fell within the range of 0.09 to 0.27 μg·kg^{-1}.

Accuracy and precision

The method's effectiveness was assessed through a three-day recovery test, involving the addition of four fortified concentration levels of compounds (5, 10, 100, and 1 000 μg·L^{-1}) to blank samples. To validate the method's precision, both intraday relative standard deviation (RSD_r) and inter-day RSD (RSD_R) were thoroughly examined. The results, as presented in Table 3, revealed that the recoveries of the five plant hormones fell within the range of 79.07% to 98.97%. The range of RSD_r ($n = 5$) and RSD_R ($n = 15$) was determined to be 2.11% to 8.47% and 1.07% to 14.64%, respectively. These values indicate a remarkable level of precision, with consistent and reliable results achieved both within the same day and over multiple days. The study demonstrated that the method performed exceptionally well in terms of recoveries, precision, and sensitivity for the determination of the five plant hormones in cotton leaves.

Application to real samples

The results at 28 days after application are shown in Fig. 6, the GA_3 content in the plant growth-regulator treated groups exhibited a notable increase, ranging from 11.37% to 42.03% compared with CK. All treatments used in combination had higher IAA content than all individually applied treatments except DPC, and DB having the highest IAA content. On day 14 after treatments, the ABA content of the DP treatment was significantly lower than that of other treatments, and this continued until day 28, when the ABA content of the CK group was higher than that of all treatment groups, but only significantly different from that of the DP group. The variations in ZT content among the treatment groups were relatively minor. Only the DB treatment in 28 days was significantly lower than the single treatment BR, but there was no significant difference between it and CK. The findings suggest that the application of plant growth regulators increases the GA_3 content of cotton while retarding its ABA content, effectively stimulates reproductive growth and impedes the senescence of cotton plants.

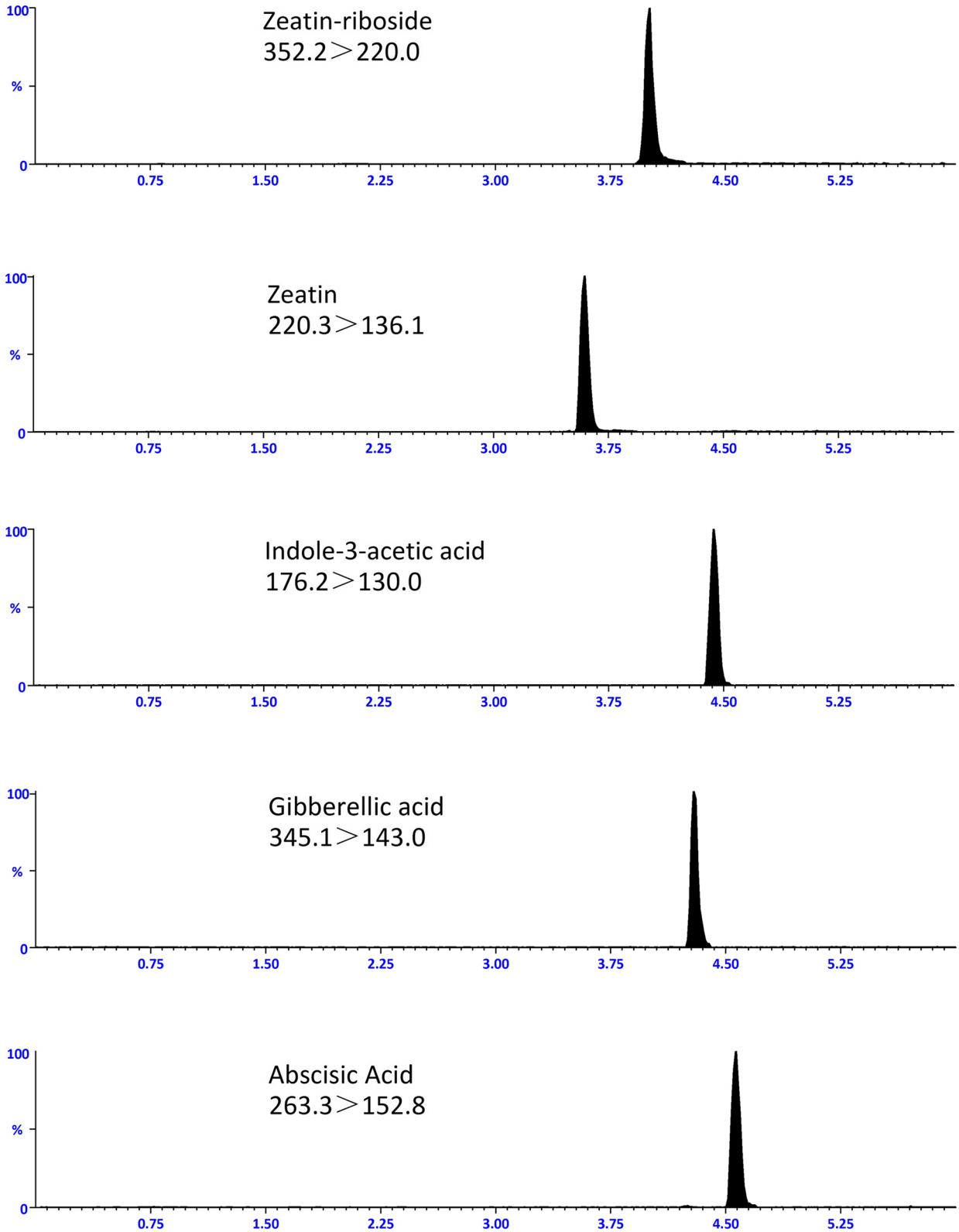

Fig. 5 Retention time and peak shape of five plant hormones. No interference peaks existed near the retention times of the five phytohormones, proving that the stability of the method met the experimental requirement

Table 2 Comparison of matrix-matched calibration and solvent calibration of 5 analytes (5 – 1000 μg·L^{-1})

PGRs	Retention time /min	Regression equations	R^2	Matrix effect /%	LOQs /(μg·kg^{-1})
ZR	3.93	y = 9 130.689 54 x - 19 001.245 95	0.997 94	- 30.06%	5
ZT	3.64	y = 5 612.367 08 x - 25 783.183 06	0.998 58	- 17.38%	5
IAA	4.52	y = 5 179.841 69 x + 6 839.084 28	0.991 75	- 37.78%	5
GA_3	4.33	y = 315.185 05 x + 523.183 00	0.990 61	- 3.35%	20
ABA	4.60	y = 557.108 70 x + 980.649 22	0.999 06	32.34%	5

Table 3 Precision and recovery of 5 analytes spiked at fresh cotton leaves

PGRs	Spiked levels / (μg·kg^{-1})	Recovery /%	RSD_r /%	RSD_R /%
ZR	0.005	91.68	5.30	4.00
	0.01	89.21	3.53	5.97
	0.1	82.84	2.79	9.37
	1	86.04	4.57	9.26
ZT	0.005	98.97	2.82	5.18
	0.01	84.03	7.11	13.12
	0.1	79.07	4.72	7.62
	1	81.77	2.11	2.83
IAA	0.005	87.58	6.46	4.33
	0.01	90.71	3.39	1.07
	0.1	87.28	3.04	4.59
	1	81.08	2.19	6.00
GA_3	0.02	92.87	7.90	14.64
	0.04	85.75	7.68	3.61
	0.1	85.41	7.06	6.89
	1	83.49	5.25	5.11
ABA	0.005	80.78	3.43	10.17
	0.01	84.25	5.02	10.57
	0.1	86.63	8.47	3.45
	1	86.95	2.28	3.66

Comparison between HPLC–MS/MS and reported analytical methods

To assess the analytical performance of the established HPLC–MS/MS methods in comparison to earlier chromatographic analysis techniques, we have detailed the parameters of the prior methods in Table 4. This analysis reveals that when contrasted with the preceding approaches, this test offers a simplified pretreatment process, a significant reduction in detection time, and the ability to separate and detect all five target compounds within a mere 6-min timeframe. Furthermore, the recoveries achieved within the linear range of 5 to 1 000 ng·mL^{-1} exhibited substantial improvement, with recovery ranging from 50.3% to 96.7% compared with the previous assay. In summary, this study presents an innovative approach that considerably reduces time required for the detection of five target compounds while delivering highly satisfactory recovery rates.

Discussion

Development of a HPLC–MS/MS method for the determination of multiple plant hormones in cotton

Prior research has underscored the significant influence of varying mobile phase compositions on ESI ionization, an effect even more substantial than the inherent detection limit of the instruments (Cho et al., 2013). The chromatographic analysis of plant hormones typically involves the amalgamation of water, methanol, or acetonitrile, with the addition of an acidic component as the mobile phase (Cai et al., 2015). Prior investigations have confirmed methanol as a suitable solvent for ESI–MS analysis of acidic compounds in negative ionization mode (Huffman et al., 2012). However, during this experiment, it was discovered that utilizing only methanol and deionized water as the mobile phase failed to achieve complete separation of the five target compounds. Notably, the addition of 0.1% formic acid to deionized water facilitated the complete separation of all five target compounds. Furthermore, the incorporation of 0.1% acetic acid into methanol led to a significant enhancement in recovery rate. Ultimately, the mobile phase of the combination of 0.1% acetic acid in methanol + 0.1% formic acid in water produced excellent separations, yielded optimal chromatograms, and exhibited high MS/MS responses.

HPLC–MS/MS systems are favored for their enhanced specificity and sensitivity when compared with other analytical instruments. As a result, they are frequently employed to determine quantities of small molecules and to ascertain critical compound parameters, such as retention time (RT), and the charge ratio of the parent ion and two daughter ions (Wu et al., 2021; Yang et al., 2021; Pan et al., 2023).

In this study, the optimization of acquisition settings and MRM transitions was carried out meticulously. To attain the most favorable mass spectrometry parameters,

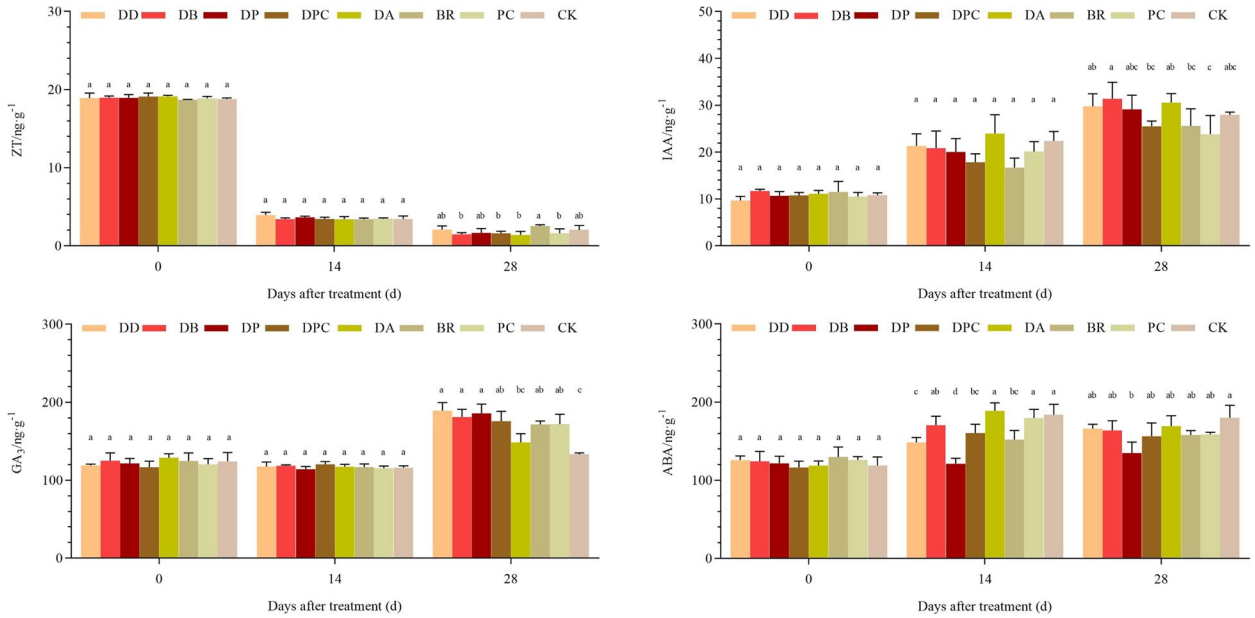

Fig. 6 Content of plant hormones in cotton leaves from the early flowering season to the stage of full bloom. Several plant growth regulator treatments commonly used in cotton fields were sprayed in the field to reveal the effects of different plant growth regulators on cotton phytohormones by detecting the dynamics of the changes in phytohormones among different treatments, and the feasibility of the methodology in this study was also validated. All plant hormones were quantified at fresh weight in this experiment

Table 4 Comparison between published chromatographic analysis methods and HPLC–MS/MS methods for plant hormones

Analytes	Plant matrix	Analytical technique	Recovery /%	Linearity	Running analysis time /min	References
IAA, GAs, *t*Z,ABA	A. thaliana	LC–ESI-IT-MS/MS	70.0–100.0	5–1 000 fmol	30	(Izumi et al., 2009)
IAA, ABA, JA, SA, IBA,GAs	Rice leaves	CE-ESI-TOF–MS	84.6–112.2	1.3–850 ng·mL^{-1}	25	(Chen et al., 2011)
ABA, IAA, IBA, GAs, SA	Green seaweeds	HPLC-ESI-QTOF-MS	80.0–92.0	0.2–100 mg·mL^{-1}	7	(Gupta et al., 2011)
tZ, K, KR	Tobacco	UHPLC-MS/MS	68.8–103.0	0.005–20 ng·mL^{-1}	17	(Du et al., 2015)
BRs	Brassica napus	UHPLC-MS/MS	30.9–88.9	0.01–10.00 pmol	9	(Oklestkova et al., 2017)
JA, ABA, SA, BA, GAS	Hamlin trees leave	LC–ESI–MS/MS	34.6–50.3	0.1–100 ng·mL^{-1}	12	(Suh et al., 2018)

five target compounds were individually detected: ZR, ZT, and IAA were analyzed in ESI + mode, while GA_3 and ABA were assessed in ESI – mode. Individualized optimization of ionization parameters for each compound, including declustering voltage (DP), collision energy (CE), and ESI source temperature, was performed. Two ions with higher abundance were chosen for both quantitative and qualitative analyses. It is worth noting that the optimized MS/MS parameters for the five compounds closely aligned with those reported in the prior study (Wang et al., 2020b).

Optimization of QuEChERS pretreatment method

For sample pretreatment in the QuEChERS method, liquid–liquid extraction with organic reagents is essential (Lakew et al., 2023). The choice of organic solvent influences the recovery of target compounds. Previous experiments have commonly employed methanol for extracting plant hormones (Du et al., 2012; Wells et al., 2013). However, the recovery using methanol alone was suboptimal, as plant hormones are susceptible to oxidative decomposition (Johnson et al., 2007). To mitigate this issue, BHT, a widely used antioxidant in the food industry to prevent lipid peroxidation, was introduced (Ramachandran et al., 2022). While the addition of BHT led to an improvement in recovery, it still fell short of expectations. To further enhance recovery, an acid solution was introduced (Jiang et al., 2020). Formic acid or acetic acid at respective concentrations of 0.1% and 1% were incorporated, and the results indicated that the extraction efficiency of a 0.1% acetic acid–methanol solution + BHT surpassed that of the other options.

Consequently, the 0.1% acetic acid–methanol solution + BHT was selected as the extraction solvent for this experiment.

In the context of the QuEChERS pretreatment, sample purification is vital to eliminate impurities that could influence the experimental results and potentially damage the instrument. In this study, the impact of $MgSO_4$ in conjunction with GCB, C18, and PSA on the recovery was explored (Musarurwa et al., 2019). $MgSO_4$, serving as a dehydrating agent, aids in water adsorption within the extract, promoting solvent distribution and enhancing the recovery rate (Abbas et al., 2017). PSA is known for forming hydrogen bonds with polar matrix components through weak ion exchange of amine groups, making it a common choice for the removal of fatty acids, sugars, organic acids, lipids, and some pigments (Tette et al., 2016). GCB is effective at eliminating nonpolar interferences and is particularly efficient in pigment and phenolic removal (Bernardi et al., 2016). C18 is also adept at adsorbing nonpolar compounds and fats from substrates (Wu et al., 2023). In this study, a thorough comparison and screening of various dosage combinations of $MgSO_4$ with three commonly used adsorbents revealed that the combination of 150 mg $MgSO_4$ and 30 mg C18 produced the highest recovery rate.

Validation of a HPLC–MS/MS method for the detection of multiple phytohormones in cotton

Creating a standard curve is an essential step in establishing a quantitative method (Rahman et al., 2018a). Quantitative analysis in ESI is primarily susceptible to signal suppression or enhancement resulting from matrix or other interferences, the phenomenon known as the "matrix effect" (ME) (Trufelli et al., 2011).

Matrix interference becomes substantial when the absolute value of ME is equal to or exceeds 50% (Li et al., 2013). Prior research has underscored the presence of a more severe matrix effect in the detection of plant hormones (Jiang et al., 2020). Consequently, in this study, the matrix effects under the MS/MS (MRM mode) were evaluated by comparing standard in the solvent with matrix-matched standard.

Application of HPLC–MS/MS on detecting dynamic of hormone concentration in response to plant growth regulator

Plant growth regulators have gained widespread application in the realm of agricultural production, effectively influencing crop growth and development while enhancing crop yield and quality (Jiang et al., 2020). In this experiment, the commonly used plant growth regulator, mepiquat chloride, was chosen for application in cotton fields. It was administered in combination with the promotive plant growth regulators diethyl aminoethyl hexanoate and 24-epibrassinolide, as well as the inhibitory plant growth regulator prohexadione calcium. The findings demonstrated that the GA_3 content in all compound treatments exceeded that of the single treatment and CK groups. It was evident that the application of mepiquat chloride led to an increase in GA_3 content, in line with previous studies (Shi et al., 2022). Notably, DP reduced the content of ABA when compared with all other treatments. ABA is a potent growth inhibitor with a pronounced inhibitory effect on cell division and elongation. It can impede the growth of various plant parts, including leaves, embryos, embryo sheaths, stems, hypocotyls, and roots. ABA is also associated with the promotion of dormancy and stomatal closure, further inhibiting plant growth (Chen et al., 2018). Moreover, All treatments applied in combination compared with those applied individually notably elevated the content of IAA. Previous research bindicated that high concentrations of IAA stimulate ethylene production and accelerate organ abscission (Mao et al., 2014). Zhu's study showed that growth hormone promotes fibre development by enhancing GA biosynthesis (Zhu et al., 2022). Most treatment combinations had no significant effect on ZT content which ensure the cell viability and delay plant senescence when treated in combination (Jiang et al., 2020). These results collectively suggest that various compounds of plant growth regulators play a pivotal role in modulating the growth and development of cotton plants by influencing GA_3 and IAA levels and reducing ABA content.

Conclusions

This study aimed to develop and validate the QuEChERS-based HPLC–MS/MS assay for the concurrent quantification of five plant hormones (ZT, ZR, IAA, ABA, and GA_3) in cotton leaves. Successful chromatographic separation and mass spectrometric detection of these plant hormones were achieved through meticulous methodology. The QuEChERS pretreatment method was employed to prepare the samples. The utilized extraction solvent was 0.1% acetic acid methanol solution + 1 $mmol{\cdot}L^{-1}$ BHT, while the adsorbent consisted of 150 mg $MgSO_4$ and 30 mg C18. The validation of the results encompassed assessments of the matrix effect, linearity, LOD, LOQ, and precision. These analyses confirmed the accuracy, sensitivity, and reproducibility of the method. Cotton leaves collected at various time points in the field were subject to examination for the presence of the five plant hormones. This method was then employed to explore alterations in plant hormones within cotton plants following treatments with various plant growth regulators. The method exhibites high sensitivity and selectivity, underscoring its utility in unraveling the mechanisms regulating cotton growth and development by plant hormones.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
Wang WH: Conceptualization, methodology, validation, Writing—original draft, Writing—review & editing. Song XP, W D, Ma YJ, and Shan YP: investigation, data curation, formal analysis and methodology. Ren XL and Hu HY: Data curation, formal analysis and supervision. Wu CC, Yang J, and Ma Y: Resources, supervision, review & editing. All authors read and approved the final manuscript.

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Declarations

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