Analytical Methods

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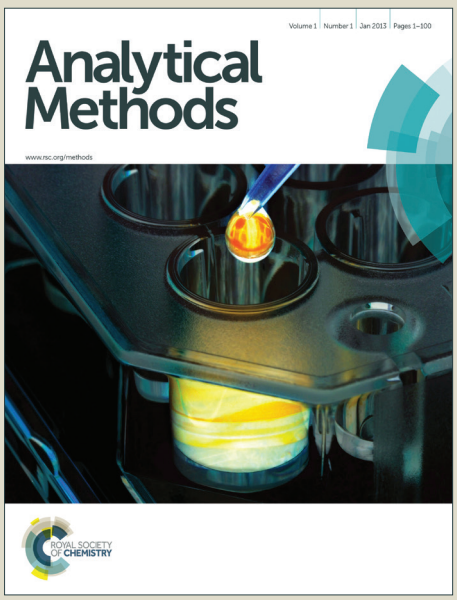

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ARTICLE

Simultaneous determination of six plant growth regulators in fruits using high performance liquid chromatography based on solid-phase extraction and cleanup with a novel mixed-mode functionalized calixarene sorbent

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In this paper, a rapid and practical high performance liquid chromatography（HPLC）method, using home-made tetraazacalix[2]arene[2]triazine-modified silica gel (NCSi) as solid-phase extraction (SPE) sorbent, was developed for the simultaneous determination of six plant growth regulators (PGRs) including thidiazuron (TDZ), naphthaleneacetic acid (NAA), p-chlorophenoxyacetic (PCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (6-BA) and forchlorfenuron (KT-30) in fruits. The NCSi is a novel multi-interaction and mixed mode functionalized calixarene sorbent, which can enrich different classes of PGRs. Prior to the HPLC separation, the key factors on influencing SPE performance were investigated in the sample pretreatment step, including the dosage of NCSi sorbent , the flow rate, volume and composition of eluant. The HPLC analysis was carried out on a Waters XBridge C18 column (250× 4.6 mm i.d., 5 μm) with a mobile phase of methanol/0.1% H_3PO_4 (55:45,v/v). With a detection wavelength of 220 nm, the good linearities of the six PGRs were obtained in the concentration ranged from 0.03 to 200 mg/kg (R^2≥0.999). The lower detection limits (4.3~20.4 μg/kg) and quantitation limits (12.9~64.0 μg/kg) of the six PGRs were also obtained, which can meet the trace analysis requirements. In addition, overall recoveries through the extraction and purification steps ranged from 72.4 to 94.9%, RSDs were less than 4.84%. The proposed method was successfully used to determine residual PGRs in real samples. It is practical and promising for the study of plant physiology by the determination of trace PGR content in the plant or fruits with complex matrix.

Introduction

Plant growth regulators (PGRs) 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. For example, Naphthaleneacetic acid is present in higher plants acting as exogenous plant hormones, and their main physiological effects include the induction of germination and flowering. KT-30 can trigger a variety of basic physiological processes including cell division, enlargement and differentiation, organogenesis. So PGRs are widely used in modern agricultural production. But the same as the other pesticides, PGRs also have a certain toxicity. Long-term consumption of PGR residues in fruits and vegetables can produce side effects on the human body, make human body secretion disorder and affect the balance of metabolism.[1,2] The hazardous effects of PGR residues for both food safety and human health have increasingly become the focus of attention worldwide.[3] So establishing a rapid, sensitive, accurate and efficient detection method for PGR residues in food has great realistic significance to ensure food safety and improve the consumers' health.

Nowadays, many methods were employed for PGRs analysis, including high performance liquid chromatography (HPLC),[4] gas chromatography-mass spectrometry (GC-MS),[5,6] liquid chromatography–mass spectrometry (LC-MS),[7-9] capillary electrophoresis (CE)[10-11] etc. Each of these methods above has its own characteristics, but they have some limitations in PGRs determination, such as bad selectivity, lowsensitivity and/or poor applicability. For example, LC-MS needs a complicated and intensive purification process, which is time-consuming and tedious. Moreover, LC-MS methods often require high resolution mass spectrometry to ensure the high detection sensitivity, not easily available in common laboratories. To GC-MS analysis, some thermally labile PGRs are likely to break down at the high temperature of the GC injector, which limits the range of PGRs fit for GC analysis. While CE with high sensitivity, lower limit of detection but poor reproducibility which goes against the detection of quantification. Based on the above reasons, the most frequently used method for PGRs

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analysis is HPLC. This is largely because HPLC is relatively simple, easy to popularize, and it has good reproducibility, which merits its choice as a better tool over others.

In order to improve the detection limit of HPLC, we pay more attention to sample pretreatment. Solid-phase extraction (SPE) is a well-established sample pretreatment method because it demands shorter processing time lower solvent consumption, simpler processing procedure and can remove interferences and preconcentrate the target analytes simultaneously.[12–15] In general, the sorbent material is the key point for SPE as similar to stationary phase for HPLC. Traditionally, SPE is mainly based on single mechanism, such as hydrophobic interaction for enriching nonpolar compounds on reversed phase sorbent and ion-exchange interaction for enriching ions compounds on ion-exchange sorbent.[16] However some complex samples of pharmacology, environment and food are often mixtures of compounds with different properties, which cannot be separated efficiently on the sorbent possessing single mechanism of action. Therefore, there are considerable interests in developing new selective sorbents for extracting and isolating components from complicated matrices.[17]

Following cyclodextrins and crown ethers, calixarenes are considered to be a typical representative of the third generation of host supramolecules. They consist of phenol units linked via methylene bridges and can also form inclusion complexes like other host supramolecules. There are a number of selective factors in the configuration of calixarenes such as cavity size, conformation, and substituents. Since Glennon and his coworkers first reported the application of calixarenes as the stationary phase, it has attracted wide attention.[18,19] Our group has been committing to the research of calixarene stationary phases in recent years, and a novel multi-interaction and mixed mode stationary phase based on tetraazacalix[2]arene[2]triazine-modified silica gel (NCSi, Fig. 1) has been synthesized in our laboratory in 2012.[20-26] Tetraazacalix[2]arene[2]triazine is different from conventional calixarenes in which the aromatic rings are linked by methylene units, and it assembles aromatic rings by –NH– and adopts a 1,3-alternate conformation with two benzene rings nearly face-to-face parallel and two triazine rings tending to an edge-to-edge orientation in the solid phase. Our previous work indicates that the NCSi exhibited high selectivity toward various compounds under different conditions with different mechanisms, including hydrophobicity, π–π stacking, hydrogen-bonding, inclusion, and anion-exchange interactions. A number of compounds, including polycyclic aromatic hydrocarbons, nitrobenzene, organic bases, phenols, and inorganic anions, have been well separated on the NCSi stationary phase.[27] Recently, a NCSi SPE sorbent was prepared utilizing preparation procedures similar to the NCSi stationary phases in our laboratory. Because of the existence of multi-interaction, it is of great application value for purification or concentration trace analyte in a complex matrix. The NCSi SPE sorbent has been shown to be a flexible and versatile method for the simultaneous enriching of highly mixed classes of analytes, especially ions and organic compounds. It has been successfully used for the extraction acrylamide in starchy foodstuffs, and tobacco-specific N-nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and a metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in rabbit plasma, for example.[28,29]

In the present work, a novel multiple retention mechanism sorbent (NCSi) was used to develop a rapid and reliable sample SPE preparation procedure for the determination of six PGRs (Fig. 2) in fruits. All of the main factors were optimized, and the results obtained by using the developed HPLC method based on NCSi SPE indicated that it is more suitable for the determination of PGRs in fruits with the complex matrices.

Experimental

Reagents , Materials and Chemicals

HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Deionized water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Tetraazacalix[2]arene[2]triazine was synthesized in accordance with the previously published procedures.[26] The polypropylene column tube and 20 μm PTFE sieve plates used for SPE were bought from DIKMA (Beijing, China). Commercially available C18 cartridges (100 mg/3 mL) were obtained from Waters (Milford, MA,USA). TDZ(≥98%), NAA (≥98%), PCPA(≥98%), 2,4-D(≥98%), 6-BA(≥98%), KT-30(≥98%) which were purchased from Zhengzhou Excellence Agriculture Technology. Phosphoric acid (≥99.5%) of HPLC grade were purchased from DIMA Technology (Richmond Hill, Ontario,Canada). Grapes, pitaya, apples, peaches were purchased from the local supermarket.

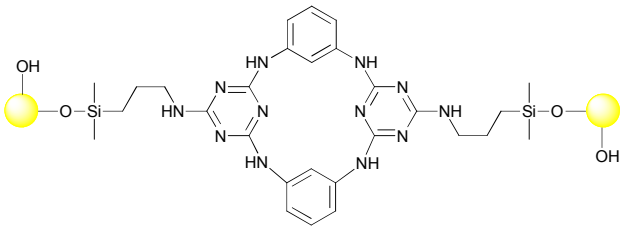

Fig. 1. Chemical structure of the NCSi stationary phase.

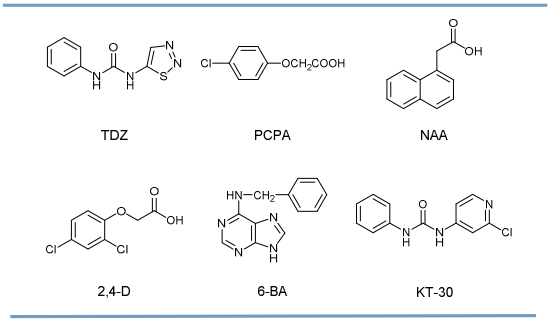

Fig. 2. Chemical structures of six target PGRs.

Instruments and Chromatographic Conditions

Chromatographic analysis were conducted using an Agilent 1260 HPLC (Agilent, USA) with VWD UV detector. A centrifuge (Zhongda Instrument Plant, Jiangsu, China) was used for centrifugal separation. A vortex mixer (Shanghai jingke industrial LTD, Shanghai, China) was used for mixing solutions. A rotary evaporator was used for enrichment (Yarong, Shanghai, China). The separation of PGRs was performed on a Waters XBridge C18 analytical column (250× 4.6 mm i.d., 5 μm). The mobile phase used in the chromatographic separation consisted of a binary mixture of eluent A (0.1% phosphoric acid in water) and eluent B (methanol) (45:55, v/v). The temperature was set at 30 °C. A flow rate of 0.7 mL/min, an injection volume of 20 μL, and a detection wavelength of 220 nm with VWD were used during the whole analyses.

Standard Solution Preparation

Mixed standard stock solution (200 mg/kg of each analyte) was prepared by dissolving PGRs in methanol. The stock solution was stored at 4 °C in the refrigerator. Working standard solutions was obtained by stepwise dilution of stock standard solutions with mobile phase.

Synthesis of NCSi SPE Sorbent

The NCSi sorbent was prepared by a two-step modification process as previously described .[26] First, aminopropyltriethoxyl-bonded silica gel (APS) was obtained. In the second step, APS was reacted with an excess of tetraazacalix[2]arene[2]triazine in anhydrous dimethylformamide (DMF) at 130 °C under nitrogen atmosphere. Different from the previous work, the spherical silica had a particle diameter of 40−60 μm instead of the previous 5 μm. The surface area is 500 m^2/g.

Sample Preparation

Extraction, and Enrichment Process. A fully homogenized sample (2 g) was weighed and put into a 50 mL plastic centrifuge tube. With the addition of 10 mL of methanol, the tube was vigorously vortexed for 1 min and centrifuged for 10 min at 2000 rpm to extract the target analytes. This process was repeated, and the two clear supernatants were combined. About 20 mL of extraction solution was rotary evaporated to about 1 mL after the addition of methanol under vacuum at 30 °C.

SPE Clean up. To investigate the availability of the NCSi SPE for the extraction of PGRs in complex matrix, 100 mg of NCSi sorbent was packed into a 3 mL SPE cartridge. The concentrated extraction solution (1 mL) was passed through the SPE cartridge, which had been previously preconditioned with 3 mL of acetonitrile and 3 mL of water, respectively. The cartridge was then washed with 1 mL of water and eluted with 5 mL of acetonitrile at the rate of 3 mL/min, collecting eluant into a 10 mL glass tube, making the final volume approximately 1 mL. The collected solution was filtered through a 0.22 μm nylon filter (Agilent, USA) prior to HPLC analysis. All tests were performed in triplicate. This process illustrated in Fig. 3.

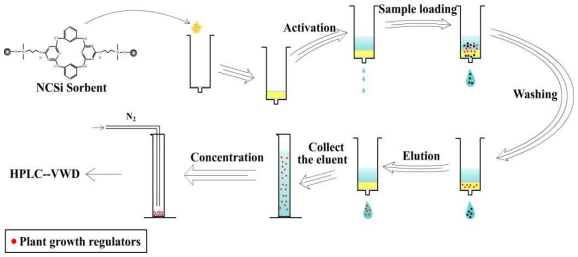

Fig. 3. Scheme of SPE process

Results and discussion

Homogenization Extraction

In the homogenization step, the collected plant samples stored at low temperature were grounded into powder in liquid nitrogen, to avoid chemical degradation or metabolic change of the hormones of interest. As for the extraction, methanol can prevent the degradation of plant growth regulators and block the extraction of too many lipids.

Optimization of SPE Condition with the NCSi Sorbent

In this paper, a practical purification procedure based on a novel homemade NCSi SPE sorbent is proposed for the enrichment of PGRs and optimization of the SPE conditions. In this section, the main influence factors (dosage and adsorption capacity of sorbent, volume and flow rate of eluant) on the SPE recoveries (n = 3) of six PGRs are evaluated in detail to obtain the optimal purification conditions.

Comparison of SPE Cleanup by NCSi Sorbent and Commercial Sorbent(C18). Octadecyl-silica (C18) is the most widely used SPE sorbent. In the present work, we compared the matrix removing effect and the enrichment effect on C18 and NCSi SPE sorbent. The peach extraction solution (1 mL) was passed through the C18 and NCSi SPE cartridge, washed with 1 mL of water and eluted with 5 mL of acetonitrile. The eluant was analysed by HPLC and chromatograms were shown in Fig. 4. The purged liquor from NCSi and C18 can minimize the matrix interference. Fortunately, the 6-BA and NAA residual targets were easily retained on the NCSi sorbert and detected in the purged liquor after the peach estraction solution passed through NCSi sorbent. This proved that NCSi sorbent is more suitable for the purification and enrichment of PGRs in fruits.

Dosage of sorbent. The dosage of sorbent is an important parameter for the extraction efficiency in SPE. Therefore, cartridges packed with different NCSi dosages of 50, 100, 150, 200 and 250 mg were respectively evaluated. The extraction efficiencies of PGRs increased as the dosages of sorbent increased from 50 to 100 mg (Fig.5). Howerver, their extraction efficiencies steadily increased from 100 to 250 mg. Thus, the optimal dosage of sorbent was 100 mg.

SPE Eluant Choice. The elution solvent used in SPE plays an important role. To maximally wash target analytes, a proper washing solvent is necessary. In this section, methanol, acetonitrile, ethanol, dichloromethane, n-hexane and isopropyl alcohol were investigated as elution solvents, and the results were shown in Fig. 6. When acetonitrile was used,

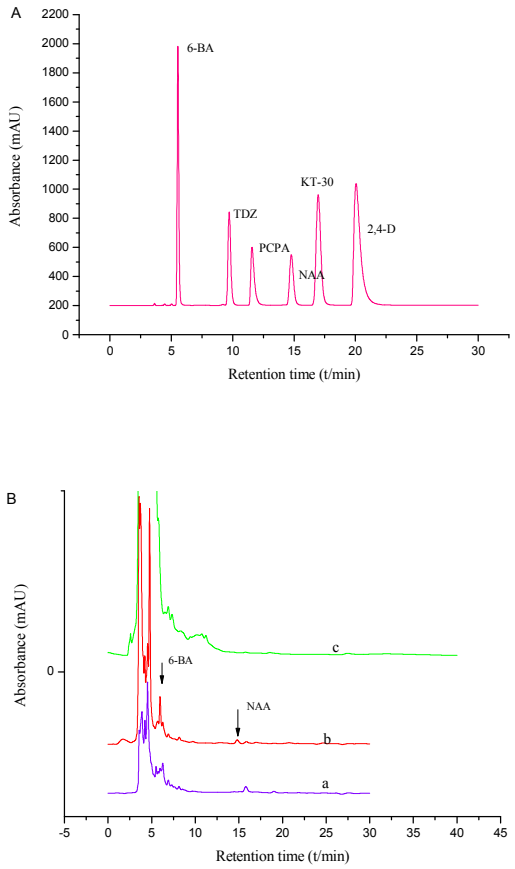

Fig. 4. (A) Separation of standard solution mixed with six PGRs (B) Chromatogram comparison of peach purged liquors from (a) C18 Sorbent and (b) homemade NCSi Sorbent and (c) without SPE

the higher recoveries for the PGRs were obtained. Therefore, acetonitrile was selected as the elution solvent in NCSi-SPE system.

Eluant Volume Optimization. The volume of eluant is another critical parameter that affects the recovery. Obviously, the affinity between analytes and sorbent is strong and analytes need more solvent for complete desorption. As displayed in Fig. 7, when volume of washing solvent changed in the range of 5-9 mL, the recovery for six analytes varied a little. Considering the solvent consumption and operation time, hence 5 mL of acetonitrile was optimized for the subsequent experiments.

Flow rate of eluant. As known, the flow rate of the eluant is another pivotal factor that not only affects the purification and enrichment of analytes but also controls the sample pretreatment time. In this section, flow rates ranging from 1 to 6 mL/min were investigated, and it was found that 3 mL/min was satisfactory to provide higher extraction recovery (Fig. 8) .

Adsorption capacity. To study the adsorption capacity of the NCSi sorbent, 10-800 mg/kg PGRs mixed standard solutions

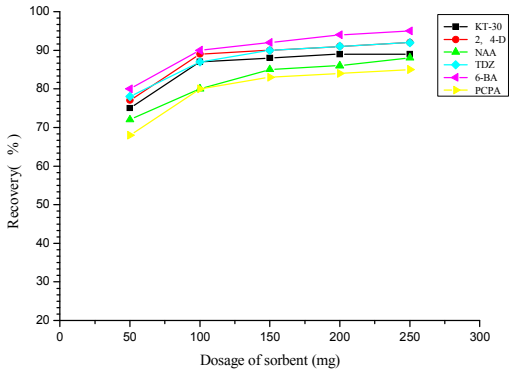

Fig. 5. Recovery efficiency with dosage of sorbent.

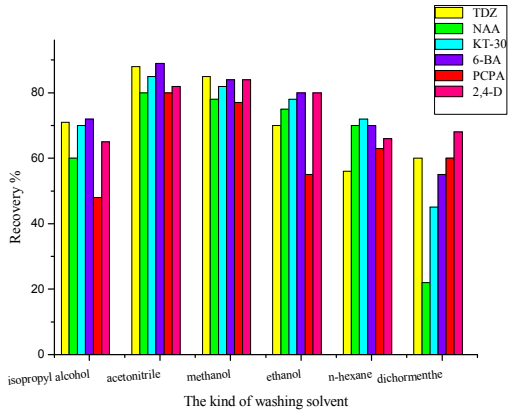

Fig. 6. Effect of SPE eluant on recovery

were investigated. As shown in Fig. 9, recovery for PGRs showed no significant decrease with the increasing of sample concentration. A observation of these profiles, KT-30, 6-BA and TDZ were better adsorbed on the NCSi sorbent because hydrophobicity effect, π–π effect, hydrogen-bonding effect. This fact suggested that the sorbent was more favorable in reacting with benzene ring or -NH_2. In conclusion, NCSi material had a large adsorption capacity, which can aviod sample's overloading during SPE processing .

Method validation

In this study, method validation such as recovery, linearity, correlation coefficients, limits of detection (LODs) and quantification (LOQs) were measured. Calibration curves were constructed by plotting peak area (y) versus the corresponding concentration of PGRs (x, mg/kg). As showed in Tab. 1, wide

linear ranges were 0.030–200 mg/kg for six PGRs and all correlation coefficients (R^2) were bigger than 0.9991.

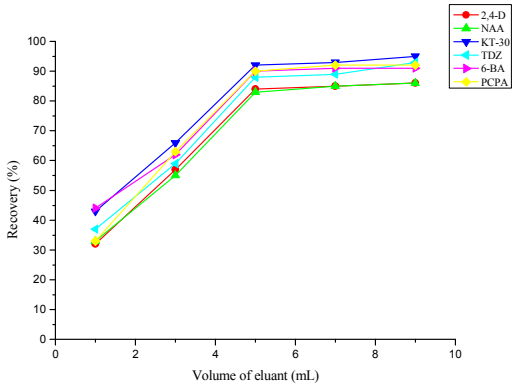

Fig. 7. Effect of eluant volume on recovery

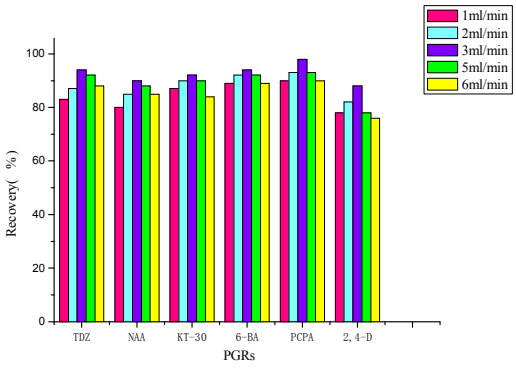

Fig. 8. The flow rate of the washing solution

LODs and LOQs were calculated on the basis of a peak–peak signal-to-noise (S/N) value that was S/N = 3 and 10, respectively. LOQs (12.9–64.0 μg/kg) were lower than the maximum limit of residues established for PGRs.[30] Particularly, the LOQ for NAA was 12.9 μg/kg for the LC-UV analysis, and the LOD of the LC-UV analysis was adequate to detect redidue four times lower than the maximum residue limit (MRL) of products. The recovery was calculated by the ratio of the concentration measured versus the concentration spiked in four kinds of fruits, and the precision expressed as the relative standard deviation (RSD). As presented in Tab. 2, satisfactory method recoveries were obtained for the PGRs spiked at three concentration levels in four kinds of fruits (72.4–94.9%, RSD <4.84%). From the results above, NCSi-SPE offers higher accuracy and better repeatability for the determination of PGRs .

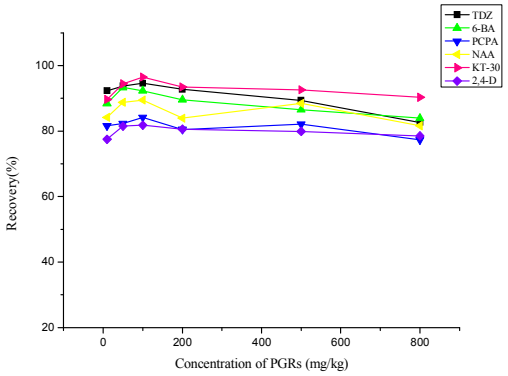

Fig. 9. The recovery for PGRs of different concentration

Application to real samples

The proposed NCSi-SPE-HPLC method was applied to determine the residues of the six PGRs in peach, grape, pitaya and apple from local supermarket in Zhengzhou. The residues of the six PGRs in fruits were listed in Tab. 3, and it was found that their residual concentrations were higher than the MRLs.[31-34] They will pose the harmful effect on the consumers' health. Thus, it is neccessary to strictly control the use of these PGRs during plant growth process.

Conclusions

In conclusion, an HPLC method based on NCSi-SPE cleanup has been successfully employed for the purification and determination of PRGs in fruits with complex matrices. The NCSi-SPE method enables a more efficient purification of PGRs from a complex matrix due to its multi-interaction ability. The SPE procedure is simple and easy to operate. Furthermore, NCSi-SPE offers higher accuracy for the trace determination of PGRs in real fruit samples with complex matrix. The proposed HPLC method based on NCSi-SPE is promising for the study of plant physiology of these PGRs by the determination of trace PGR content during their growing.

Acknowledgements

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Tab. 1. Linear Regression Equation, LOD and LOQ of Six kinds of PGRs .

PGRs	linearity range (mg/kg)	Linear Regression Equation	R^2	LOQ (μg/kg)	LOD (μg/kg)
KT-30	0.03 ~ 200	Y = 125.51X - 251.94	0.9999	32.1	10.7
2,4-D	0.1 ~ 200	Y = 49.221X - 317.63	0.9999	61.2	20.4
PAPC	0.1 ~200	Y = 25.869X - 149.74	0.9994	64.0	21.3
TDZ	0.03 ~ 200	Y = 40.486X + 32.139	0.9999	32.1	16.1
NAA	0.03 ~ 200	Y = 118.03X - 95.923	0.9991	12.9	4.3
6-BA	0.03 ~ 200	Y = 35.554X + 213.78	0.9998	31.9	10.6

Tab.2 .The recovery experiments of PGRs in four kinds of fruits

PGRs	spiking levels (mg/kg)	Recovery percentage (% RSD)			
		apple	peach	grape	pitaya
KT-30	0.03	85.6 (2.51)	84.2 (1.71)	92.4 (1.03)	77.7 (2.43)
	0.1	85.1 (4.84)	88.8 (1.11)	94.3 (1.54)	88.6 (1.46)
	0.5	86.1 (2.36)	91.5 (3.16)	86.6 (1.57)	94.4 (1.83)
2,4-D	0.1	75.9 (3.96)	76.6 (1.88)	75.0 (4.27)	81.5 (2.43)
	0.2	73.8 (2.03)	83.8 (2.73)	72.4 (1.36)	83.6 (1.18)
	0.5	76.1 (2.66)	76.7 (2.04)	81.0 (3.94)	83.0 (2.30)
PAPC	0.1	74.5 (1.02)	73.5 (1.51)	78.4 (0.96)	78.5 (4.28)
	0.2	77.2 (3.19)	79.1 (0.86)	74.8 (4.13)	83.5 (1.28)
	0.5	83.8 (0.96)	76.2 (3.67)	73.5 (4.89)	86.9 (2.71)
TDZ	0.03	76.7 (2.85)	86.3 (1.32)	88.4 (2.64)	85.1 (0.18)
	0.1	92.2 (2.07)	90.7 (0.78)	93.1 (2.22)	80.4 (1.30)
	0.5	90.0 (3.53)	93.5 (0.91)	95.0 (2.55)	91.6 (2.81)
NAA	0.03	84.2 (0.27)	75.37 (2.79)	79.6 (1.70)	84.4 (1.26)
	0.1	85.9 (1.80)	81.7 (1.43)	90.3 (2.84)	93.0 (1.91)
	0.5	80.83 (1.80)	82.6 (2.07)	87.7 (2.55)	92.8 (1.95)
6-BA	0.03	82.6 (2.22)	85.7 (0.84)	85.9 (2.25)	78.8 (4.57)
	0.1	79.5 (1.71)	86.6 (3.07)	88.3 (1.16)	80.4 (1.62)
	0.5	89.4 (1.29)	90.1 (1.24)	94.9 (3.36)	91.6 (2.81)

a N.D: not detected.

Tab. 3. Concentration levels of target PGRs in four kinds of fruits (mg/kg)

Fruits	concentration detected (mg/kg)					
	6-BA	PAPC	TDZ	NAA	KT-30	2,4-D
peach	0.0623	N.D	0.0665	0.1345	0.0494	0.0837
grape	N.D	N.D	0.0422	0.0198	0.0407	0.0755
pitaya	0.1802	0.3865	N.D	0.2285	N.D	0.2735
apple	0.0873	N.D	0.0406	0.0504	0.0590	N.D

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