RESEARCH ARTICLE

The role of matrix effects on the quantification of abscisic acid and its metabolites in the leaves of *Bauhinia variegata* L. using liquid chromatography combined with tandem mass spectrometry

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ABSTRACT

Phytohormone analysis using liquid chromatography combined with tandem mass spectrometry is a very important tool for studies involving plant metabolism; however, this analysis can be affected by matrix composition. Although it is necessary to understand this effect to produce reliable results, it has been widely neglected in analyses of plant hormones. Leaf extracts from *Bauhinia variegata* were obtained by solvent extraction followed by solid-phase cleanup. The extract was analyzed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in negative ionization mode, using the multiple reaction monitoring mode with two or three transitions to enhance analytical quality control. Although deuterated standards were used as surrogates, pronounced matrix effects were detected for phaseic acid (PA), abscisic acid (ABA) -glycosyl ester (ABA-GE) and dihydrophaseic acid (DPA), whereas ABA, neoPA and 7`-hydroxy-ABA (7′OHABA) showed negligible matrix effects. The method was validated using spiked samples and was applied to monitor ABA, PA, DPA, neoPA, 7´OHABA and ABA-GE on a daily basis. Analyte recoveries from spiked samples ranged from 67% to 87%. The highest leaf concentration of phytohormones was found at 2:00 pm and 5:00 pm, which represent typical day times related to the decrease of stomatal conductance. Despite the use of deuterated phytohormones as internal standards, we showed that the use of a calibration curve constructed with a matrix extract is mandatory for the reliable quantification of ABA and its metabolites, especially PA, ABA-GE and DPA. Daily variations in endogenous ABA leaf concentration were discussed.

Keywords: ABA-β-D-glucosyl ester, ABA daily variations, dihydrophaseic acid, LC-ESI-MS/MS, phaseic acid.

INTRODUCTION

Plants exposed to daily increase in air temperature, typically peaking at midday, also experience an elevation in vapor pressure deficit (VPD) (Habermann and Rodrigues, 2009). Because stomatal conductance (gs) diminishes with increasing VPD, even well-hydrated plants of some species tend to transpire more under these conditions (Habermann et al., 2003). Plants can minimize such excessive transpiration rates (E) by reducing gs, which is regulated by abscisic acid (ABA), a plant hormone found in the apoplast of cells in the mesophyll (Assmann, 2010).

The biosynthesis of ABA occurs in chloroplasts and other plastids (Schwartz and Zeevaart, 2010). The metabolism of ABA differs among species and also

according to plant development stage and tissue type (Schwartz and Zeevaart, 2010). The catabolism of ABA is based on three reactions: oxidation, conjugation and reduction. The main oxidative route of natural ABA is 8` hydroxylation, which results in the production of 8`-hydroxyABA and isomerizes spontaneously to phaseic acid (PA). Then, it may be reduced to form dihydrophaseic acid (DPA), the main product, and lesser quantities of epidihydrophaseic acid (epiDPA) (Saika et al., 2007).

A second oxidative route involves oxidation of the 9-methyl group of ABA, resulting in a cyclic product called neophaseic acid (neoPA). The minor oxidative route results in the formation of (+)-7`-hydroxy-ABA (7`OHABA), whereas the minor reductive pathway produces the unstable 1`,4`-diol ABA. ABA and its metabolites can also be conjugated with glucose to form glycosylated esters corresponding to C1, ABA-β-D-glucosyl ester (ABA-GE) or glycosides at C-1`or C-4` (Zaharia et al., 2005).

It is widely recognized that any deficiency in ABA biosynthesis can lead to plant desiccation (Schwartz and Zeevaart, 2010; Assmann, 2010). Therefore, analytical methods that are capable of determining variations in the leaf ABA content with high sensitivity under varying conditions are of scientific interest. In many cases, satisfactory analytical results for ABA in plant tissues can be obtained using gas chromatography (Duffield and Netting, 2001; Birkemeyer et al., 2003), immunoassays or liquid chromatography (LC) as separation methods. These are always used in combination with an identification tool, typically mass spectrometry (MS). Derivatization of the compounds is a critical step that is essential for gas chromatography (GC) analyses (Pan and Wang, 2009). Because immunoassays generally display high error rates due to cross-reactivity, liquid chromatography is the preferred analytical separation method for hormonal analysis in plant tissues (Chiwocha et al., 2003; Schwartz and Zeevaart, 2010; Susawawngsup et al., 2011).

The complexity of the matrix and the small amounts of analytes present in the plant turn the study of matrix effects into an important step to enable the collection of reliable data (Thompson et al., 2002). When using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) for plant hormone analysis, co-extracted constituents in the matrix can interfere with the analysis and cause ionization enhancement or suppression (Fan et al., 2011). The type and magnitude of the matrix effect may vary for different concentration ranges (Zrostlíková et al., 2001); thus, the use of surrogates (typically deuterated phytohormones) cannot effectively correct for interference in all cases. In general, specific surrogates are added to the samples at a single concentration; therefore, the nature and magnitude of the matrix effect must be determined for each compound to be quantified.

We developed a LC-ESI-MS/MS method for the rapid determination of the quantities of ABA and its metabolites, which are present in *Bauhinia variegata* leaves sampled at different times over a 24-h period. We investigated the effects of the matrix on the analytical results to determine whether any corrective precautions were necessary to yield an accurate analysis of ABA and its metabolites. Based on the hypothesis that *Bauhinia variegata* leaves show higher levels of ABA in the afternoon compared with the night and predawn periods, we evaluated variations in the concentrations of ABA and its metabolites over the course of the day.

MATERIAL AND METHODS

Plant material: To evaluate the ability of LC-ESI-MS/MS to discriminate between ABA and its metabolites (ABA-GE, DPA, PA, 7′-OHABA and neoPA) and to detect trace quantities of these compounds, we used leaf samples from three *Bauhinia variegata* plants harvested at 2:00 am, 5:00 am, 8:00 am, 11:00 am, 2:00 pm, 5:00 pm, 8:00 pm and 11:00 pm during the dry season (August) of 2011. The plants were grown at the Universidade Estadual Paulista (UNESP), campus Rio Claro (22°25' S, 47°33' W) in Brazil. The canopy of each plant was subdivided into quadrants (North, South, East and West), and leaves were randomly harvested from each quadrant. After harvesting, the leaves were wrapped immediately in aluminum foil, frozen in liquid nitrogen and stored at -80°C until analysis.

Reagents and standards: Methanol and formic and acetic acids (all HPLC grade) were purchased from J.T.Baker (Xalostoc, Mexico). High-purity water was produced by the Millipore Milli-Q System (Billerica, MA, USA). Solid-phase extraction cartridges (Strata X, 200 mg, 6 mL) were purchased from Phenomenex (Torrance, CA, USA), and membrane filters (Nylon™, 0.22 µm porosity) were purchased from Sartorius (Goettingen, Germany).

Authentic ABA was purchased as a solid standard from Sigma-Aldrich (Oakville, Canada, 98.5% purity). The standards for DPA, ABA-GE, PA, 7′-OHABA and neoPA and the deuterated surrogates d_3-PA, d_4-7′-OHABA, d_5-ABA-GE, d_3-DPA, d_3-neoPA and d_6-ABA were purchased from the Plant Biotechnology Institute (Saskatoon, Saskatchewan, Canada) and were synthesized as necessary to at least 98% purity.

Extraction and purification: The used method was first proposed by Chiwocha et al. (2003) and modified by Tureckova et al. (2009). In our analysis, we used an adapted version of Tureckova's method. Leaves were ground using a porcelain mortar and pestle. The sample (50 mg) was extracted with 750 µL of a methanol:water:acetic acid (10:89:1) solution for 1h at 4°C. A solution containing each labeled standard (30 ng each of d_6-ABA, d_5-ABA-GE, d_3-DPA, d_3-PA, d_4-7′-OHABA and d_3-neoPA) was added as an internal standard and surrogate. The solvent was subsequently removed with a syringe, and the plant tissue was extracted again for 30 min in the same conditions as the initial extraction. Both extracts were combined [to avoid leaf particles in the extract, we filtered the solvent in a 0.45 mm x 47 mm glass microfiber filter (Sartorius AG, Germany) membrane] and loaded onto a solid phase extraction (SPE) cartridge that had been conditioned with 2 mL methanol and balanced with 2 mL of a methanol:water:acetic acid (10:89:1) solution. After the sample was loaded, the cartridge was washed with 1 mL of the methanol:water:acetic acid (10:89:1) solution and eluted using 3 mL methanol:water:acetic acid (80:19:1). The eluate was dried under a gentle nitrogen flow and reconstituted with 300 µL of methanol:water (30:70) containing 0.1% formic acid, filtered through a 0.22-µm nylon membrane filter and analyzed. The extraction was performed in duplicate, and the LC-ESI-MS/MS analyses were performed in triplicate for each extract.

LC-ESI-MS/MS analysis: Abscisic acid and its metabolites were analyzed with a liquid chromatograph (Agilent 1200 series) coupled to an ESI-MS/MS system (3200 QTrap; Applied Biosystems/MDS Sciex) using a reversed-phase column (Agilent, C18, 150 mm × 4.6 × 5 µm). The mobile phase consisted of methanol (A) and water (B), both containing 0.1% formic acid in the gradient elution mode. The A:B elution started with 50% B and increased to 80% B over 7.5 min, and then switched to 100% A over 2.5 min and remained at 100% A for 2.0 min. The re-equilibration time was 5.0 min. The flow rate was 500 µL min^{-1}, and the injection volume was 20 µL. To prevent the contamination of the MS detector, a divert valve was used, which directed the column elution flow directly to the waste before 4 min and after 12 min in each sample run. The instrument detection limit (the concentration that results in a signal:noise ratio above 1:3) was determined. The limit of quantification (LOQ) was defined as the lowest concentration in the linear response portion of the calibration curve (Eurachem, 1998).

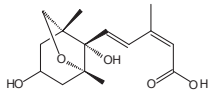
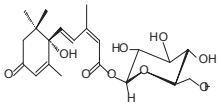
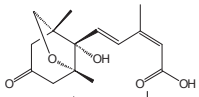
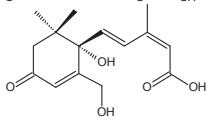
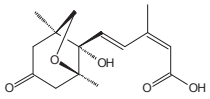
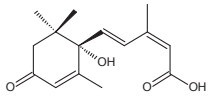
Mass spectrometry operating parameters: To select suitable diagnostic precursor-to-product ion transitions, standard solutions with 200 ng mL^{-1} of each compound in methanol:water (1:1) containing 0.1% formic acid were injected directly into a spectrometer (API 3200, Applied Biosystems/MDS Sciex) equipped with a TurboV™ ionization source operating in a negative ionization mode. All data were processed by the Analyst software (version 1.5.1). A Harvard infusion apparatus (Holliston, MA, USA) was used to optimize the MS conditions. A hybrid triple quadrupole/linear ion trap mass spectrometer with an electrospray (ESI) ion source was used.

MS parameters were optimized by a flow injection analysis (FIA) of the solutions containing DPA, ABA-GE, PA, 7′-OH-ABA, neoPA, and ABA and their respective deuterated standards. The optimized ion source parameters were as follows: curtain gas (CUR), 10 psi; ion spray voltage (IS), -4,000 V; nebulizer gas (GS1), 45 psi; turboheater gas (GS2), 45 psi; and temperature (TEM), 450 °C. CAD (collisionally activated dissociation) gas was set to medium pressure. In Table 1, we list the optimized MS parameters; the declustering potential (DP), the entrance potential (EP, Q_0 lens), the collision entrance potential (CEP), the collision energy (CE) and the cell exit potential (CXP) were optimized for each compound. N_2 (99.999% purity) was used as the collision gas. The transitions selected for the analysis are also described in Table 1.

Method performance evaluation: Method performance assessment was conducted with spiked samples. Because all plant matrices contain these compounds, a sample extract was analyzed, and the area obtained for each compound was subtracted from the corresponding areas obtained for the spiked samples. The spiking solution contained all of the compounds (natural and deuterated) at the same concentration.

Matrix effect: To evaluate the effect of the leaf compound matrix extracted with ABA and its metabolites, dry extracts were prepared containing the same concentration of standard mixture as used in the calibration curve prepared from neat reagent. The extracts were reconstituted in methanol:water (30:70) containing 0.1% formic acid. As noted by Thompson (2002), the matrix effect can influence the sensitivity of the analytical response either positively or negatively. Thus, the effect was calculated as the percentage difference between the slopes of the analytical curves that were constructed for the matrix and solvent. Using this approach, the effect can be considered as important when greater than 10%. The calibration curve in solvent was prepared using eight concentrations diluted in a methanol: water (30:70) solution with 0.1% formic acid. Data were obtained by injecting 10 µL of each solution

Table 1. Specific ion ratios or area ratios collected by MS/MS for accurate confirmation.

Retention Time (min)	Analyte	Structures	MRM transitions		Calculated Ion Ratio* MRM1/MRM2 MRM1/MRM3
4,65	DPA		MRM1	281 > 237	1.35
			MRM2	281 > 171	
4,93	ABAGE		MRM1	425 > 261	1.02
			MRM2	425 > 148	
5,60	PA		MRM1	279 > 139	4.44
			MRM2	279 > 205	
			MRM3	279 > 168	4.35
7,08	7-OH-ABA		MRM1	279 > 151	1.50
			MRM2	279 > 217	
			MRM3	279 > 205	3.60
7,51	neo-PA		MRM1	279 > 205	1.55
			MRM2	279 > 122	
			MRM3	279 > 139	3.49
8,61	ABA 1		MRM1	263 > 153	2.30
			MRM2	263 > 219	

*n=3 (spiked samples); Coefficient of variation ranged from 3 to 12%

containing 1, 2.5, 5, 10, 25, 50, 100 or 250 ng mL^{-1} of the standard mixture. The response was calculated as the ratio between the natural standard peak area and the labeled standard peak area.

Recovery study: Labeled and unlabeled standards were added at four concentrations (0.3, 1.5, 15 or 75 ng) to 50 mg of ground plant tissue at the beginning of the extraction. In parallel, another portion of the plant tissue was extracted without spiking, and its final dry extract was reconstituted with a standard solution containing the compounds at the same concentration as that used to spike the original samples. All procedures were performed in triplicate, and the extracts were also analyzed in triplicate. The recovery and the coefficient of variation were determined by comparing the peak areas of the analytical standard spiked before and after extraction.

Precision and accuracy: Precision was evaluated with the relative standard deviation determined from repeated extract injections and triplicate extractions. Accuracy was evaluated as the difference between the spiked analyte mass and the analyte mass obtained on analysis, i.e., the recovery.

To quantify ABA and its metabolites from the sample calibration curve, the calibration curve prepared using the matrix extract was used instead of the calibration curve prepared with the neat solvent when the calculated matrix effect was higher than 10% (Thompson et al., 2002). The method quantification limit was set as the lowest spiking concentration that yielded recoveries between 70–120% and a CV below 20% (Brito et al., 2003).

Data analysis: A one-way analysis of variance (ANOVA) was performed to test the significance of variations in the leaf contents of each ABA metabolite over the course of the day (at 2, 5, 8, 11, 14, 17, 20 and 23 h). For each ABA metabolite, Dunn's method (α=0.05) was used to perform *post-hoc* mean result comparisons.

RESULTS

The optimized chromatographic conditions avoided coelution of the analytes, especially PA, neoPA and 7'OHABA, which have similar structures (Figure 1). A comparison between their retention times showed that the analytes

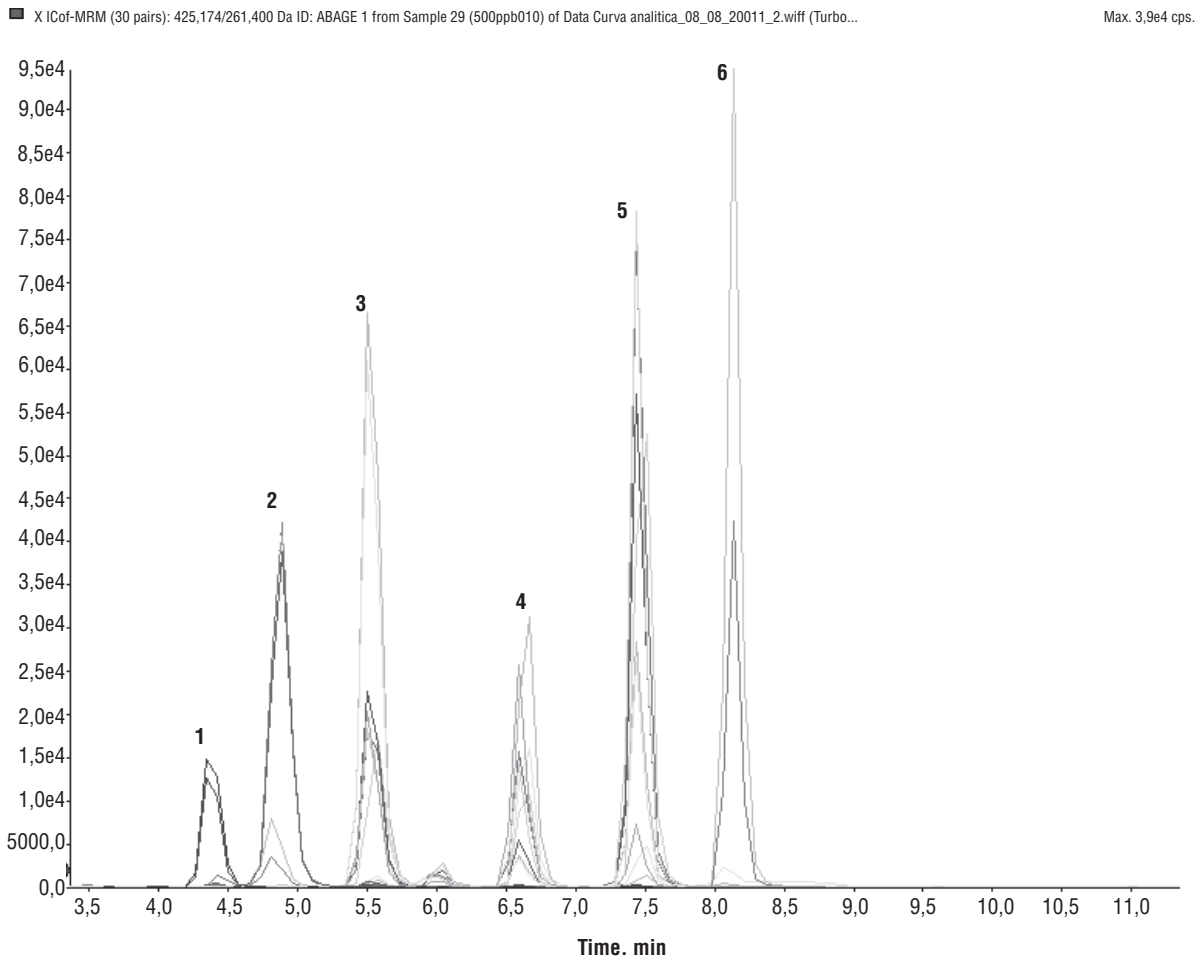


Figure 1. LC-ESI-MS/MS chromatogram for a standard solution of each compound (500 ng mL^{-1}). Different lines represent the transitions monitored for each substance. Peaks in elution order: 1, DPA; 2, ABA-GE; 3,PA; 4, 7´OH ABA; 5, neoPA; 6, ABA.

were clearly differentiated in the chromatogram. However, due to the similarities of the compounds, another form of identification, such as mass spectrometry, was necessary (Pan and Wang, 2009). Depending on the analyte, the method described here uses two or three multiple reaction monitoring (MRM) transitions, which includes additional confirmatory information to our results (Table 1).

In this study, we established ratios among transitions as the key for the reliable identification of these phytohormones. We determined these ratios using surrogate compounds in standard solutions (for all analytical curves) and using spiked samples. For the confirmation of analytes in plant samples, the chromatographic retention time of the sample plant hormones could not vary by more than 2%, and the relative abundance of the monitored MRM transitions was required to be within ± 25% of both the calibration standards and the spiked samples (European community, 2002).

As recommended by Eurachem (Eurachem, 1998), our quantification data are within the recovery limits for the given concentration zone (Table 2). An additional recovery study of a cluster spiked with 75 ng of a standard mixture, which yielded a coefficient of variation (CV) of approximately 40% (data not shown), indicated that this concentration zone was not validated.

The most significant matrix effects were observed for ABA-GE, DPA and PA, with 63%, 29% and 27%, respectively (Table 3). On inspection of these relationships, if a solvent curve had instead been used for quantification in these cases, the results would have been grossly inaccurate.

Due to the presence of natural ABA and its metabolites in plant tissues, the detection limit cannot be used as a parameter to validate this method (Eurachem, 1998). Thus, we obtained limits of quantification of 4 ng g^{-1} for ABA, neoPA and 7′-OHABA and 20 ng g^{-1} for PA, DPA and ABA-GE.

Table 2. Recovery percentage and precision data obtained with spiked samples in the recovery study.

Compound	Spiked samples[a]					
	6 ng g^{-1}		30 ng g^{-1}		300 ng g^{-1}	
	Recovery	CV	Recovery	CV	Recovery	CV
DPA	76	20	72	36	73	15
ABA-GE	68	31	74	13	75	21
PA	84	11	72	11	79	11
7′OH-ABA	75	18	67	12	75	18
neoPA	79	8	70	8	79	10
ABA	87	6	68	5	73	7

The method used for the recovery study is described in the text (Method Performance Evaluation Section, n=9). [a] ng each compound per g plant tissue (dry mass)

Table 3. Matrix effect results showing curve equations in the solvent and in the matrix extracts, differences between angular coefficients and curve intervals

	Solvent		Matrix extract		Difference in angular coefficients (%)	Concentration range (ng mL^{-1})
	R^2	Curve equation	R^2	Curve equation		
DPA	0.9988	y=1.6925x + 0.0394	0.9794	y=1.1959x + 0.2083	29	5–250
PA	0.9474	y=0.882x + 0.1074	0.9739	y=1.1231x + 0.048	27	5–250
ABA-GE	0.9985	y=1.2888x + 0.0135	0.8789	y=0.4779x + 0.4151	63	5–250
neoPA	0.9981	y=2.9893x + 0.0576	0.9968	y=2.7x + 0.0598	9.7	1–250
7′OHABA	0.9992	y=5.5002x + 0.0589	0.9993	y=5.7308x – 0.1091	4.2	1–250
ABA	0.9989	y=0.1484x + 0.0022	0.9998	y=0.1386x + 0.0007	6.6	1–250

As anticipated, the concentration of ABA in *B. variegata* leaves ranged with the time of day. The highest leaf concentrations were found at 2:00 pm and 5:00 pm, intermediate concentrations were observed at 800h, 8:00am, 8:00 pm, 11:00 pm and 2:00 am, and the lowest concentrations were observed at 5:00 am and 11:00 am (Figure 2A). There were high leaf concentrations of PA at 2:00 pm and 5:00 pm (Figure 2C), and the concentrations of neoPA, 7′-OHABA, ABA-GE and DPA showed no significant variations according to the time of day (Figure 2B, D-F).

DISCUSSION

Chromatography and mass spectrometry: One should consider that coelution of substances with similar fragmentation profiles can result in false positives. Tandem analysis with mass spectrometry is widely used to identify ABA and its metabolites, with one (Turecková et al., 2009; Zhou et al., 2003; Ross et al., 2004; Lopez-Carbonell et al., 2009) or at most two transitions (Gómez-Cadenas et al., 2002; Vilaró et al., 2006) for each compound. However, even with different retention times, the use of only one MRM transition for each analyte may also be problematic.

Phaseic acid and neo-PA, under our optimized chromatographic conditions, showed good peak resolution, but it demonstrated to have MRM transitions with identical fragmentation patterns: PA, 1st transition 279 > 139; neo-PA, 3rd transition 279 > 139; PA, 2nd transition 279 > 205; neo-PA, 1st transition 279 > 205 (Figure 3). Thus, in this case, it is necessary to use at least two transitions along with retention time for a reliable identification of these analytes.

When determining analytes (pollutants) in environmental water, it is recommended that one use retention time plus at least two transitions, with a suitable ion-intensity ratio between them, for robust compound identification; in addition, the use of as many specific transitions as possible may help avoid false positives due to common losses (e.g., H_2O or CO_2) (Pozo et al., 2006). Therefore, the same can be applied to plant tissues, because the achievement of reliable identification of compounds at low concentrations may be much more complex in plant matrices.

During the recovery study using spiked samples, we observed peaks that coeluted with the phytohormones, especially with PA at m/z 279.0, neo-PA at m/z 279.0 and 7′-OH-ABA at m/z 279.0. These peaks may have been other plant constituents with similar structures. One of the most abundant chemical classes in plant tissues is phenolics. Phenolic compounds show nonspecific losses of CO_2, CO and

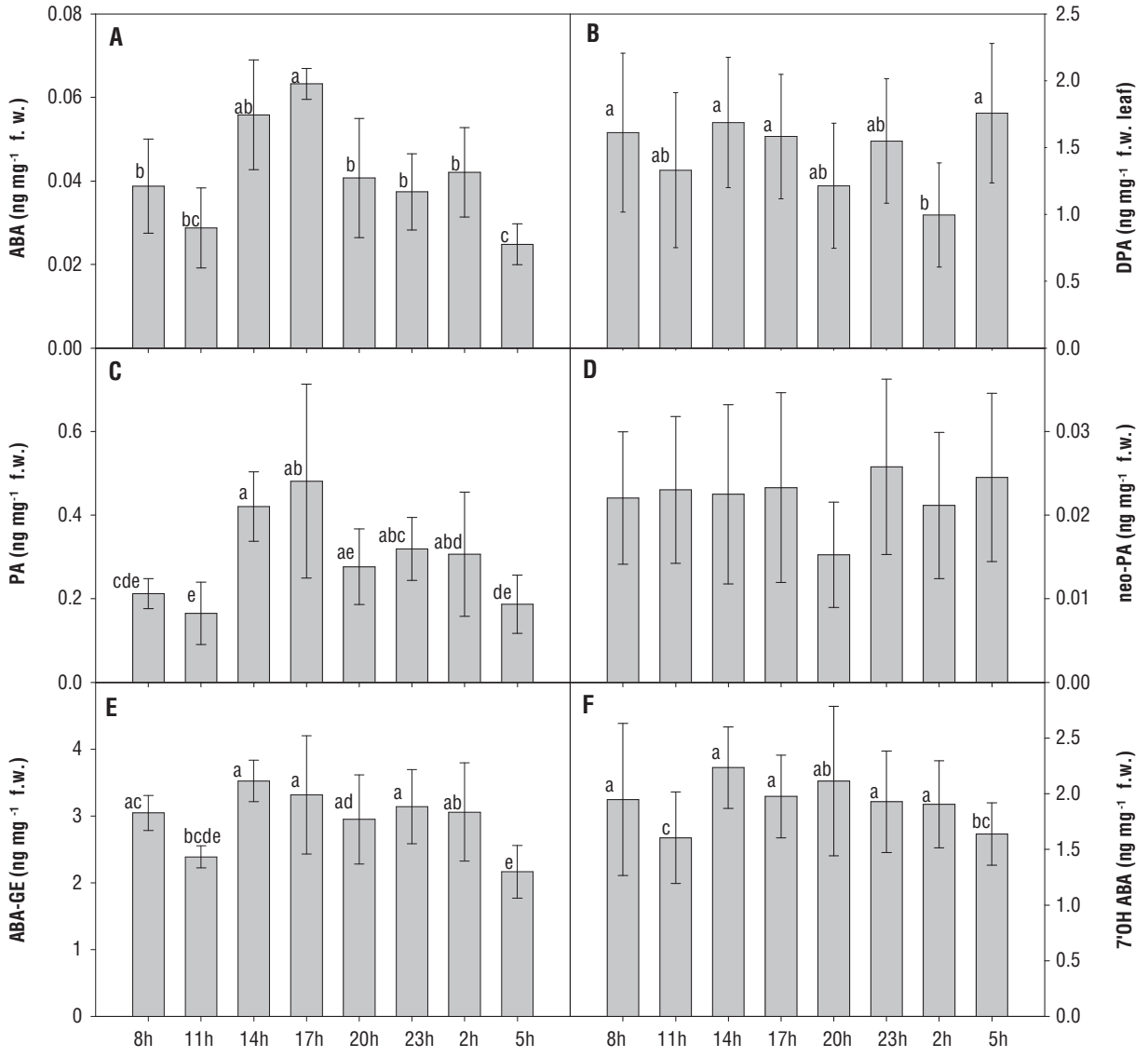


Figure 2. *Bauhinia variegata* leaf contents of ABA (A), DPA (B), PA (C), neoPA (D), ABA-GE (E) and 7′-OHABA (F) over the course of a day. Similar lowercase letters or absence of letters indicates no significant differences ($p < 0.05$) among day times. Bars=SD

H_2O that are widely used for their identification in plant extracts (Ma et al., 1997; Vessecchi et al., 2011). However, using only these losses could lead to mistaken compound identifications.

Matrix effects: Given that coeluting compounds can interfere with the ionization process, which is essential for quantification, the matrix effect is directly affected by the composition of the tissue under analysis. Therefore, it is essential to perform a similar study using additional tissues from the same species or tissues from a different species.

In contrast to our results, Fan et al. (2011) reported no matrix effects on the analysis of several phytohormones (including ABA) in canola. Their results are in accordance with our findings, because ABA showed a 6.6% matrix effect (Table 3), allowing its quantification by the solvent curve method (i.e., a matrix effect <10%). This lack of matrix effect may underlie the common practice of quantifying ABA without analyzing other compounds from the ABA metabolic route, which are of similar importance for understanding water stress.

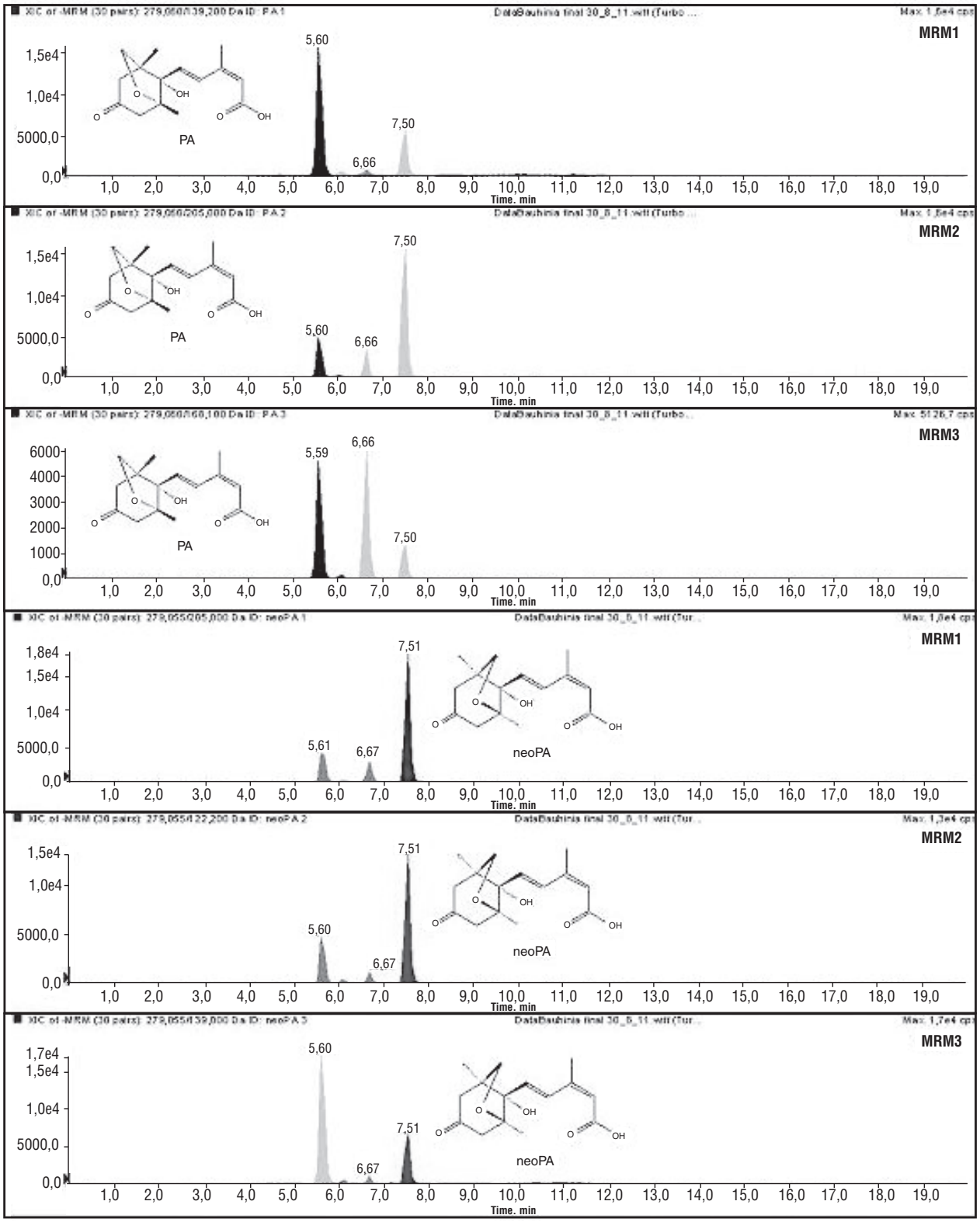


Figure 3. Chromatogram of a sample – PA (t_r=5.60 min) and neoPA (t_r=7.51 min) have identical fragmentation patterns at 279 >139 and at 279>205, which can lead to identification errors.

Considering quantification limits, our results are consistent with previously published values (Zhou et al., 2003; Turecková et al., 2009; Lopez-Carbonell et al., 2009). However, in several reports, the authors did not confirm if the reported limit of quantification referred to the analytical system or to the entire method.

The matrix effect in most cases depends on the amount of sample used, because in more concentrated extracts coextracted compounds it will be, consequently, more concentrated, and increased effects on ionization processes will also be expected, generating unreliable results. On the other hand, in the present study the relationship between the amount of sample used and consequences for matrix effects was not the main objective.

Hormone quantification and physiological implications: The abscisic acid leaf content reported over the course of the day are rare for native plants, and for crop species ABA leaf contents are described following water deficient treatments, instead of during the course of the day. On the other hand, our findings fit the physiologically expected pattern of variation in the concentration of this plant hormone throughout the day. As established in other studies, the stomatal conductance (gs) of leaves of many species diminishes when exposed to a high vapor pressure deficit, or VPD (Khairi and Hall, 1976; Levy, 1980; Sinclair and Allen, 1982; Grantz, 1990; Assmann, 2010). Citrus plants respond to elevated VPDs by lowering both transpiration rate and gs (Habermann et al., 2003). In this study, we did not measure gs, but the VPD was highest at midday and early afternoon and decreased in the evening in correlation with air temperature (data not shown). However, in maple plants ABA leaf content has been suggested as a substitute for gs (Bauerle et al., 2006).

The leaf content of PA exhibited a daytime pattern similar to that of ABA (Figure 2C). Although both exert physiological effects, PA is considered as the first compound to be degraded in a biochemical pathway that ends with the biosynthesis of the inactive DPA (Schwartz and Zeevaart, 2010). The inactive compounds (DPA, neoPA, 7′-OHABA and ABA-GE) measured in *B. variegata* leaves appeared to be maintained in high concentrations throughout the day (Figure 2B,D-F).

Because we did not measure these compounds in root samples or in xylem exudates, it was not possible to determine whether the root system contributed to the changes in concentration of these compounds. However, ABA leaf concentration measured in maple plants was described as a better indicator of gs responses to soil water deficit, in comparison with xylem collected ABA (Bauerle et al., 2006).

Although we were unable to determine the specific mechanisms underlying the fluctuations in the level of each measured compound, we performed an accurate quantitative analysis of the compounds in the abscisic acid biosynthetic pathway. Moreover, the endogenous concentrations of ABA and PA fit the physiologically expected daytime variations accurately.

In prior literature, a discussion of the matrix effect on phytohormone quantification has focused on ABA or other plant hormones exclusively (Fan et al., 2011). In our study, we included all ABA metabolites. Plant tissue is a very complex matrix, and studies of matrix effects are critical for hormone quantification. ABA does not present a significant matrix effect; however, if these analyses were not performed, we would have obtained PA values smaller than the actual ones. For ABA-GE and DPA, the concentrations obtained without accounting for the matrix effect would have been greater than the actual values. To our knowledge, no other study has analyzed daytime variation in ABA and its metabolites. We selected *Bauhinia variegata* as a subject for the profiling of ABA hormones. Although used only as a confirmation of well known ABA daily leaf concentrations, since our investigation was mainly devoted to the ABA methodological concepts, including the matrix effects, our results corroborate the fact that the highest ABA and PA leaf concentrations were found at 14:00 and 17:00 h, when VPD typically increases and stomatal conductance normally decreases, which is in accordance with classical ecophysiological response in plants.

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