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Modified QuEChERS method for 24 plant growth regulators in grapes using LC-MS/MS

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ABSTRACT

A multiresidue analytical method was developed for grapes for the following 24 plant growth regulators: 1-naphthylacetamide, 2,3,5-triiodobenzoic acid, 2,4,5-T, 2-naphthoxyacetic acid, 3-indolylacetic acid, 4-(3-indolyl)-butyric acid, 4-chlorophenoxyacetic acid, 4-nitrophenol, 6-benzylaminopurine, N6-isopentenyladenine, butralin, chlormequat chloride, chlorphonim-Cl, cloprop, forchlorfenuron, gibberellic acid 3, gibberellic acid 4, gibberellic acid 7, inabenfide, mepiquat chloride, paclobutrazol, prohydrojasmon, thidiazuron and uniconizole-P. The compounds were extracted from grape samples using an extraction method modified from the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method. Liquid chromatography – tandem mass spectrometry was used for the detection and quantification of the compounds. Validation of the method was performed by using recovery studies at both intra-day and inter-day intervals, as well as by evaluation of the matrix effect, limit of quantification, trueness and precision. We used matrix-matched calibrations for the quantification of the compounds, which all resulted in determination coefficients (r^2) higher than 0.995. The limit of quantification ranged from 0.1 to 5 ng/mL. Recovery studies using three spiking concentrations at varying levels showed recoveries of 70.2–112.6% and 67.5–101.8% at intra-day and inter-day intervals, respectively. Relative standard deviations were below 20% for the recovery studies. The extraction method were further validated by performing recovery study and matrix effect test in six different grape varieties from Taiwan and the United States and all resulted in comparable results. Application of the established method to 50 grape samples, resulted in the detection of chlormequat chloride and forchlorfenuron residues in the tested grapes. The results of the method validation and real sample analysis shows the extraction method is therefore suitable for routine monitoring of residue in grapes.

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1. Introduction

Plant growth regulators (PGRs) are natural or synthetic chemical compounds that regulate plant physiologies at minimal amounts. PGRs have been widely used in agricultural practices, such as grape cultivation, to achieve desirable traits for high quality and production. Studies on grape cultivation have indicated that regular usage of gibberellins

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and cytokinins promote floral cluster elongation [1–3]. The use of abscisic acid has also been found to improve grape color [2].

PGRs have specific functions and can mainly be classified into auxins, cytokinins, gibberellins and inhibitors [4,5]. Auxin indole compounds such as 3-indolylacetic acid (IAA), 4-(3-indolyl)-butyric acid (IBA), 2-naphthoxyacetic acid (2-NOA), 1-naphthylacetamide (1-NAD), atonik, 4-chlorophenoxyacetic acid (4-CPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) can be used in fruits to promote cell enlargement and differentiation, root formation and fruit enlargement [4,6]. Cytokinins such as N6-isopentenyladenine (2iP), forchlorfenuron (CPPU), 6-benzylaminopurine (6-BAP) and thidiazuron (TDZ) are N6-substituted adenine derivatives that stimulate cell division and growth [6,7]. Gibberellic acids (GAs) such as GA3, GA4, and GA7 are terpenoids that promote seed dormancy breakage and flower induction; studies have shown that GAs are used to promote cluster loosening in seedless grapes [5,6,8]. Inhibitors of GA biosynthesis include chlorphonium chloride, chlormequat chloride (CCC), mepiquat chloride, paclobutrazol (PBZ) and uniconazole-P [8,9]. CCC promotes crop production during periods of moisture stress, but can inhibit crop production during periods of drought stress [5]. Auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) have been found to affect crop growth, flowering and production [5,10–13].

Nevertheless, the application of chemicals in agricultural practices has led to concerns regarding consumer health and environmental contamination. Studies have shown that CCC may affect mammalian fertility [14] and that GA may increase mast cell recruitment and affect the level of Substance P [15]. An analysis of residues of atonik and 4-nitrophenol in the urine of adults living in the United States had a detection rate and residue mean of 41% and 1.6 ng/mL, respectively [16,17]. Thus, international and national regulatory agencies for pesticide residues such as Codex, as well as from those from Taiwan, the European Union (EU) and the United States (US) have developed PGR maximum residues limits (MRLs) in order to monitor and regulate PGR residues in crops.

Multiresidue analysis methods are commonly used in routine residue monitoring to ensure compliance with MRLs. The development of the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method shortens the multiresidue extraction to less than 2 h, it requires small amounts of solvents, and it uses simple procedures to achieve favorable extraction results [18]. Studies on methods for PGR analysis have focused either on analysis of single compounds such as prohexadione and 6-benzylaminopurine [19,20] or on multiple compounds analysis [21–24]. An analytical method for rapeseed encompassing 12 PGRs using ultrasound-assisted extraction and liquid chromatography – tandem mass spectrometry (LC-MS/MS) was established [23]. The analytical method for rapeseed requires a further re-extraction procedure and rotatory evaporation, which would require more time than would the QuEChERS method. A modified QuEChERS method was developed for 5 and 15 PGRs in bean sprouts, tomatoes, oranges, and peaches [21,24]. The modification methods mainly changed the extraction solvent that was used or excluded the cleanup procedure. Published PGR multiresidue analytical methods either are suitable for a few compounds, require complex procedures, or exclude cleanups to compensate for adequate extraction recoveries. However, complex monitoring analysis procedures have disadvantages due to increased time consumption, and the removal of cleanup procedures may easily lead to instrumental contamination after routine monitoring.

Grapes are a highly preferred fruit in Taiwan: their production and their production value reached 85,434 metric tons and five million New Taiwan dollars, respectively, in 2015. Imported grapes are the seventh highest fruit import products in Taiwan, reaching 57,761 metric tons in 2015 [25]. The production of grapes is known to regularly make use PGRs. However, PGRs residues are not regularly monitored in Taiwan; therefore, PGR usage in grape production and residues in grapes remain unclear. This study aims to develop a modified QuEChERS method for PGRs analysis in grapes that includes various PGR classifications. The established method in this study was then used to analyze 50 grape samples in Taiwan in order to evaluate PGR residues in the grapes.

2. Materials and methods

2.1. Chemicals and standard solutions

Analytical-grade ammonium acetate (98%), magnesium sulfate anhydrous (≧98.0%), trisodium citrate dihydrate (≧99.0%), disodium hydrogen citrate sesquihydrate (≧99.0%), sodium chloride (≧99.5%), formic acid (FA, 98–100%) and HPLC-grade methanol (≧99.8%) were purchased from Merck. Primary secondary amine (PSA) was purchased from Agilent Technologies, and HPLC-grade acetonitrile (≧99.9%) was purchased from J.T.Baker. HPLC-grade acetone (99.98%) was from Burdick & Jackson. Highly purified water (Milli-Q, Millipore) was used in the mobile phase.

The chemical structures of the 24 PGRs are shown in Fig. 1. Certified standards of 1-NAD (99.0%), IAA (99.3%), 4-nitrophenol (99.9%) and 2iP (>90%) were purchased from Sigma–Aldrich/Fluka (St. Lousis, MO, USA). Certified standards of TIBA (99.0%), 2,4,5-T (99.0%), 2-NOA (96.5%), IBA (99.0%), 4-CPA (99.5%), 6-BAP (99.0%), butralin (99.0%), CCC (99.0%), chlorphonim-Cl (99.0%), cloprop (99.0%), CPPU (99.2%), GA3 (98.0%), inabenfide (98.0%), mepiquat chloride (99.0%), PBZ (98.5%) and TDZ (99.0%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Certified standards of GA4 + GA7 (90.0%), prohydrojasmon (99.8%) and uniconizole-P (99.5%) were purchased from Duchefa Biochemie (Haarlem, The Netherlands), Wako (Osaka, Japan) and Chem Service (Pennsylvania, USA), respectively. Standard stock solutions at concentrations of 1000 μg/mL in solvents (mainly methanol, acetone, or acetonitrile) were prepared and stored at −20 °C.

2.2. Mass instrument

Chromatography analysis was performed with an AQUITY UPLC® (Waters, USA). PGRs were separated with a BEH C18 1.7 μm pre-column (AQUITY UPLC® VanGuard™, 2.1 mm diameter, 5 mm length) linked to a BEH C18 1.7 μm column (AQUITY UPLC®, 2.1 mm diameter, 100 mm length). Ammonium acetate (1 mM) dissolved in 0.1% FA solution in H_2O and

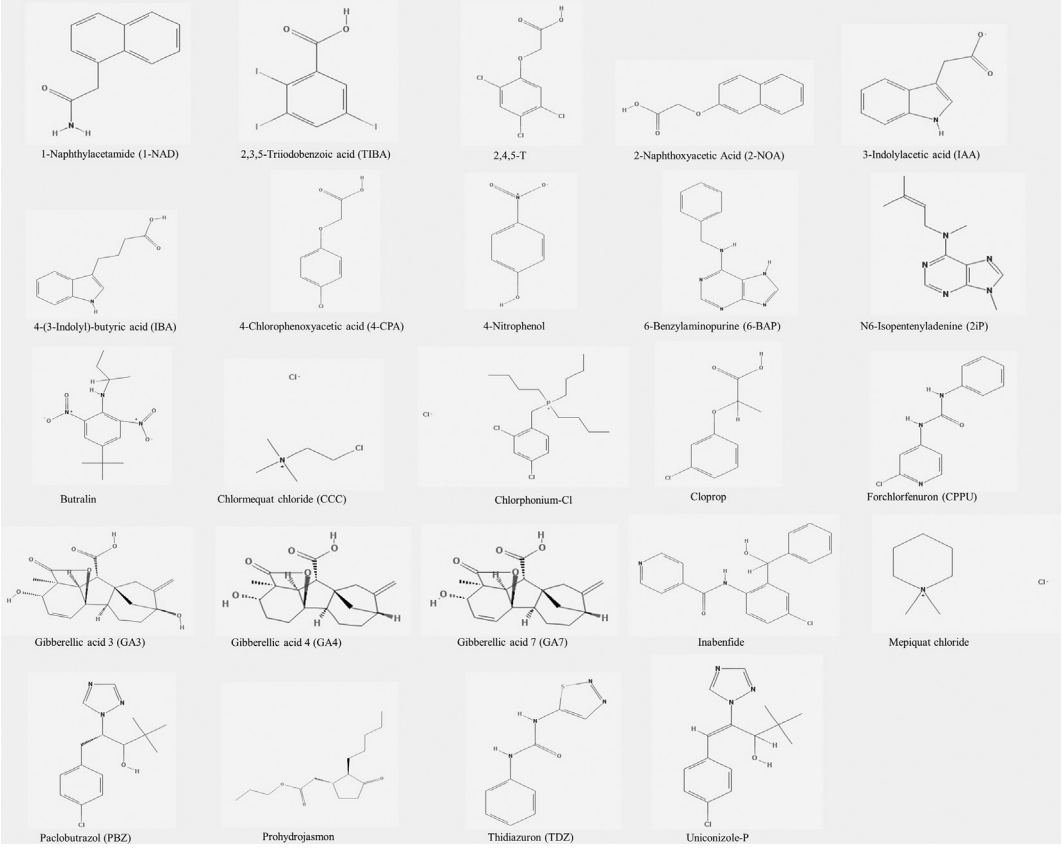

Fig. 1 – Schematic structure of the 24 PGRs. Original chemical structures were obtained from the PubChem Database [42].

1 mM ammonium acetate dissolved in methanol was prepared for use as the mobile phase. The mobile phase gradient is shown in Table 1. The flow rate and injection volume were set at 0.3 mL/min and 5 μL, respectively, and the column temperature was set at 50 °C. MS analysis was performed with a tandem quadrupole Xevo™ TQ-S (Waters, USA) using electrospray ionization as the interface, as well as nitrogen gas and argon gas as the nebulizer and collision gas, respectively. Parameters were set as follows: a source temperature of 150 °C, desolvation temperature of 500 °C, desolvation flow rate of 1000 L/hr and capillary voltage of 3 kV for positive ions and 2.8 kV for negative ions. Instrumental control was performed using Waters MassLynx™ software.

Table 1 – UPLC gradient condition for the 24 PGRs.

Time (min)	Flow rate (mL/min)	1 mM ammonium acetate and 0.1% FA in H_2O (%)	1 mM ammonium acetate in methanol (%)
0	0.3	99	1
2	0.3	50	50
8	0.3	30	70
10	0.3	10	90
12	0.3	1	99
13	0.3	1	99
13.5	0.3	99	1
15	0.3	99	1

2.3. *Sample preparation*

The extraction method used was modified from the QuEChERS method, DIN EN 15662:2009 [26]. Grape samples were frozen at −20 °C for at least 2 h and then homogenized with dried ice using a blender. Each homogenized sample (10 g) was mixed vigorously with 1% FA in acetonitrile/methanol solution (4:1, v/v) for 1 min, and then 6.5 g of a salt mixtures (magnesium sulfate anhydrous, sodium chloride, trisodium citrate dehydrate and disodium hydrogen citrate sesquihydrate (8:2:2:1, w/w/w/w)) was added and the mixture was then mixed vigorously for 1 min. The mixed samples were centrifuged at 3000 × g at 15 °C for 5 min to obtain the supernatant. The cleanup of 6 mL of supernatant was performed by vigorously mixing the supernatant with 150 mg of PSA and 900 mg of magnesium sulfate anhydrous for 2 min. The cleanup solution was then centrifuged at 3000 × g and 15 °C for 5 min and 1 mL of supernatant was collected and dried with nitrogen gas. Samples were dissolved using 1 mL of 0.05% FA solution in methanol. The samples were filtered through a 0.2 μm PVDF filter prior to instrumental analysis.

3. Results and discussion

3.1. *LC-MS/MS optimization*

To obtain MS/MS parameters for the 24 PGRs, working standard solutions (0.1 μg/mL) were prepared from stock standard

solutions with methanol and were infused into the mass spectrometer at a flow rate of 10 μL/min. The optimum sensitivity was obtained for 12 PGRs in negative mode and for 12 PGRs in positive mode (negative mode: TIBA, 2,4,5-T, 2-NOA, 4-CPA, 4-nitrophenol, 6-BAP, cloprop, GA3, GA4, GA7, inabenfide and TDZ; positive mode: 1-NAD, 2iP, IAA, IBA, butralin, CCC, chlorphonium-Cl, CPPU, mepiquat chloride, PBZ, prohydrojasmon and uniconizole-P). MS scans were then applied in the search for appropriate precursor ions, and the selected precursor ions were used to produce daughter scans for the selection of product ions. The Waters IntelliStart™ software was then used for the optimization of the collision energy and cone energy. Ion detection was performed in multiple reaction monitoring (MRM) mode with the highest response of precursor-product ion transition selected as quantitative ions (Table 2). The ion modes and quantitative ions of TIBA, 2-NOA, 4-CPA, 6-BAP, 1-NAD, IAA, IBA, CPPU, GA3, GA4, PBZ, butralin and uniconizole-P were consistent with previous studies [21,23,24,27–30].

Chromatogram separation is a considerable factor in instrumental analysis in which one of its major influences is the additives in the mobile phases. A mobile phase using methanol and H_2O and without additives resulted in poor separation and peak shapes for mepiquat chloride, CCC, IAA, IBA, prohydrojasmon, uniconizole-P, chlorphonium-Cl, 4-nitrophenol, 4-CPA, cloprop, 2-NOA, 2,4,5-T, GA4, GA7 and TIBA. Several of these compounds showed poor peak characteristics such as split peak or fat peak width. The addition of 1 mM ammonium acetate in the mobile phase enhanced by at least 10-fold the response for mepiquat chloride, IAA, IBA, prohydrojasmon, 1-NAD, uniconizole-P, butralin, chlorphonium-Cl and 4-nitrophenol. With the combination of 1 mM ammonium acetate and 0.1% FA added in the mobile phase, the obtained peak shapes and retention behaviors of CCC, mepiquat chloride, IBA, prohydrojasmon, 4-nitrophenol, 4-CPA, cloprop, 2-NOA, 2,4,5-T, GA3, GA4, GA7, chlorphonium-Cl and TIBA were greatly improved. The addition of 1 mM ammonium acetate and 0.1% FA resulted in an acidic environment (around a pH of 3) in the mobile phase. An acidic environment along with the addition of a buffer, improved the ionization efficiency and stabilized ions such as CCC, 4-CPA and mepiquat chloride, eventually resulting in sharper and narrower peak shapes as well as stable retention behaviors. Mobile phase with addition of 1 mM ammonium acetate and 0.1% FA resulted in acceptable retention behavior in terms of stable retention time, sharp peak shape and narrow peak width and was therefore further used as the mobile phase for PGRs analysis in this study.

3.2. *Optimization of sample extraction*

Several PGRs in this study possess carboxyl groups or a pKa value lower than 4, such as TIBA, 2,4,5-T, 2-NOA, IBA, 4-CPA, cloprop, GA3, GA4, GA7 and 1-NAD [29,31,32]. The extraction results of these PGRs may be affected by the condition of the extraction buffers and cleanup procedures due to its carboxyl group [33]. To obtain the optimal extraction conditions for the 24 compounds, extraction buffers of acetonitrile, methanol, 1% FA in acetonitrile and 1% FA in methanol/acetonitrile (1:4) each followed by a clean-up procedure using 150 mg of PSA and 900 mg of magnesium sulfate anhydrous were examined for the recoveries of the 24 PGRs (Fig. 2).

Acetonitrile used as an extraction buffer resulted in poor recovery (<10%) of over half of the PGRs, namely GA3, GA4, GA7, IAA, IBA, 2-NOA and 4-CPA. In particular, it resulted in

Table 2 – Optimized MS/MS parameters for the 24 PGRs.

Compound name	Molecular formula	Ion mode	RT (min)	MRM transition (*m*/z)	Cone (V)	Collision (V)
2,3,5-Triiodobenzoic acid (TIBA)	$I_3C_6H_2CO_2H$	Negative	7.23	498.8 > 455[a], 498.8 > 127	16, 16	20, 20
2,4,5-T	$C_8H_5Cl_3O_3$	Negative	6.59	255.1 > 196.9[a], 255.1 > 160.9	44, 44	14, 28
2-Naphthoxyacetic acid (2-NOA)	$C_{12}H_{10}O_3$	Negative	4.81	201 > 143.1[a], 201 > 115	26, 26	14, 34
4-Chlorophenoxyacetic acid (4-CPA)	$C_8H_7ClO_3$	Negative	4.19	184.9 > 127[a], 187 > 129	24, 20	12, 16
4-Nitrophenol	$C_6H_5NO_3$	Negative	3.36	138 > 108.1[a], 138 > 92	22, 22	14,20
6-Benzylaminopurine (6-BAP)	$C_{12}H_{11}N_5$	Negative	3.42	224 > 133[a], 224 > 106	34, 34	20, 30
Cloprop	$C_9H_9ClO_3$	Negative	5.03	199 > 127[a], 199 > 71	20, 20	10, 10
Gibberellic acid 3 (GA3)	$C_{19}H_{22}O_6$	Negative	3.08	345 > 239[a], 345 > 143	28, 28	14, 28
Gibberellic acid 4 (GA4)	$C_{19}H_{24}O_5$	Negative	6.12	331.3 > 243.2[a], 331.26 > 257.2	15, 15	20, 20
Gibberellic acid 7 (GA7)	$C_{19}H_{22}O_5$	Negative	5.74	329 > 223[a], 329 > 211	20, 20	15, 20
Inabenfide	$C_{19}H_{15}ClN_2O_2$	Negative	6.19	337 > 122[a], 337 > 231	32, 32	14, 20
Thidiazuron (TDZ)	$C_9H_8N_4OS$	Negative	4.01	219 > 100[a], 219 > 71.2	22, 20	10, 30
1-Naphthylacetamide (1-NAD)	$C_{12}H_{11}NO$	Positive	3.81	186.32 > 141.1[a], 186 > 115.1	30, 16	15, 28
N6-isopentenyladenine (2iP)	$C_{10}H_{13}N_5$	Positive	3.41	204 > 136.1[a], 204 > 148	16, 16	14, 12
3-Indolylacetic acid (IAA)	$C_{10}H_9NO_2$	Positive	3.33	176 > 130[a], 176 > 103	28, 28	23, 45
4-(3-Indolyl)-butyric acid (IBA)	$C_{12}H_{13}NO_2$	Positive	4.27	204 > 130[a], 204 > 186	30, 30	35, 22
Butralin	$C_{14}H_{21}N_3O_4$	Positive	10.94	296 > 240[a], 296 > 222	16, 16	15, 20
Chlormequat chloride (CCC)	$C_5H_{13}Cl_2N$	Positive	0.97	122.14 > 59.1[a], 124 > 65	37, 32	20, 18
Chlorphonium-Cl	$C_{19}H_{32}Cl_3P$	Positive	6.09	361.2 > 159.2[a], 361.2 > 76.0	25, 25	35, 35
Forchlorfenuron (CPPU)	$C_{12}H_{10}ClN_3O$	Positive	5.12	248 > 129[a], 248 > 93	25, 25	15, 35
Mepiquat chloride	$C_7H_{16}ClN$	Positive	0.96	114.01 > 98.2[a], 114.01 > 58.4	20, 20	22, 22
Paclobutrazol (PBZ)	$C_{15}H_{20}ClN_3O$	Positive	6.78	294.1 > 70.2[a], 294.1 > 125.1	27, 27	38, 20
Prohydrojasmon	$C_{15}H_{26}O_3$	Positive	9.62	255.43 > 135.2[a], 255 > 194.5	27, 26	10, 8
Uniconizole-P	$C_{15}H_{18}ClN_3O$	Positive	8.09	292 > 70[a], 292 > 125	38, 38	20, 22

[a] Quantitative ion.

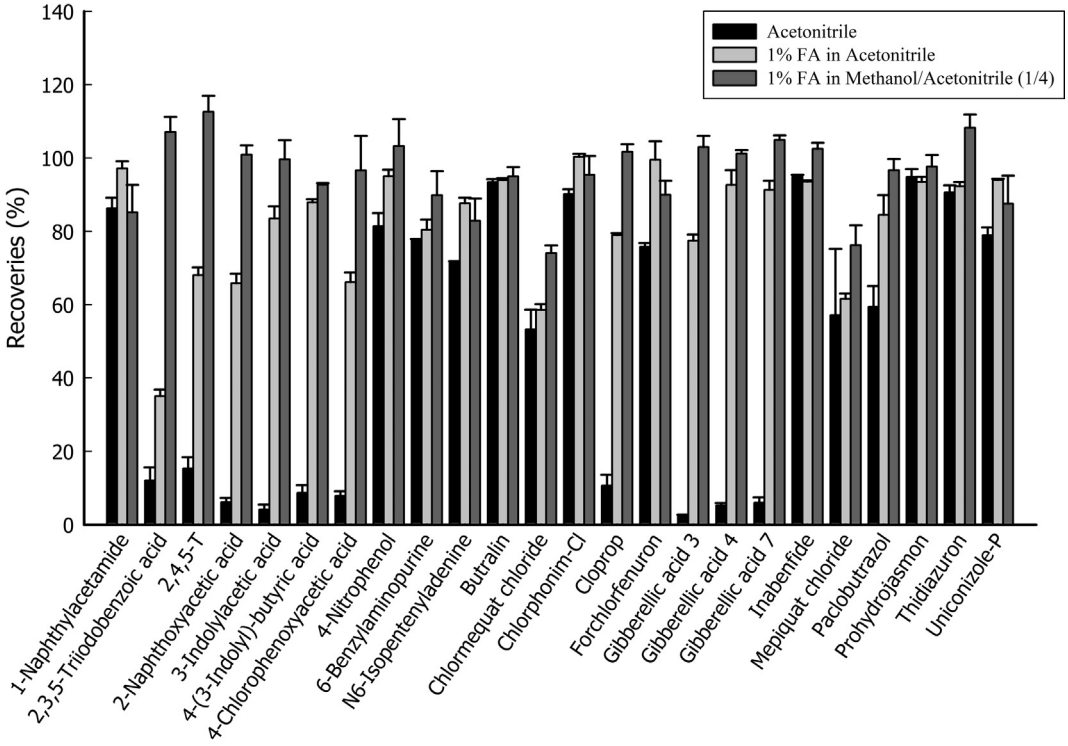

Fig. 2 – PGRs recoveries in the grape matrix obtained using different extraction buffers.

<70% recoveries for cloprop, TIBA, 2,4,5-T, CCC, mepiquat chloride and PBZ. The extraction buffer using methanol showed high miscibility with water and poor partitioning. After mixing 10 mL methanol with the homogenized grape samples, 6.5 g of salt mixtures was added and further vigorously mixed. The extracts were then centrifuged, but the result showed only single phase extract with volume of 15 mL (compared to a phase separation when using the acetonitrile as extract buffer, with the upper phase volume of around 10 mL). The presence of water in the extract resulted in difficulty of drying up with the use of nitrogen gas in the final procedure of quantifying the volume. Therefore, recovery results using the methanol extraction buffer were not obtained.

A previous study has shown that pH adjustment of the extraction buffer improved the extraction recoveries of acidic compounds [24]. In this study, the addition of 1% FA in acetonitrile resulted in the improvement of recoveries by up to 70% for most of the studied PGRs except for TIBA, CCC, mepiquat chloride, 2-NOA, 4-CPA and 2,4,5-T. The extraction buffer composed of 1% FA in methanol/acetonitrile (1:4) resulted in improved recoveries in the range of 70–120% for all 24 PGRs. The composite solvent mixture (methanol/acetonitrile, 1:4) along with the addition of acids (1% FA) led to a pH of 3 (tested with Whatman pH indicator paper), which may have resulted in an appropriate extraction conditions with regards to polarities. The pH value may also have stabilized acidic compounds such as IAA, IBA, 4-CPA, cloprop, GA3, GA4 and GA7, thereby preventing absorption of these compounds by the clean-up compounds, and resulting in the recovery improvement for all 24 PGRs. Thus, the extraction method using 1% FA in methanol/acetonitrile (1:4) as an extraction buffer followed by cleanup using 150 mg of PSA and 900 mg of magnesium sulfate anhydrous was adopted for further analysis.

3.3. *Method validation*

The validation of the extraction method was done by determining the matrix effect (ME), calibration, limit of quantitation (LOQ), trueness and precision. Chromatogram patterns of the 24 PGRs spiked into the grape matrices are shown in Fig. 3. The ME of the 24 PGRs in the grape matrix was evaluated through 5 repetitions of a matrix matched standard at the concentration of 5 ng/mL for 1-NAD, 2iP, chlorphonium-Cl, CPPU, PBZ and, uniconizole-P and 50 ng/mL for the other standards. The ME (%) was calculated as follows: ME = (area of standard spiked in grape matrix - area of grape matrix)/(area of standard in solvent) × 100 [23]. A distinct ME was observed in compounds at an early retention time (RT), whereas a lesser ME was observed after an RT of 4 min (Table 3). Ion suppression was observed in CCC (27.3%), 6-BAP (39.0%), 2iP (45.4%), mepiquat chloride (52.8%), 4-nitrophenol (63.3%) and IAA (71.8%), whereas ion enhancement was observed for 4-CPA (110.4%) and GA3 (138.3%). Previous studies have found that compound polarity has a significant influence on ME [34]; this was also observed for the compounds of the present study. Taking into account of the observed ME, we therefore used matrix-matched calibration for qualitative and quantitative analysis in the validation and analysis of the 24 PGRs.

The matrix-matched calibration curve for 1-NAD, 2iP, chlorphonium-Cl, CPPU, PBZ and uniconizole-P covers 0.1–50 ng/mL, whereas that of other compounds ranged from 1 to 500 ng/mL. All determination coefficients (r^2) for the

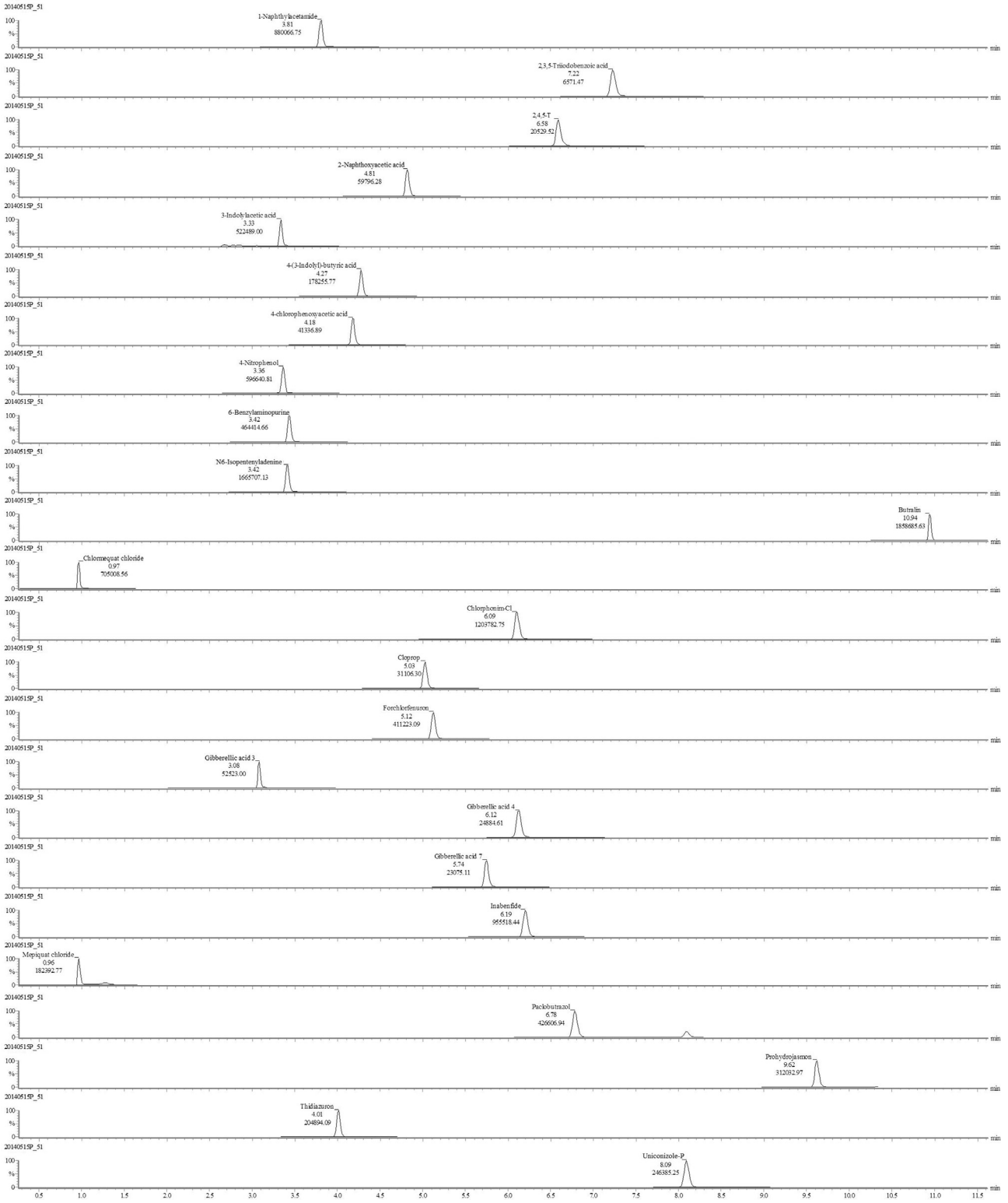

Fig. 3 – MRM chromatogram of the grape matrix that has been spiked with the 24 PGRs. Standards of 1-NAD, 2iP, chlorphonium-Cl, CPPU, PBZ and uniconizole-P were spiked at 5 ng/mL, while other standards were spiked at concentrations of 50 ng/mL. The chemical compound names, retention times and peak areas are shown beside each chromatogram peak (from top to bottom).

Table 3 – ME, determination coefficient, and LOQ for the 24 PGRs.

Plant growth regulators	ME (% ± RSD%) (n = 5)	Matrix matched linear range (ng/mL)	Determination coefficient (r^2)	LOQ (ng/mL)
1-Naphthylacetamide	82.4 ± 2.7	0.1–50	0.9967	0.1
2,3,5-Triiodobenzoic acid	104.1 ± 4.6	2–500	0.9997	2
2,4,5-T	108.4 ± 4.0	2–500	0.9997	2
2-Naphthoxyacetic acid	107.8 ± 2.7	1–500	0.9999	1
3-Indolylacetic acid	71.8 ± 2.3	1–500	0.9986	1
4-(3-Indolyl)-butyric acid	100.3 ± 2.7	1–500	1.000	1
4-Chlorophenoxyacetic acid	110.5 ± 2.4	1–500	0.9998	1
4-Nitrophenol	63.3 ± 3.8	5–500	1.000	5
6-Benzylaminopurine	39.0 ± 2.6	1–500	0.9991	1
N6-isopentenyladenine	45.4 ± 1.9	0.1–50	1.000	0.1
Butralin	89.2 ± 3.8	1–500	0.9999	1
Chlormequat chloride	27.3 ± 7.5	1–500	1.000	1
Chlorphonium-Cl	97.6 ± 2.5	0.1–50	0.9996	0.1
Cloprop	106.1 ± 2.2	2–500	0.9999	2
Forchlorfenuron	94.9 ± 2.8	0.1–50	0.9999	0.1
Gibberellic acid	138.4 ± 3.1	1–500	0.9994	1
Gibberellic acid 4	100.3 ± 3.0	1–500	0.9996	1
Gibberellic acid 7	101.4 ± 2.1	2–500	0.9999	2
Inabenfide	99.5 ± 2.0	1–500	0.9985	1
Mepiquat chloride	52.8 ± 12.4	1–500	0.9997	1
Paclobutrazol	93.3 ± 2.4	0.1–50	0.9999	0.1
Prohydrojasmon	90.5 ± 3.9	1–500	0.9997	1
Thidiazuron	97.4 ± 2.3	1–500	0.9987	1
Uniconizole-P	91.5 ± 3.3	0.1–50	0.9999	0.1

calibration curves were higher than 0.995. The LOQ values were determined at the lowest concentration that yielded the response with signal to noise ratio higher than 10 (Table 3). Trueness and precision were evaluated by recovery studies in three repetitions of recovery studies at intra-day periods over three consecutive days. Three concentrations were spiked with the PGRs in each study to determine the recoveries at low, medium and high concentrations. Spiking with 2iP and CPPU was done at concentrations of 0.01, 0.02 and 0.04 μg/mL, 1-NAD, chlorphonim-Cl, PBZ and uniconizole-P were spiked at concentrations of 0.01, 0.02 and 0.05 μg/mL; and other standards were spiked at concentrations of 0.02, 0.05 and 0.1 μg/mL. Intra-day recoveries of low, medium and high concentrations of the 24 PGRs were 70.2–107.2%, 74.1–112.6% and 76.5–109.0%, respectively. Inter-day recoveries of low, medium and high concentrations of the 24 PGRs were 67.5–98.9%, 71.2–100.5% and 70.4–101.8%, respectively (Table 4). Recovery results of the 24 PGRs were all acceptable, with relative standard deviations (RSDs) lower than 20%.

To validate whether the extraction method is consistent in different grape varieties, six grape samples of different varieties/sources were used to perform additional recovery studies and matrix effect tests (Tables 5 and 6). The six grape samples represents grapes of different purpose use, variety, and production region, which includes two wine grapes from Taiwan (Golden Muscat and Black Queen, which has yellow-green color and purple-black color, respectively), one table grape from Taiwan (Kyoho grape, which has purple-black color) and three seedless table grapes imported from the US (green, red, and black color grape, respectively). The retention time of the matrix-matched standards of 24 PGRs in these different grape varieties stayed consistent with the retention time in Table 2. Matrix effect test results were also comparable with the previous result in Table 3, where there was more matrix effect in early retention times compared with later retention time. Ion suppression was seen in CCC, 6-BAP, 2iP, mepiquate chloride, 4-nitrophenol, and ion enhancement was observed for GA3. The ion enhancement of 4-CPA was only seen more obvious in Black Queen and black seedless grapes. Recovery studies of the six grape varieties were done at low and high concentration and the recoveries were 60.6–120.7% and 67.6–128.7%, respectively. Relative standard deviations (RSDs) were lower than 20%. Most of the PGRs resulted in recoveries with a range of 70–120% and RSD lower than 20%, meeting with SANTE/11945/2015 [35]. Recoveries of IBA, 4-nitrophenol, 6-BA, CCC, GA7, and mepiquat chloride in some of the grape varieties resulted in lower recovery (60.6–70%) or higher recovery (120–128.7%), but the precision were all satisfying (RSD lower than 20%). The recovery study of the different grape varieties shows that the extraction method is generally suitable for various varieties and production region of grapes.

3.4. *Application of analysis of PGR residues in grapes*

Previous studies on PGR usage have shown that IBA, 2-NOA, 1-NAD, 4-CPA, 2,4,5-T, GA, CCC and atonik may be commonly used during grape cultivation [4,6]. The multi-residue analysis of the 24 PGRs established in this study was applied to 50 grape samples collected in 2014 from Taichung City and Changhua County, which are the main grape cultivated areas in Taiwan. Analysis results of the 50 grape samples showed the detection of 1-NAD, IAA, 2iP, 4-nitrophenol, CCC and CPPU (Table 7). Since IAA and 2iP are also naturally occurring plant hormones [36–40], it was not possible to distinguishing whether the detected residues resulted from natural occurrence or PGR usage application in the present study.

Table 4 – Inter-day and intra-day recoveries of the 24 PGRs in the grape matrix.

Plant growth regulator	Spiked conc. (μg/mL)	Intra-day recovery (%, n = 3)	RSD (%)	Inter-day recovery (%, n = 3)	RSD (%)	Spiked conc. (μg/mL)	Intra-day recovery (%, n = 3)	RSD (%)	Inter-day recovery (%, n = 3)	RSD (%)	Spiked conc. (μg/mL)	Intra-day recovery (%, n = 3)	RSD (%)	Inter-day recovery (%, n = 3)	RSD (%)
1-Naphthylacetamide	0.01	87.2	3.0	91.2	4.1	0.02	85.2	8.7	82.8	11.0	0.05	84.4	1.6	84.8	2.3
2,3,5-Triiodobenzoic acid	0.02	72.3	3.3	74.2	12.7	0.05	107.1	3.8	96.6	10.2	0.1	99.1	2.5	100.6	1.8
2,4,5-T	0.02	78.2	8.8	77.0	10.0	0.05	112.6	3.9	98.4	13.4	0.1	95.8	4.2	100.8	4.7
2-Naphthoxyacetic acid	0.02	84.5	6.4	81.1	4.3	0.05	100.9	2.5	94.8	7.8	0.1	97.1	5.0	98.2	2.6
3-Indolylacetic acid	0.02	81.7	6.0	75.3	7.4	0.05	99.6	5.2	94.1	6.0	0.1	94.7	2.4	95.3	0.8
4-(3-Indolyl)-butyric acid	0.02	70.5	3.0	70.7	0.2	0.05	92.7	0.5	89.4	3.7	0.1	84.0	6.6	78.3	6.3
4-Chlorophenoxyacetic acid	0.02	85.2	4.1	81.0	8.3	0.05	96.6	9.7	90.6	13.1	0.1	93.7	2.8	95.8	2.1
4-Nitrophenol	0.02	82.6	5.8	85.7	8.0	0.05	103.2	7.2	99.6	3.5	0.1	101.4	0.9	101.0	2.6
6-Benzylaminopurine	0.02	76.5	5.5	73.5	7.8	0.05	89.9	7.2	90.3	1.6	0.1	96.8	1.2	94.8	2.8
N6-isopentenyladenine	0.01	83.5	3.3	83.7	3.2	0.02	82.9	7.4	79.7	6.0	0.04	90.7	0.7	84.1	7.0
Butralin	0.02	87.7	5.4	82.7	5.4	0.05	95.0	2.6	90.0	6.2	0.1	91.6	3.9	88.3	4.1
Chlormequat chloride	0.02	79.1	1.9	74.2	6.8	0.05	74.1	2.8	72.0	6.1	0.1	78.7	1.8	73.6	12.3
Chlorphonium-Cl	0.01	71.5	7.9	73.2	3.9	0.02	95.4	5.3	94.8	1.2	0.05	85.4	0.5	83.9	3.6
Cloprop	0.02	88.0	4.9	85.2	5.3	0.05	101.7	2.0	93.9	10.1	0.1	94.2	4.4	95.9	3.0
Forchlorfenuron	0.01	78.0	11.4	75.6	2.8	0.02	90.0	4.2	87.9	2.4	0.04	99.0	1.2	95.6	3.2
Gibberellic acid 3	0.02	93.2	8.1	90.2	5.2	0.05	103.0	3.0	99.9	5.2	0.1	99.5	0.6	97.0	2.4
Gibberellic acid 4	0.02	88.7	4.5	82.8	11.2	0.05	101.2	0.9	98.4	3.7	0.1	95.6	2.3	94.2	3.3
Gibberellic acid 7	0.02	85.4	7.2	82.4	7.3	0.05	104.9	1.2	100.5	4.7	0.1	94.4	3.5	93.5	2.1
Inabenfide	0.02	88.7	4.5	79.7	10.6	0.05	102.5	1.6	99.3	3.3	0.1	91.9	1.3	96.5	15.6
Mepiquat chloride	0.02	79.1	3.7	67.5	14.9	0.05	76.2	7.1	71.2	6.7	0.1	76.5	0.6	70.4	8.5
Paclobutrazol	0.01	70.2	15.5	76.3	7.5	0.02	96.7	3.1	91.2	6.5	0.05	92.7	3.4	94.4	3.5
Prohydrojasmon	0.02	85.3	7.3	80.3	5.8	0.05	97.7	3.2	90.9	7.8	0.1	97.3	5.1	96.9	8.4
Thidiazuron	0.02	107.2	5.7	98.9	7.2	0.05	108.3	3.3	99.3	9.7	0.1	109.0	5.8	101.8	7.5
Uniconizole-P	0.01	70.9	5.1	76.3	6.5	0.02	87.6	8.7	85.5	5.4	0.05	96.4	0.5	90.4	9.9

Table 5 – Recoveries and ME of the 24 PGRs in the grape matrix of 3 different grape varieties (Golden Muscat, Black Queen and green color seedless grape).

Plant growth regulator	ME (%)			Spiked conc. (μg/mL)	Golden Muscat		Black Queen		Green seedless grape		Spiked conc. (μg/mL)	Golden Muscat		Black Queen		Green seedless grape	
	P01	P02	P03		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
1-Naphthylacetamide	92.5	88.9	94.2	0.01	92.0	5.6	96.6	1.7	91.3	7.1	0.05	100.1	3.5	104.6	1.4	98.5	3.5
2,3,5-Triiodobenzoic acid	95.9	96.1	96.9	0.02	89.4	13.2	89.0	10.9	92.2	10.4	0.10	104.2	4.8	113.7	1.7	106.1	3.9
2,4,5-T	96.6	100.8	97.0	0.02	93.6	10.6	90.2	4.9	100.1	2.0	0.10	103.4	3.0	116.1	2.6	106.2	2.2
2-naphthoxyacetic acid	95.2	97.4	95.3	0.02	91.4	9.4	96.9	1.0	95.9	2.4	0.10	104.6	1.6	114.7	1.1	104.0	4.9
3-Indolylacetic acid	98.6	98.7	102.4	0.02	82.0	10.5	88.7	0.8	92.9	4.4	0.10	95.0	3.6	115.8	5.9	103.2	1.3
4-(3-Indolyl)-butyric acid	97.6	101.0	99.6	0.02	61.7	1.9	74.7	2.9	88.4	8.7	0.10	68.1	1.2	94.3	3.6	91.4	3.7
4-chlorophenoxyacetic acid	100.7	109.3	98.1	0.02	92.8	3.2	85.0	6.9	90.3	6.2	0.10	102.8	3.7	120.1	1.0	108.2	3.0
4-Nitrophenol	79.7	64.5	89.9	0.02	66.7	8.9	93.8	6.0	86.7	8.4	0.10	115.9	2.9	125.4	1.4	116.2	4.2
6-benzylaminopurine	69.5	48.4	84.6	0.02	88.0	8.1	86.1	0.6	86.9	4.4	0.10	103.1	2.4	105.7	1.5	101.6	1.7
6-isopentenyladenine	78.1	75.4	67.0	0.01	94.5	1.4	83.6	0.1	88.8	4.9	0.04	97.0	2.7	91.4	2.3	93.5	1.0
Butralin	95.5	93.3	96.3	0.02	87.0	6.9	91.6	0.7	89.5	1.6	0.10	103.7	3.2	111.0	0.8	104.4	3.7
Chlormequat chloride	60.6	60.1	65.6	0.02	79.3	4.2	64.0	3.9	67.2	0.5	0.10	81.5	2.3	78.2	2.5	69.6	1.4
Chlorphonim-Cl	97.8	98.2	99.3	0.01	101.7	1.0	94.2	7.4	96.3	6.9	0.05	105.2	2.5	107.0	2.0	104.1	2.1
Cloprop	97.1	102.8	100.7	0.02	90.2	4.7	100.2	3.4	104.2	6.9	0.10	98.2	2.4	116.2	1.6	103.9	3.7
Forchlorfenuron	99.3	101.2	101.0	0.01	100.4	2.4	93.9	1.7	91.7	2.3	0.04	104.9	3.5	103.6	2.7	101.8	2.9
Gibberellic acid	116.2	147.0	98.1	0.02	100.2	3.5	85.1	6.3	104.5	9.7	0.10	98.3	3.1	105.4	4.6	101.9	7.0
Gibberellic acid 4	100.6	96.7	99.0	0.02	83.3	5.1	101.7	11.2	102.4	13.8	0.10	96.7	3.4	93.8	10.2	105.1	4.5
Gibberellic acid 7	98.4	99.7	96.1	0.02	62.6	8.2	77.3	3.7	99.5	2.1	0.10	74.5	14.7	91.8	12.1	104.3	2.5
Inabenfide	95.4	91.7	97.1	0.02	88.8	9.0	93.8	1.9	100.8	3.4	0.10	105.3	2.7	110.6	1.2	107.6	2.3
Mepiquat chloride	79.9	77.3	85.3	0.02	70.1	3.5	61.8	2.1	64.2	1.4	0.10	74.5	3.6	74.4	1.9	67.6	1.4
Paclobutrazol	97.7	98.0	99.4	0.01	93.3	5.2	95.7	3.1	92.4	8.5	0.05	102.3	4.2	103.4	1.5	100.3	2.2
Prohydrojasmon	94.8	92.7	96.2	0.02	89.4	5.7	103.8	4.2	92.7	3.6	0.10	102.3	2.9	116.8	2.6	101.8	5.3
Thidiazuron	94.4	90.6	93.7	0.02	91.9	9.6	107.2	0.7	98.4	2.7	0.10	106.4	2.0	128.7	0.5	110.2	3.6
Uniconizole	98.9	101.6	99.6	0.01	92.7	5.7	93.9	2.9	92.3	8.8	0.05	101.5	3.7	102.7	1.7	99.9	2.5

Table 6 – Recoveries and ME of the 24 PGRs in the grape matrix of 3 different grape varieties (Red color seedless grapes, black color seedless and Kyoho grapes).

Plant growth regulator	ME (%)			Spiked conc. (μg/mL)	Red seedless grape		Black seedless grape		Kyoho		Spiked conc. (μg/mL)	Red seedless grape		Black seedless grape		Kyoho	
	P04	P05	P06		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
1-Naphthylacetamide	96.2	101.1	94.5	0.01	88.2	2.1	89.5	8.8	89.7	2.7	0.05	102.0	2.7	100.0	2.3	100.3	3.0
2,3,5-Triiodobenzoic acid	101.7	103.3	97.0	0.02	82.6	14.1	94.7	12.2	79.6	16.2	0.10	112.6	3.7	108.4	2.8	108.0	5.7
2,4,5-T	96.3	98.9	94.6	0.02	98.8	5.8	102.0	13.3	73.6	12.4	0.10	114.7	2.4	112.2	2.4	116.5	2.8
2-naphthoxyacetic acid	98.5	103.8	97.7	0.02	100.8	12.6	94.0	19.4	78.1	8.5	0.10	113.2	4.2	106.9	5.2	110.0	2.7
3-Indolylacetic acid	103.8	93.9	108.2	0.02	90.4	12.9	105.1	17.6	72.2	8.4	0.10	103.4	4.2	104.7	4.8	110.2	0.3
4-(3-Indolyl)-butyric acid	101.7	106.8	102.3	0.02	69.9	12.8	95.3	13.5	64.2	9.7	0.10	84.5	4.8	99.3	2.3	96.9	1.9
4-chlorophenoxyacetic acid	102.9	112.2	103.0	0.02	103.8	9.1	99.2	18.9	75.0	15.6	0.10	111.3	2.9	111.9	2.6	117.7	1.3
4-Nitrophenol	97.3	54.0	75.4	0.02	62.2	16.3	89.3	2.8	63.8	8.7	0.10	122.5	2.2	115.9	12.3	120.2	1.2
6-benzylaminopurine	83.0	37.7	62.0	0.02	92.3	13.1	97.2	19.7	68.4	9.3	0.10	105.0	3.2	100.2	7.6	107.3	1.0
6-isopentenyladenine	82.4	53.7	49.5	0.01	82.0	9.2	80.8	3.6	82.9	3.5	0.04	91.6	3.8	85.2	5.5	90.7	2.3
Butralin	99.6	104.3	99.6	0.02	93.5	12.8	94.7	19.8	73.6	7.9	0.10	115.7	4.2	105.6	4.6	110.3	0.7
Chlormequat chloride	58.8	57.6	52.8	0.02	69.8	9.4	66.0	18.9	65.7	16.5	0.10	72.4	4.6	76.8	8.5	82.2	6.2
Chlorphonim-Cl	99.9	104.0	98.5	0.01	94.2	3.2	88.5	2.2	92.6	1.7	0.05	106.2	2.3	106.1	4.9	104.5	0.9
Cloprop	96.1	102.8	95.6	0.02	100.0	11.0	102.5	17.3	77.4	10.4	0.10	111.9	2.7	106.4	3.7	109.4	2.2
Forchlorfenuron	100.1	105.1	98.2	0.01	90.1	7.2	85.8	4.6	88.7	3.0	0.04	100.9	4.1	96.8	2.1	96.9	1.2
Gibberellic acid	102.7	151.6	153.1	0.02	120.7	3.8	94.4	18.4	75.3	3.9	0.10	119.6	5.2	110.5	6.2	107.2	2.1
Gibberellic acid 4	99.0	106.9	96.8	0.02	102.1	14.9	104.4	12.5	73.1	11.1	0.10	119.5	1.5	110.7	2.5	114.5	0.1
Gibberellic acid 7	98.6	102.6	97.2	0.02	101.2	13.0	96.3	18.3	74.6	5.7	0.10	115.6	4.9	109.5	3.0	112.0	3.0
Inabenfide	98.1	101.5	96.1	0.02	105.6	12.0	96.1	18.7	76.8	10.3	0.10	116.5	3.1	109.6	4.1	109.3	0.6
Mepiquat chloride	80.0	78.2	80.2	0.02	65.4	9.8	65.1	19.3	60.6	7.7	0.10	72.4	0.8	73.9	6.7	72.7	1.2
Paclobutrazol	100.3	104.3	98.1	0.01	87.9	3.1	87.7	8.5	85.7	2.8	0.05	102.6	3.7	101.5	2.7	95.6	2.3
Prohydrojasmon	97.3	101.9	98.1	0.02	99.5	12.5	96.1	15.0	73.7	2.1	0.10	109.9	5.1	104.1	4.7	110.6	1.2
Thidiazuron	99.1	101.0	94.8	0.02	98.1	15.9	93.6	19.7	83.7	8.1	0.10	113.4	4.5	113.2	3.4	118.0	1.8
Uniconizole	99.9	104.1	98.4	0.01	89.2	1.5	88.3	9.2	87.2	2.9	0.05	103.2	2.7	102.4	3.6	99.0	1.9

Table 7 – Residues of the 24 plant growth regulators in the 50 grape samples.

Compound	Residues (mg/kg)	MRLs in grapes (mg/kg)
1-Naphthylacetamide	0.0003 (n = 1)	EU (0.06[a])
2,3,5-Triiodobenzoic acid	-	–
2,4,5-T	-	EU (0.05[a])
2-Naphthoxyacetic acid	-	EU (0.01[b])
3-Indolylacetic acid	0.0010–0.1417 (n = 6)	EU (0.1[a]), US (exempted)
4-(3-Indolyl)-butyric acid	-	Taiwan (exempted), EU (0.1[a]), US (exempted)
4-Chlorophenoxyacetic acid	-	EU (0.01[b])
4-Nitrophenol	0.0016–0.0583 (n = 31)	EU (0.03[a]), Taiwan (exempted), US (exempted)
6-Benzylaminopurine	-	EU (0.01[b]), Japan (0.02), US (exempted)
N6-isopentenyladenine	0.0001–0.0188 (n = 39)	Taiwan (exempted), US (exempted)
Butralin	-	EU (0.01[a]), Taiwan (0.01[a])
Chlormequat chloride	0.0019–0.8470 (n = 44)	EU (0.05[a]), Japan (1), Korea (1)
Chlorphonium-Cl	-	EU (0.01[b])
Cloprop	-	–
Forchlorfenuron	0.0017 (n = 1)	EU (0.01[a]), Japan (0.1), Korea (0.05), US (0.03)
Gibberellic acid 3	-	EU (exempted), Japan (0.2), Taiwan (5), US (exempted)
Gibberellic acid 4	-	EU (exempted), US (exempted)
Gibberellic acid 7	-	EU (exempted), US (exempted)
Inabenfide	-	–
Mepiquat chloride	-	EU (0.02[a]), US (1), Japan (2), Korea (0.5)
Paclobutrazol	-	EU (0.05), Taiwan (0.5)
Prohydrojasmon	-	EU (0.01[b]), Japan (0.01), US (exempted)
Thidiazuron	-	EU (0.01[b]), Korea (0.2)
Uniconizole-P	-	EU (0.01[b])

"-"; Below LOQ; "–": currently there are no MRLs set in grapes in Codex, the EU, Japan, Korea, Taiwan and the US.
[a] limit of determination.
[b] default MRL value set by the EU.

Residues of 1-NAD and CPPU were detected each in one of the 50 grape samples, with 1-NAD and CPPU residue concentration of 0.0003 mg/kg and 0.0017 mg/kg, respectively. Both of the PGR residues are lower than the MRLs established in the EU, the US, Japan and Korea. Currently, the MRL of 1-NAD in the EU is set at its limit of determination (0.06 mg/kg), whereas the MRLs of CPPU in grapes in the EU, US, Japan and Korea are 0.05 (limit of determination), 0.03, 0.1 and 0.05 mg/kg, respectively. The detection rate of IAA and 2iP were 12% and 78%, respectively, with the residues in range of 0.0010–0.1417 mg/kg and 0.0001–0.0188 mg/kg, respectively. IAA and 2iP are also natural occurring phytohormones in plants [36] and currently MRLs of IAA are exempted from tolerance in the US. In the EU, a limit of determination at 0.1 mg/kg for IAA in all crops was set since 2016. However, it should be noted that the European Food Safety Authority published report in the review of IAA MRLs and concluded that because enforcement laboratories can't distinguish between residues from natural occurring and IAA usage application, establishment of IAA MRLs may not be appropriate [41]. Cytokinins such as 2iP are also currently exempted from MRLs in Taiwan and the US, whereas no MRLs for 2iP are set in the EU. The residue of atonik, 4-nitrophenol, was detected in 31 samples with residues ranging from 0.0016 to 0.0583 mg/kg. Currently MRLs for atonik in Taiwan and the US are exempted whereas in the EU it is set at a limit of determination of 0.03 mg/kg. Detection rate of CCC in the 50 grape samples was at 88% (i.e. 44 samples), with residues ranging from 0.0019 to 0.8470 mg/kg. Previous studies have shown that the application of CCC to grapes can inhibit shoot growth and promote fruit setting [6]. At present, Taiwan has not set a CCC MRL in grapes, whereas the MRLs for grapes in Japan, Korean, Australia and the EU are 1.0, 1.0, 0.75 and 0.05 (limit of determination) mg/kg, respectively. In conclusion, the analytical results for the 50 grape samples show the effectiveness of the established method and the current PGR residues of grapes in the market in Taiwan.

4. Conclusions

This study presents an analytical method for the detection of 24 PGRs in grapes using LC/MS–MS. The analytical method is fast and easy, and is suitable for grapes in terms of calibration linearity, ME, LOQ, specificity, trueness and precision. The analysis of 50 samples collected from main cultivation areas in Taiwan showed that PGRs are commonly applied to grapes and should therefore be regularly monitored with consideration of residue regulations.

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