RESIDUES AND TRACE ELEMENTS

Multiresidue Analysis of Multiclass Plant Growth Regulators in Grapes by Liquid Chromatography/Tandem Mass Spectrometry

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A selective and rapid multiresidue analysis method is presented for simultaneous estimation of 12 plant growth regulators (PGRs), namely, auxins (indol-3-acetic acid, indol-3-butyric acid, and naphthyl acetic acid), cytokinins (kinetin, zeatin, and 6-benzyladenine), gibberellic acid (GA_3), abscisic acid, and synthetic compounds, namely, forchlorfenuron, paclobutrazole, isoprothiolane, and 2,4-dichlorophenoxy acetic acid (2,4-D) in bud sprouts and grape berries at the development stages of 2–3 and 6–8 mm diameters, which are the critical phases when exogenous application of PGRs may be necessary to achieve desired grape quality and yield. The sample preparation method involved extraction of plant material with acidified methanol (50%) by homogenization for 2 min at 15000 rpm. The pH of the extract was enhanced up to 6 by adding ammonium acetate, followed by homogenization and centrifugation. The supernatant extract was cleaned by SPE on an Oasis HLB cartridge (200 mg, 6 cc). The final extract was measured directly by LC/MS/MS with electrospray ionization in positive mode, except for 2,4-D, GA_3, and abscisic acid extracts, which required analysis in negative mode. Quantification by multiple reaction monitoring (MRM) was supported with full-scan mass spectrometric confirmation using "information-dependent acquisition" triggered with MRM to "enhanced product ionization" mode of the hybrid quadrupole-ion trap mass analyzer. The LOQ of the test analytes varied between 1 and 10 ng/g with associated recoveries of 80–120% and precision RSD <25% (n = 8). Significant matrix-induced signal suppression was recorded when the responses for pre- and postextraction spikes of analytes were compared; this could be resolved by using matrix-matched calibration standards. The method could successfully be applied in analyzing incurred residue samples and would, therefore, be useful in precisely deciding the necessity and dose of exogenous applications of PGRs on the basis of measured endogenous levels.

Plant growth regulators (PGRs) are hormones that alter plant growth patterns and cellular activities through the regulation of various life cycle processes, such as cell division, cell differentiation, cell elongation, dormancy, bud break, seed germination, flowering, fruit setting, ripening, etc. Every plant produces a number of PGRs, such as auxins, gibberellins, cytokinins, and abscisic acid (ABA), endogenously; their levels vary with different growth stages (1). The endogenous secretion and relative proportion of these hormones are largely responsible for regulation of various biochemical processes at the cellular level. In addition to natural products, a number of synthetic PGRs are also available and regularly used in agriculture. These include synthetic cytokinins, such as 6-benzyl aminopurine (6-BA) and forchlorfenuron (CPPU), the growth retardant paclobutrazol (PBZ), and the plant growth promoter isoprothiolane (IPT). The PGRs are applied in viticulture through foliar spray or dipping of grape bunches to optimize vine growth and quality of grape berries (2) in terms of the desired berry size, shape (e.g., elongated, round), bunch weight, etc., to comply with the requirements of various national and international quality standards. In most cases, the necessity of any exogenous application of PGRs at any specific growth stage depends on visual observations by farmers or previous experience. Such decisions could be misleading, as similar symptoms

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indicating the necessity of exogenous application of any PGR might appear due to multiple reasons, such as nutritional deficiencies or physiological disorders. To achieve the best response from PGR applications, therefore, it is necessary to determine the stage and optimum concentration of the PGR treatments, as excess application of any such chemical might cause either no effect or adverse effects on plant health. Thus, precise quantification of the endogenous level of such chemicals can justify the requirement and dosage to be applied, especially at critical growth stages like bud sprout and berry development. Furthermore, monitoring the residues of PGRs is also relevant in the context of food safety. Considering the exogenous treatment of gibberellic acid (GA_3) and PBZ in viticulture, regulatory bodies like the European Commission have fixed their maximum residue limit (MRL) in grapes at 5 and 0.05 mg/kg (3), respectively, which signifies the requirement of their residue monitoring in mature fruits.

Multiresidue monitoring of PGRs is challenging because of the diversity in their chemical properties. Due to thermal instability, these compounds are not amenable to GC analysis without derivatization. In addition, their analysis by HPLC suffers from masking of the target HPLC-UV signals by matrix coextractives, and moderate to high aqueous solubility restricts their multiresidue recovery by phase separation with organic solvents, which also renders the sample cleanup difficult because of variable affinity of different PGRs toward different cleanup agents/adsorbents. Lu et al. (4) applied the electrospray ionization ion (ESI) trap MS in estimating four auxins in Chinese cabbage. Hou et al. (5) and Kelen et al. (6) reported simultaneous analysis of GA_3, indol-3-acetic acid (IAA), and ABA with SPE followed by estimation by LC/MS/MS. Chiwocha et al. (7) reported simultaneous analysis of several auxins, gibberellins, and their metabolites in lettuce by LC/MS/MS. Forcat et al. (8) reported an efficient method for rapid quantitative determination of the abundance of three acidic plant hormones from a single crude methanol extract directly by LC/MS/MS. All these reported methods essentially establish the suitability of LC/MS/MS as the best choice that offers high selectivity and sensitivity in trace-level residue analysis in complex matrixes. However, all of the above methods mainly targeted multiresidue analysis of compounds of similar chemical nature, and none attempted to combine all the classes of compounds having relevance in viticulture, which is otherwise essential in deciding the requirement and dose of any exogenous PGR application in viticulture. Unnecessary or excess application of PGRs could adversely affect the quality of grape berries, and create food safety concerns. Additionally, precise applications of PGRs based on measured endogenous levels can substantially reduce the cost of cultivation.

MS has become a powerful technique for quantitative profiling of plant hormones at trace level (9), and by applying advanced strategic techniques, such as hybrid triple quadrupole linear ion trap (10), it is now possible to quantify a target molecule by multiple reaction monitoring (MRM) with simultaneous nontarget screening of chemicals based on library-based matching confirmations. This is possible through a combined approach, incorporating the conventional MRM scan and the MRM-triggered information-dependent acquisition (IDA) within the same scan cycle during an LC/MS run. We envisioned the usefulness of a multiclass, multiresidue analysis method to accommodate 12 frequently used PGRs (natural and synthetic) of different chemistries in a single chromatographic run. Efforts were undertaken to develop a simple and straightforward sample preparation procedure for their simultaneous estimation at different plant growth stages by using hybrid quadrupole linear ion trap LC/MS/MS that involves quantification by tandem MS and confirmation through comparison with a self-created, library-based matching of full-scan mass spectra as well as ion ratios of different MRM transitions.

Experimental

Chemicals

(**a**) *Pesticide standards*.—Certified reference standards of the test compounds, namely, auxins [indole acetic acid (IAA) and indole butyric acid (IBA)], naphthyl acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), cytokinins [kinetin (KT), zeatin (ZT), 6-benzyl aminopurine (6-BA)], gibberellins (GA_3), ABA, and synthetic plant growth regulators [forchlorfenuron or 1-(2-chloro-4-pyridyl)-3-phenylurea (CPPU), paclobutrazole (PBZ), and isoprothiolane (IPT)] were of >98% purity and purchased from Sigma Aldrich (Mumbai, India) and Dr. Ehrenstorfer GmbH (Augsburg, Germany).

(**b**) *Solvents*.—Other chemicals, such as gradient-grade acetonitrile, methanol, ammonium acetate, ammonium formate, hydrochloric acid, and formic acid, were purchased from Merck India Ltd (Mumbai, India). Sodium acetate, sodium sulfate, and magnesium sulfate anhydrous were purchased from Sisco Research Laboratory (Mumbai, India). The HPLC-grade water (≤18 ΩMcm) was obtained through a Sartorius (Göttingen, Germany) water purification system.

Apparatus

(**a**) *LC/MS/MS*.—An API 4000 Qtrap MS (AB Sciex, Concord, Ontario, Canada) hyphenated to (Agilent Technologies, Waldbronn, Germany) 1200 series HPLC was used for analysis.

(**b**) *Mixer with grinder*.—The apparatus used included

a mixer of 1 L capacity with grinder (Model GX7) manufactured by Bajaj India Ltd (Mumbai, India).

(**c**) *Other equipment*.—Precision and rough balance (Adair Dutt, Mumbai, India), homogenizer (Diax 900, Heidolph, Schwabach, Germany), vortex mixer (Geni2T, Imperials Biomedicals, Mumbai, India), ultrasonic bath (Oscar Electronics, Mumbai, India), tabletop nonrefrigerated centrifuge (Remi, Mumbai, India), microcentrifuge (Microfuge Pico, Kendro D-37520, Osterode, Germany), low-volume concentrator (TurboVap, Caliper Life Sciences, Russelsheim, Germany), vacuum manifold, SPE cartridges, and Oasis HLB 6 cc/200 mg (Waters India Ltd, Bangalore, India).

Preparation of Standard Solutions

An accurately weighed 10 mg (±0.1 mg) reference standard of each analyte was taken in a calibrated 10 mL volumetric flask (certified "A" class) and dissolved in 10 mL methanol. These were stored in dark vials in a refrigerator at 4°C. An intermediate stock standard mixture of 10 mg/L was prepared by mixing the appropriate quantities of the individual stock solutions. A working standard mixture of 1 mg/L was prepared from the above stock. Calibration standards within the range 1–50 ng/mL were prepared by serial dilution of 1 g/L working standard with methanol–water (1:1, v/v). Matrix-matched standards were prepared separately using untreated (control) grapes at respective stages of maturity.

Standardization of Sample Preparation Technique

(**a**) *Sample size*.—Samples of bud sprouts and grape berries (at development stages of 2–3 and 6–8 mm diameters) were collected from the vineyards of National Research Centre for Grapes (latitude 18.31 N, longitude 73.55 E) and preserved at –20°C before analysis. The samples were cut into small pieces before homogenization. The mature and soft berries analyzed for food safety assessment (MRL compliance) could be homogenized directly. The homogenized sample (200 g) was spiked with the test compound mixture at 25 ng/g and further homogenized to achieve uniform distribution of analytes. Finally, from the homogenized mass, 2.5, 5.0, and 10.0 g samples were drawn in 10 replicates each (n = 10) and analyzed.

(**b**) *Selection of extraction solvent*.—*Methanol extraction*: The homogenized leaf and berry samples (5.0 ± 0.1 g) were weighed accurately in a 50 mL polypropylene centrifuge tube containing 5 mL 1% HCl in water, vortexed for 1 min, followed by the addition of 0.5 g sodium acetate/ammonium acetate for pH adjustment (pH = 6). Methanol (5 mL) was used as the extraction solvent. This mixture was homogenized thoroughly for 2 min at 15 000 rpm and then centrifuged at 6000 rpm for 5 min. The supernatant was separated, passed through a 0.2 µm nylon membrane filter (Pall Life Sciences, Mumbai, India), and analyzed by LC/MS/MS without any cleanup. In a different set, the above extract was subjected to cleanup by SPE with Oasis HLB cartridges. The cartridges were preconditioned with 5 mL methanol and 5 mL water, and subsequently 5 mL extract was loaded and allowed to pass through. The cartridges were dried by passing nitrogen for 1 min and then the elution was performed with 5 mL methanol. The final extract was filtered through a 0.2 µm nylon membrane filter and analyzed by LC/MS/MS.

Acetonitrile extraction: A homogenized sample (5.0 ± 0.1 g) was mixed with 5 mL acetonitrile (+5 mL 1% formic acid) followed by the addition of 0.5 g sodium acetate/ammonium acetate and 2 g anhydrous $MgSO_4$. This mixture was vortexed for 2 min and then centrifuged at 5000 rpm for 5 min. The lower aqueous extract was injected to LC/MS/MS directly. The upper acetonitrile phase was separated and 1 mL of it was cleaned by dispersive SPE (DSPE) with a combination of 50 mg primary secondary amine (PSA) + 50 mg C18 powder and 150 mg $MgSO_4$, followed by vortexing and centrifugation at 10 000 rpm for 5 min. The supernatant was diluted by adding 1 mL water and measured by LC/MS/MS.

Acetonitrile-tertiary butyl methyl ether (TBME) extraction: A homogenized sample (5 ± 0.1 g) was extracted with 5 mL water (acidified with 1% HCl) plus 5 mL acetonitrile and 5 mL TBME, along with 0.5 g sodium acetate/ammonium acetate and 2 g anhydrous magnesium sulfate followed by vortexing for 2 min and subsequent centrifugation at 5000 rpm for 5 min. The supernatant was separated and cleaned by DSPE with PSA (50 mg) and C18 (50 mg) sorbents. The cleaned extract was evaporated to dryness and redissolved in methanol water (1:1, v/v). The final extract was centrifuged at 10 000 rpm for 5 min. The supernatant was filtered through a 0.2 µm membrane filter and measured by LC/MS/MS.

LC/MS/MS Optimization

The MRM parameters for each molecule were optimized at both positive and negative polarities on an API 4000 Qtrap mass spectrometer (AB Sciex) hyphenated to Agilent 1200 series HPLC. In MRM mode, the most prominent ion with the highest S/N was selected for quantitation and the subsequent ions were used for confirmation in the unknown sample. The chromatographic separation was carried out on an Atlantis dC_{18} (Waters India Ltd) column (150 × 2.1 mm id, 5 µm). The mobile phase was composed of A = methanol–water–formic acid (99:900:1) and B = methanol–water–formic acid (900:99:1); gradient program: 0–1 min 98% A, 1–8 min 98–5% A, 8–12 min 5% A, 12–13 min 5–98% A, and 13–20 min 98% A. The effect of 5 mM ammonium formate in place

Table 1. Instrument parameters with LOQ, slope ratio, Horwitz ratio, and correlation coefficient (R2)

Serial No.	Name of chemical	Molecular formula	ESI [±][a]	RT[b] (min)	Q1[c] (amu)	Q2[d] (amu)	DP[e] (V)	CE[f] (V)	CXP[g] (V)	Q3[h] (amu)	CE (V)	CXP (V)	LOQ (ng/g)	Slope ratio (M/S)	HorRat[i]	R^2
1	2,4-D (I)	$C_8H_6Cl_2O_3$	[M–H]-	8.02	219	161	–35	–17	–8	125	–38	–6	5.0	1.74	0.39	0.9990
2	6-BA (II)	$C_{12}H_{11}N_5$	[M+H]+	5.88	226	148	22	24	8	91	42	8	2.5	0.65	0.21	0.9988
3	ABA (III)	$C_{15}H_{20}O_4$	[M–H]-	7.27	263	153	–49	–16	–8	219	–19	12.5	5.0	2.03	0.48	0.9999
4	CPPU (II)	$C_{12}H_{10}ClN_3O$	[M+H]+	9.28	248	155	67	20	7	129	25	6.3	1.0	0.64	0.25	0.9999
5	GA3 (III)	$C_{19}H_{22}O_6$	[M–H]-	5.97	345	143	–75	–40	–7	239	–20	–15	5.0	0.30	0.25	0.9980
6	IAA (I)	$C_{10}H_9NO_2$	[M+H]+	6.77	176	130	52	23	6	103	45	5	5.0	0.59	0.15	0.9989
7	IBA (I)	$C_{12}H_{13}NO_2$	[M+H]+	8.48	204	186	30	22	10	130	35	5.7	5.0	0.40	0.43	0.9979
8	IPT (III)	$C_{12}H_{18}O_4S_2$	[M+H]+	10.30	291	189	38	19	11	189, 145	34, 49	9,6	1.0	0.78	0.15	0.9999
9	KT (II)	$C_{10}H_9N_5O$	[M+H]+	3.11	216	148	60	19	7	81	31	6	5.0	0.43	0.44	0.9991
10	NAA (I)	$C_{12}H_{10}NO$	[M+H]+	8.47	186	143	92	32	6	—	—	—	10.0	0.34	0.47	0.9927
11	PBT (III)	$C_{15}H_{20}ClN_3O$	[M+H]+	9.94	294	70	60	40	10	125, 165, 207	40, 30, 22	10, 8, 10	2.5	0.74	0.22	0.9990
12	ZT (II)	$C_{10}H_{13}N_5O$	[M+H]+	1.40	220	136	10	26	6	119	45	5	2.5	0.34	0.36	0.9967

[a] ESI = Electrospray ionization.

[b] RT = Retention time.

[c] Q1 = Precursor ion.

[d] Q2 = Product ion for quantification.

[e] DP = Declustering potential.

[f] CE = Collision energy.

[g] CXP = Collision cell exit potential.

[h] Q3 = Product ion for confirmation.

[i] HorRat = Horwitz ratio.

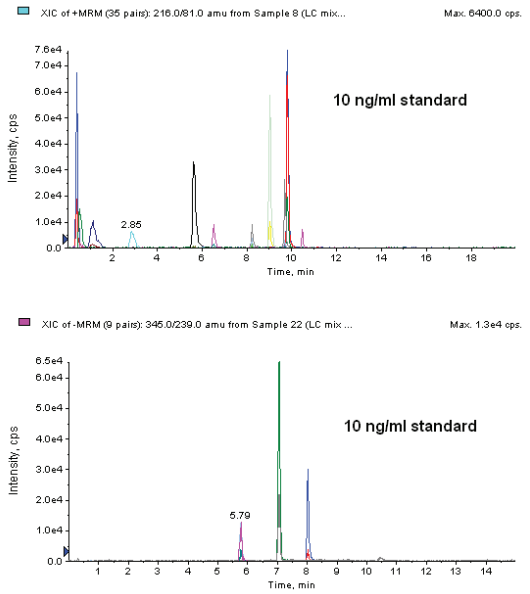

Figure 1. Multiresidue chromatogram at 10 ng/mL level at positive and negative polarities.

of formic acid was separately evaluated. The column oven temperature was set at 35°C with a flow rate of 0.4 mL/min. An aliquot of 10 µL was injected through an auto sampler. MS was performed with ESI in both positive (5500 V) and negative (4500 V) modes for each sample. The nebulizer and heater gases were adjusted at 30 and 55 psi, respectively. The ion source temperature was set at 500°C. For each ion, different voltages were applied (Table 1) to achieve the highest stable signal. ABA, GA_3, and 2,4-D were estimated in negative polarity, while the rest of the compounds were monitored in positive polarity by MRM, with a scan time of 50 ms (Figure 1). The probable structure (Figure 2) of each of the product ions was established on the basis of their fragmentation patterns at different collision energies (Table 1).

A hybrid triple quadrupole linear ion trap mass spectrometer (QqQ_{LIT}) could achieve simultaneous quantitative and qualitative analyses by integrating MRM and MRM-triggered IDA-enhanced product ion (EPI), resulting in enhanced sensitivity (11–13) at trace level. The MRM was used as survey scan to trigger an EPI scan for simultaneous quantitative and qualitative MS. In survey scans, the MRM signals exceeded a predefined threshold limit. IDA-EPI experiments were automatically triggered to obtain product ion mass spectra of these peaks. It has been demonstrated that MRM plus the MRM-trigged EPI strategy does not have any negative impact on sensitivity in quantitative analysis (14, 15). In the IDA experiment, the parameters included collision energy (CE) of 35 eV with CES (collision energy spreading) of 15 eV, scan speed of 4000 amu/s, and dynamic trap fill time as a dependent scan. Mass spectra at these CE levels were recorded and saved independently in a library. Identification of compounds in the unknown samples was based on matching the full scan spectra with the reference library spectra.

Method Validation

The analytical method was validated as per the single laboratory validation approach of Thompson et al. (16), and performance was evaluated through the following features:

Precision and Accuracy

Untreated control samples were selected for the method validation. While estimating the performance of the method for the naturally occurring PGRs through recovery experiments, the concentrations naturally present in the samples were subtracted from the test results of the spiked samples. The validation study was done at 1.0, 2.5, 5.0, and 10.0 ng/g levels with eight replicates at each level (Table 2). Similar recovery experiments were repeated on two different days to account for the inter- and intraday repeatability (17) in terms of the Horwitz ratio (HorRat). The linearity of the calibration curve was determined in the concentration range of 1–50 µg/L. The calibration curves were obtained by plotting the peak area against the concentration of the corresponding calibration standards at five calibration levels. The correlation coefficient (R^2) of calibration lines was ≥0.99 for individual compounds in solvent as well as in matrix.

LODs and LOQs

The LODs of the test compounds were determined by considering three times the average SD of the peak area of all the calibration levels in solvent (methanol–water) divided by the slope of the calibration equation. The LOQs were determined by considering 10 times the average SD of all the calibration levels divided by the slope in matrix (obtained through the optimized sample preparation method). At the LOQ level, the S/N of at least one of the confirmatory MRMs was >3:1.

Evaluation of Matrix Effect

The matrix effect in terms of signal suppression or enhancement due to coeluting matrix components was evaluated by postextraction spiking and compared with the solvent standards. Oasis HLB-cleaned extract was also used for matrix-matched calibration and compared with the solvent standard (methanol–water, 1:1) and matrix calibration without HLB cleanup. A higher and lower slope of matrix calibration equation with reference to the solvent-based calibration equation represented the matrix-induced signal enhancement and suppression, respectively. The slope ratios of matrix to solvent standards is presented in Table 1.

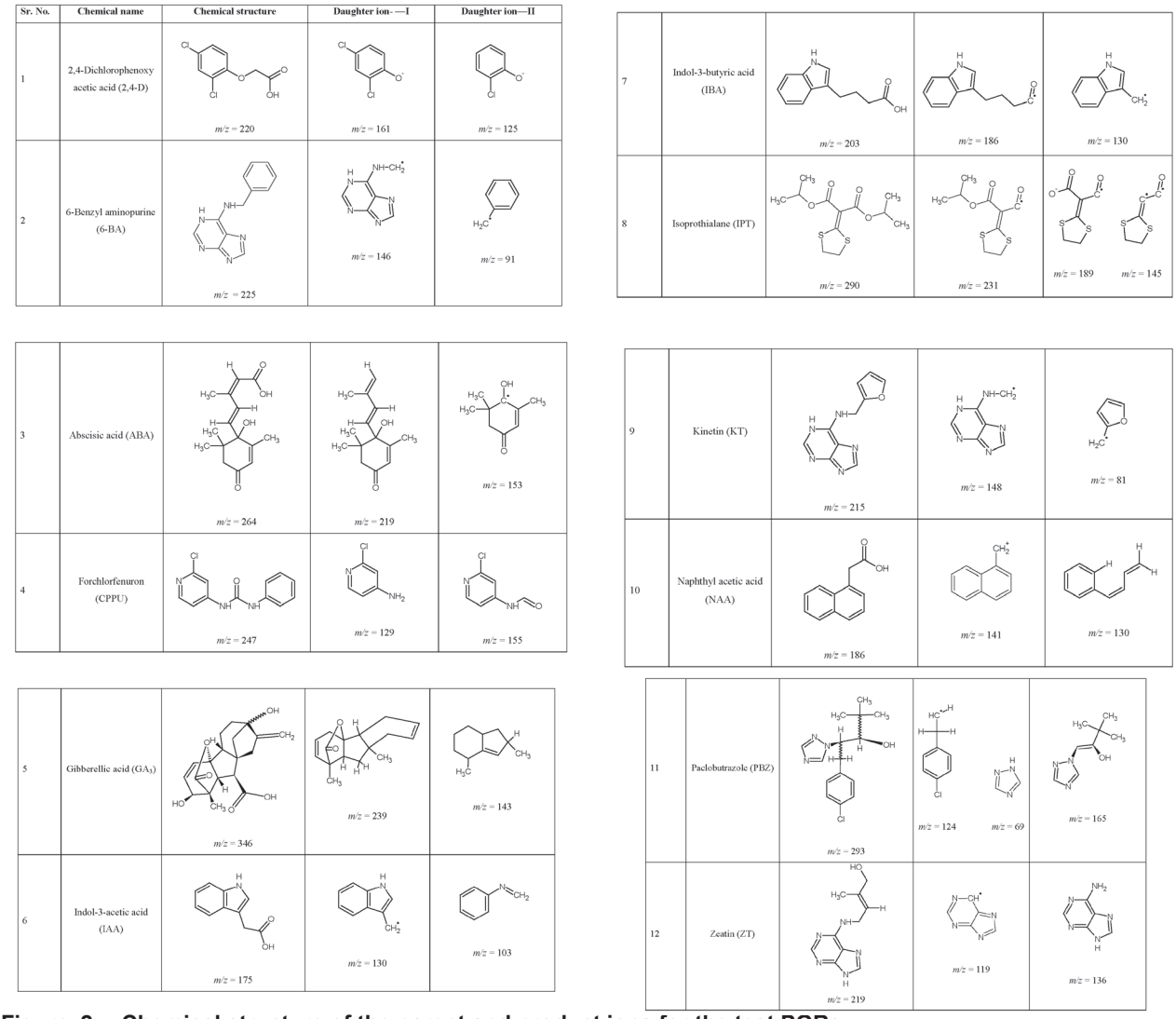

Figure 2. Chemical structure of the parent and product ions for the test PGRs.

Incurred Samples

Incurred grape samples were collected from vines that received exogenous application of CPPU, GA3, and PBZ. All incurred samples were analyzed in six replicates.

Results and Discussion

Sample Preparation

(**a**) *Sample size*.—Of the three different sample sizes (2.5, 5.0, and 10.0 g) tested, recoveries for the 5.0 g and 10.0 g samples were statistically similar (at 5% level of significance), above 80% with good repeatability, in comparison to 2.5 g, where precision was unacceptably low. Hence, a 5.0 g sample size was selected. Since the premature berries at 2–3 and 6–8 mm diameters were hard in texture, it was difficult to achieve satisfactory homogenization unless the berries were first chopped into small pieces. In all cases, the extent of homogenization was quite satisfactory, with RSD <5% for each compound when analyzed in 10 replicates over 3 different days. Replacing the high-speed homogenization step with vortexing or shaking resulted in lower precision (RSD >10%, n = 10); these were, therefore, not adopted.

(**b**) *Selecting extraction solvent*.—When acetonitrile was used as the extraction solvent, there was phase separation between aqueous and organic layers. The polar analytes, such as ZT and KT, remained in the aqueous phase to the extent of 70 and 25%, respectively. Thus, for estimation of all the test compounds, it was necessary to analyze both the organic and aqueous layers separately as two injections into the LC/MS/MS, increasing the analysis time. The addition of TBME to acetonitrile increased the extraction efficiency of highly polar compounds like ZT with >80% recovery and good

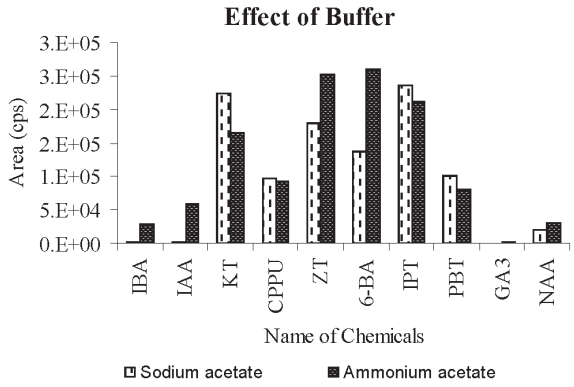

Figure 3. Comparative effects of sodium acetate and ammonium acetate.

repeatability. Conversely, in the case of extraction by methanol, there was no phase separation as methanol and water are highly miscible with each other; thus, a single injection could estimate all 12 compounds. Furthermore, due to heat generation during the mixing of methanol and acidified water, the temperature of the system increased to 35°C (±2), which increased the extraction efficiency; thus, all of the compounds showed >80% recovery. When TBME was added to methanol, the extraction efficiency for CPPU, IPT, and PBZ was increased by 10–15%, but this caused phase separation and, hence, required analysis of both phases separately. Thus, the addition of TBME was avoided, and methanol was selected as the extraction solvent.

(**c**) *Effect of modifier*.—The pH of the sample plays an important role in the extraction of PGRs. The treatment of samples with 1% HCl (5.0 mL for leaf and berry development stages, and 2.5 mL for berries) showed a better result with an increase in stability of the analytes in extract. On the addition of 0.5 g sodium acetate or ammonium acetate to the same sample, the pH shifted from 2 to 6. This treatment helped to minimize matrix-interfering peaks coeluting with polar analytes, such as IAA, IBA, and NAA, at a retention time (RT) of 1–2 min without loss of any target signals. This also increased the intensity of the signal for KT, ZT, 6-BA, and GA_3 by about 40% with better peak shapes. The addition of ammonium acetate in place of sodium acetate improved the signal intensity of ZT, IAA, IBA, 6-BA, and NAA (Figure 3). This could also avoid the formation of sodium adducts during ESI.

(**d**) *Effect of SPE cleanup*.—Cleanup on Oasis HLB cartridges reduced the color intensity of the extracts, resulted in better peak shapes, and reduced the noise level for most of the compounds, except ZT and KT. In other cases, on average there were enhancements in S/N by 10–15%, which in turn resulted in the corresponding lower LOQs.

LC/MS/MS Optimization

(**a**) *Chromatography conditions*.—Ammonium formate and formic acid were used as modifiers in the mobile phase. In comparison to 0.1% formic acid, enhancement in S/N was observed for ZT (50%), KT (50%), IAA (15%), and IPT (30%) when 5 mM ammonium formate was used. But the remaining analytes, namely, IBA, IAA, CPPU, 6-BA, and PBZ had 10–20% suppressions in signal intensity. There was RT shifting for several analytes (IAA, IBA, and NAA), which eluted around 2 min earlier; ZT eluted 2 min late when ammonium formate was used under the same chromatographic conditions as formic acid. Furthermore, some matrix interferences for IAA could not be resolved, rendering its identification difficult in ammonium formate. Therefore, 0.1% formic acid was selected to resolve the above issues. In addition, the gradient conditions were optimized to separate the target analytes from nontarget matrix compounds.

(**b**) *Selection of polarity*.—The literature suggests that compounds such as IAA, IBA, NAA, 6-BA, and KT be analyzed in negative polarity of ESI (9). But in our analysis, most of the test molecules gave greater S/N in positive mode, as compared to negative. Furthermore, in negative polarity, in many cases, only quantitative MRM could be detected, with low intensity, and the confirmatory MRM was not detectable. In comparison to negative polarity, the S/N of the compounds in positive polarity were 5, 6, 5, 8, and 4 times higher for IAA, IBA, KT, ZT, and 6-BA, respectively (Figure 4). Since the extraction was performed at an acidic pH, it favored ionization in the positive mode for all analytes except three, GA3, 2,4-D, and ABA, that were optimized in negative polarity.

(**c**) *Determination*.—All compounds were analyzed by a single chromatographic run of 20 min by using gradient chromatographic conditions. The dwell time of 50 ms was found to be optimum for all compounds. In matrix blanks, the peaks of the same mass transition were observed for

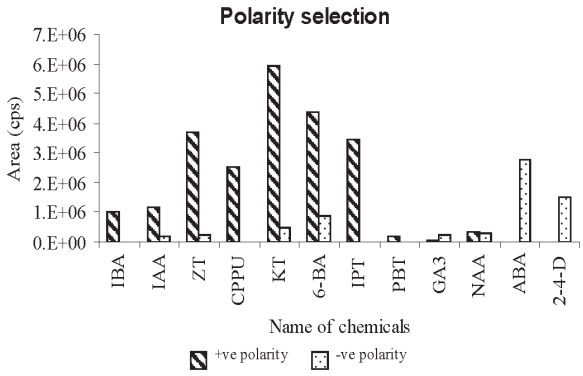

Figure 4. Selection of polarity.

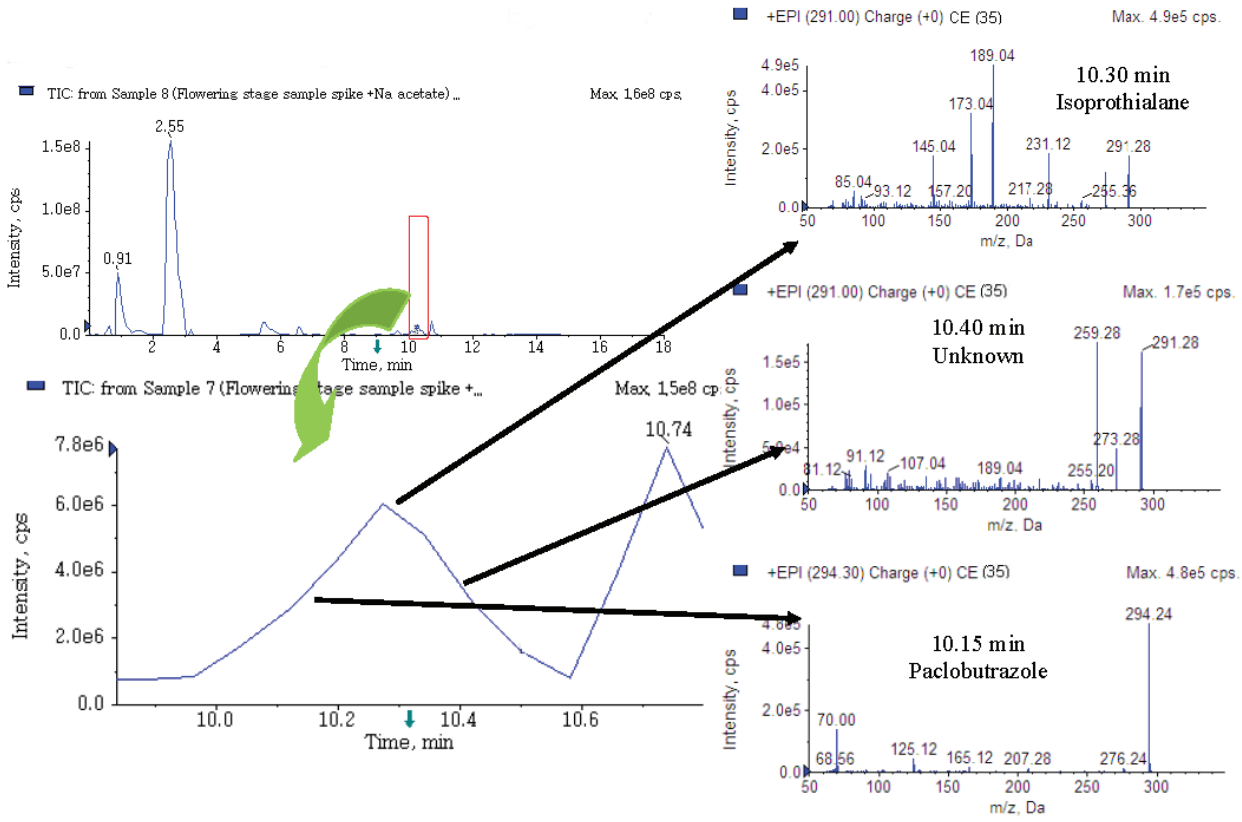

Figure 5. Full scan of the spiked flowering stage sample for the target and nontarget screening.

PBZ at 10.6 min (MRM 294>165). These peaks were separated chromatographically and were found to be of a different compound upon examination of the ion ratio of quantifier-to-qualifier MRMs.

In general, the MS/MS helped in quantitation accuracy, but at trace levels it was difficult to confirm the positive detection of some compounds (e.g., IPT and PBZ) in sample, as the confirmatory (qualifier) MRM was either not detectable or had such a low intensity that it was very difficult to calculate their ion ratio. For IPT in berry samples, two matrix peaks eluted at very close RTs, which affected its ionization (Figure 5). In this study, we used a QqQ_{LIT} system for the accurate identification and quantitation. In IDA, by using the intensity criteria 2000 cps, it was possible to scan enhanced product ion spectra of an analyte at CE 35 eV (CES = ±15 eV) and identify any compound on the basis of EPI library-based matching. In berry samples, the matrix interference peaks eluting at 4.5, 6.5, and 10.3 min matched with the MRM of IPT. These interfering compounds could be differentiated on the basis of the EPI spectra obtained at 35 eV with a CES of 15 eV. The matrix peaks matching with IPT was differentiated in MRM to IDA-EPI mode. With the help of survey scans, the spectra of the molecules were compared with the reference analyte spectra (created previously using reference standards) and could easily be differentiated (Figure 6). In addition, they were separated chromatographically by a gradient mobile phase program. On a C18 column, the elution pattern was obtained in the order of catechin, epicatechin, PBZ, and IPT. On the basis of the mass spectral matching, the above unknown peaks were identified as (A) catechin at 4.5 min, and (B) epicatechin at 6.5 min, both of which are phenolic compounds naturally present in grapes.

In the flowering or berry formation stages, IPT and PBZ peaks were of low intensity and it was very difficult to confirm their identity. By MRM triggered to EPI mode, all three compounds could be differentiated very well at RTs of 10.15, 10.30, and 10.45 min for PBZ, IPT, and the interfering matrix peak, respectively. The *m/z* values of 291, 259, and 273 for coeluting peaks were also present in IPT (Figure 5). The scheduled MRM improved the detection limits of all analytes significantly (Figure 7). The enhancement in S/N was from 2 to 40%, with >10% enhancement for IPT and NAA; 10–20% for IBA, CPPU, and PBZ; and 20–40% for IAA, KT, ZT, 6-BA, and GA_3 (Figure 7).

Method Validation

(**a**) *Recovery, repeatability, and within-laboratory*

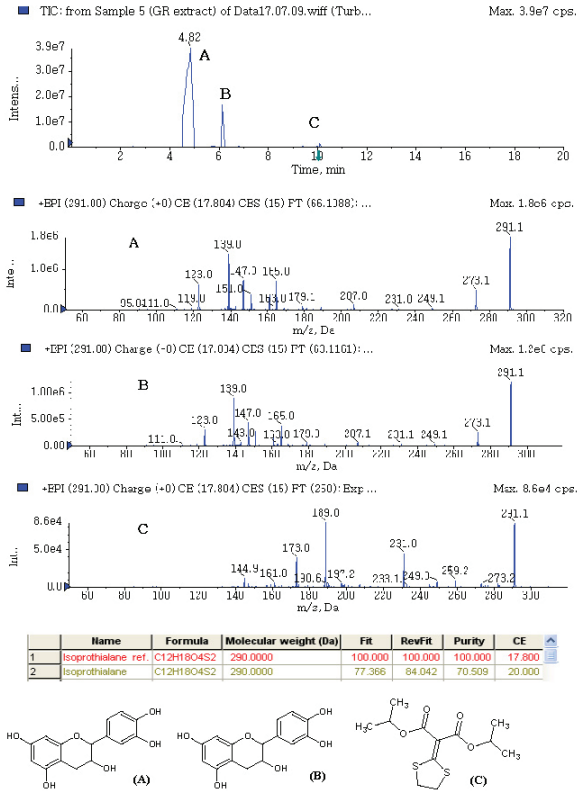

Figure 6. Full scan analysis of the spiked berry sample for the target and nontarget screening.

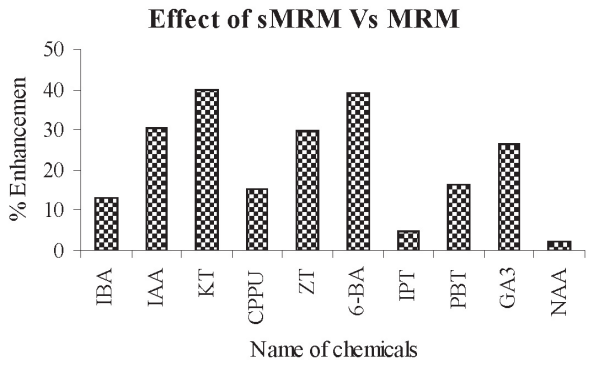

Figure 7. Comparative enhancement in S/N over the scheduled multireaction monitoring (sMRM) against multiple reaction monitoring (MRM).

reproducibility.—The recovery of all the compounds at 1.0, 2.5, 5.0, and 10.0 ng/g was within 70–120%, with RSDs varying from 8 to 35% (Table 2). The recovery experiments at the 10.0 ng/g fortification level were repeated on three different days to evaluate reproducibility and ruggedness of the method. The interday reproducibility varied between 8 and 25%, whereas the intraday repeatability was 12–30%. The intralaboratory precision in terms of HorRat (17) of the compounds ranged between 0.10 and 0.44 and, hence, was satisfactory (Table 1).

(**b**) *LOD, LOQ, and linearity*.—The test PGRs were detectable at 1–10 ng/mL (or even at lower levels), with a 50 ms dwell time. The R^2 of the calibration curve, both pure solvent-based as well as matrix-matched, was >0.99 for all compounds (Table 1). The LOQ of the analytes is presented in Table 1, and at this concentration level, the S/N of all the analytes was greater than 10 in matrix.

Table 2. Recovery data for berry, flowering stage, and small leaves (*n* = 6)

Name of chemicals	Berry (2–4 mm)				Flowering stage		Small leaf	
	1.0	2.5	5.0	10.0	5.0	2.5	5.0	2.5
2,4-D	78 (±19)	84 (±11)	93 (±4)	99 (±5)	107 (±10)	50 (±20)	92 (±15)	76 (±14)
6-BA	107 (±35)	120 (±6)	108 (±4)	116 (±11)	88 (±7)	87 (±6)	81 (±16)	89 (±20)
ABA	—	109 (±15)	117 (±12)	117 (±7)	90 (±6)	—	95 (±15)	101 (±20)
CPPU	161 (±13)	114 (±10)	110 (±11)	120 (±8)	83 (±14)	100 (±19)	80 (±14)	52 (±22)
GA3	93 (±11)	103 (±6)	80 (±4)	85 (±9)	—	—	98 (±25)	—
IAA	—	130 (±15)	104 (±12)	112 (±8)	89 (±6)	102 (±10)	106 (±10)	92 (±23)
IBA	108 (±10)	116 (±10)	105 (±7)	100 (±6)	107 (±20)	105 (±29)	103 (±10)	98 (±5)
IPT	122 (±8)	124 (±4)	109 (±4)	109 (±5)	92 (±6)	92 (±10)	86 (±7)	91 (±16)
KT	—	80 (±57)	87 (±16)	92 (±20)	90 (±8)	89 (±6)	99 (±2)	102 (±12)
NAA	—	—	—	99 (±14)	—	—	—	—
PBZ	111 (±24)	109 (±8)	97 (±7)	93 (±9)	80 (±10)	81 (±17)	97 (±20)	77 (±13)
ZT	121 (±32)	120 (±7)	95 (±6)	105 (±5)	98 (±5)	94 (±9)	115 (±9)	75 (±16)

(**c**) *Matrix effect evaluation*.—The matrix effect was prominent for most of the compounds. An overall suppression of the detector response by >80% was observed for ABA, KT, and ZT, whereas for the rest of the compounds, the matrix-induced signal suppressions ranged between 10 and 30%. The matrix effect was also evaluated by comparing the S/N in matrix-matched standards against the corresponding solvent standards. The slope of the matrix-matched calibration equation was less than the solvent standards (Table 1) for all analytes except for 2,4-D. The ratio of slope values for the matrix to solvent calibration equation was less than 1; it was 1.7 for 2,4-D, indicating matrix-induced signal enhancement for this specific analyte. Although the cleanup on the HLB cartridge improved the S/N of most of the compounds, the slopes of the calibration equations with and without HLB cleanup were statistically similar.

Incurred Samples

In the incurred grape samples, the concentrations (mg/kg) of CPPU, GA_3, and PBZ were 0.02 (±5%), 0.05 (±4%), and 0.02 (±6%), respectively. This establishes repeatability and ruggedness of the method. The concentrations were less than their European Union MRLs (3), ensuring food safety. From the same treated vines, mature grape samples were collected at harvest and screened, and were found free of any residues.

Conclusions

The final multiresidue method involved extraction of a 5 g sample with 5 mL methanol containing 1% HCl, followed by the adjustment of pH with 0.5 g ammonium acetate. The sample was homogenized for 2 min and centrifuged for 5 min. A preconditioned Oasis HLB cartridge was used for cleanup, where elution was performed with 5 mL methanol. The extract was slowly evaporated to dryness and reconstituted in methanol–water (2 mL, 1:1). The supernatant was passed through a 0.2 µm membrane filter and analyzed by LC/MS/MS. Identification and quantification of 12 PGRs were done by QqQ_{LIT} without sacrificing any sensitivity. Thus, this method could successfully estimate all 12 multiclass PGRs using a single multiresidue method. This method did not require any derivatization, which has been reported in previous literature (18, 19).

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