

## Simultaneous Determination of Multiple Phytohormones in Plant Extracts by Liquid Chromatography–Electrospray Tandem Mass Spectrometry

ABHILASHA DURGBANSHI,<sup>†,‡</sup> VICENT ARBONA,<sup>†,‡</sup> OSCAR POZO,<sup>†</sup> OTTO MIERSCH,<sup>§</sup>  
 JUAN V. SANCHO,<sup>†</sup> AND AURELIO GÓMEZ-CADENAS<sup>\*,†</sup>

Departamento de Ciencias Experimentales, Universitat Jaume I, Campus Riu Sec,  
 E-12071 Castelló, Spain, and Department of Natural Product Biotechnology, Leibniz Institute of  
 Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany

A rapid multiresidue method to quantify three different classes of plant hormones has been developed. The reduced concentrations of these metabolites in real samples with complex matrixes require sensitive techniques for their quantification in small amounts of plant tissue. The method described combines high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Deuterium-labeled standards were added prior to sample extraction to achieve an accurate quantification of abscisic acid, indole-3-acetic acid, and jasmonic acid in a single run. A simple method of extraction and purification involving only centrifugation, a partition against diethyl ether, and filtration was developed and the analytical method validated in four different plant tissues, citrus leaves, papaya roots, barley seedlings, and barley immature embryos. This method represents a clear advantage because it extensively reduces sample preparation and total time for routine analysis of phytohormones in real plant samples.

**KEYWORDS:** Abscisic acid; barley; citrus; indole-3-acetic acid; jasmonic acid; LC–ESI-MS/MS; papaya

### INTRODUCTION

Plant hormones play a crucial role in controlling plant growth and development. These groups of naturally occurring substances influence physiological processes at low concentration. They serve as mediators of endogenous developmental programs (1, 2) and integrate extracellular signals to regulate and optimize plant growth and performance. To sum up, they control the balanced response of plants to adverse environmental conditions (3) or biological threats (4). To achieve a precise regulation of these essential processes, the biosynthetic and catabolic pathways of the different hormonal groups have to be highly responsive and adaptable to changing conditions. Comprehensive considerations on hormone biosynthesis, signaling, and control of gene expression have been presented recently (5, 6). For example, it is not surprising that abscisic acid (ABA) signaling can act on a target shared with other response pathways for, e.g., ethylene (7), jasmonates (8), and gibberellins (5).

However, the knowledge of this intimate relation among phytohormones has not avoided the fact that most of the work published until now has focused on a specific compound or, at the most, on a group of hormones but ignored the others. This

lack of studies approaching multiple plant hormone groups is due, at least in part, to the complexity of plant extracts where multiple difficulties are encountered in achieving an accurate analysis to determine components present at concentrations lower than 50 ng/g. Therefore, the classical methods for hormone determination involved several steps of intensive purification and large amounts of plant tissue (9).

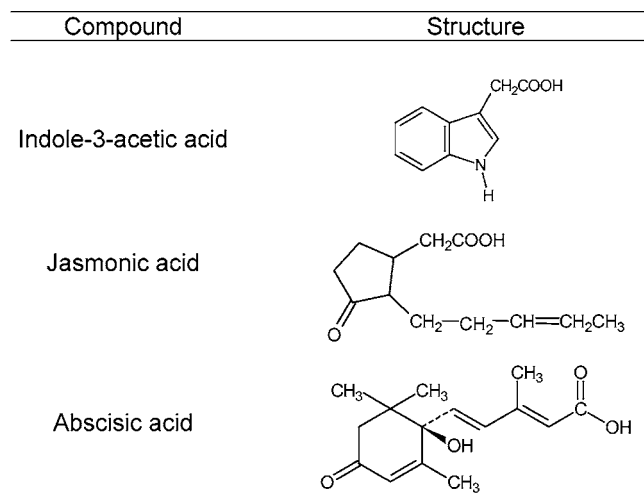
Several analytical techniques have been used in plant hormone analysis (10–14). While methods based on ultraviolet or electron capture detectors are almost abandoned, indirect methods such as enzyme-linked immunosorbent assays are still used frequently (10, 11). Recently, determination of ABA by capillary electrophoresis with laser-induced fluorescence detection has been achieved (14). Among the methods available to quantify plant hormones, mass spectrometry is the most powerful due to its high sensitivity and selectivity (15, 16). By using gas chromatography coupled to mass spectrometry, many plant hormone groups have been quantified including auxins (17), ABA (18), and jasmonic acid (JA) (19). Recently, methods for profiling several plant hormones by GC–MS/MS have been reported (16, 20, 21). Although strategies to reduce the purification steps have been developed, samples must necessarily be derivatized prior to analysis by GC–MS. Such extensive purification protocols may consume many hours of tedious work despite the possible breakdown of labile compounds (discussed in refs 13 and 20), due to the high temperatures reached in the GC injector and columns.

\* To whom correspondence should be addressed. Phone: 34-964729402. Fax: 34-964728066. E-mail: cadenas@exp.uji.es.

<sup>†</sup> Universitat Jaume I.

<sup>‡</sup> A.D. and V.A. contributed equally to this work.

<sup>§</sup> Leibniz Institute of Plant Biochemistry.



**Figure 1.** Schematic structures of the three phytohormones studied.

The application of liquid chromatography–tandem mass spectrometry (LC–MS/MS) to the analysis of plant hormones is of particular interest because it offers an opportunity to quantify several compounds simultaneously. Unobserved interference with the analysis will only occur when an impurity not only coelutes with the compound of interest but also induces a detector response at the  $m/z$  values monitored that is indistinguishable from that of the endogenous compound. Distinct procedures have been published for the quantification of several phytohormones including auxins (22), ABA and catabolites (12, 23), and jasmonates (24). Recently, a method to profile several plant hormones and their metabolites at a time have been reported (13). The electrospray ionization (ESI) process by itself is extremely gentle, optimizing the probability of obtaining molecular mass information (25), and is therefore very suitable for the analysis of phytohormones.

The main objective of the present work was to develop a sensitive and selective analytical method for the determination of three acidic plant hormones (see **Figure 1** for the structures) in a given sample and in a single experiment. In this sense, different plant materials have been used to confirm the validity of the method including citrus leaves, barley seedlings, barley immature grains, and papaya roots. In addition, the efficiency of two methods for rapid phytohormone extraction and purification are discussed.

## MATERIALS AND METHODS

**Plant Material.** Plant material was obtained from the following. (A) Clementine plants (*Citrus clementina* Hort ex Tanaka): Only mature leaves were used in the experiments described. After separation from the plant, the leaves were immediately frozen in liquid nitrogen, lyophilized, and kept at room temperature until extraction. (B) Barley seedlings: Uniform *Hordeum vulgare* L. (cv. Himalaya) seeds were germinated in vitro on sterilized vermiculite in plastic Petri dishes (14 × 14 × 2 cm). Before germination, the seeds were surface disinfected with 2% sodium hypochlorite for 20 min followed by 10 cycles of washing with sterile deionized water. The seeds were kept for germination in the dark in a versatile environmental test chamber. The temperature of the chamber was maintained at 25 °C. After being kept in the dark for 2 days, the seedlings were pricked out. The first 2 cm of the epicotyl was used for the analyses. (C) Barley immature embryos: Nonmature ears were collected from plants of the Himalaya cultivar grown in a greenhouse. The seeds were then cut, and the embryo-containing half was used for the analysis without further manipulation. (D) Papaya roots: Young roots from *Carica papaya* L. (cv. Sunrise) were harvested, washed, frozen in liquid nitrogen, lyophilized, and kept at room temperature until the analyses.

**Reagents and Standards.** Acetonitrile, acetic acid, methanol, and water were HPLC grade (purchased from Scharlab, Barcelona, Spain). Diethyl ether was from Panreac, Barcelona, Spain. Indole-3-acetic acid (IAA), (±)-2-*cis*,4-*trans*-abscisic acid (ABA), and (±)-jasmonic acid (JA) standards were from Sigma-Aldrich (Madrid, Spain). [ $^2\text{H}_2$ ]Indole acetic acid was obtained from Isotech (Sigma-Aldrich). [ $^2\text{H}_6$ ]Abscisic acid was synthesized as described in ref 12 and [ $^2\text{H}_6$ ]jasmonic acid as described in ref 28.

**Extraction and Purification Procedures.** Different amounts of tissues were weighed depending on the plant material. For barley tissues, fresh material was frozen in liquid nitrogen and ground to a fine powder, and 0.5 g was weighed. For citrus and papaya tissues, 0.25 g of previously lyophilized and powdered material was directly weighed. In all cases, before any extraction was performed, 50  $\mu\text{L}$  of a mixture of internal standards containing 5 ng of [ $^2\text{H}_2$ ]IAA, 100 ng of [ $^2\text{H}_6$ ]ABA, and 100 ng of [ $^2\text{H}_6$ ]JA was added. The tissue was immediately homogenized in 5 mL of either ultrapure water or a mixture of methanol/water (80:20). In both cases, centrifugation (5000g, 10 min) followed to pellet debris. When water was used as an extractant, the pH of the supernatant was adjusted to 2.8 with 15%  $\text{CH}_3\text{COOH}$  and the supernatant partitioned twice against an equal volume of diethyl ether. After the aqueous phase was discarded, the organic fraction was evaporated in a vacuum at room temperature and the solid residue resuspended in 1 mL of a water/methanol (90:10) solution which was filtered through a 0.22  $\mu\text{m}$  cellulose acetate filter. A 20  $\mu\text{L}$  aliquot of this solution was then directly injected into the HPLC system. When methanol/water (80:20) was used as an extractant, the supernatant was vacuum evaporated to remove methanol. The aqueous residue was then adjusted to 1 mL with water. From this point the following purification steps were identical to those described before, i.e., pH adjustment to 2.8, partitioning against diethyl ether, evaporation of the organic phase, and resuspension in water/methanol (90:10).

**Chromatographic and Mass Spectrometry Procedures.** High-performance liquid chromatography was performed using a Waters (Milford, MA) Alliance 2690 system, which consists of an autosampler and a quaternary pump. Aliquots (20  $\mu\text{L}$ ) were injected on a Nucleosil ODS reversed-phase column (100 × 2 mm i.d., 5  $\mu\text{m}$ ; Scharlab, Barcelona, Spain). Phytohormones were eluted with a gradient of methanol and 0.01%  $\text{CH}_3\text{COOH}$  in water that started from 10:90 (v/v) and linearly reached 60:40 (v/v) in 10 min. In the following 4 min, the gradient increased to 80:20 (v/v). Isocratic conditions of 80:20 were then retained during the last 2 min of the run. The initial conditions were restored and allowed to equilibrate for 5 min, giving a total time of 21 min per sample. The solvent flow rate was 0.3 mL/min with working pressures around 70–100 bar.

Using an orthogonal Z-spray electrospray interface (Micromass, Manchester, U.K.), the effluents from the HPLC were introduced into a triple-quadrupole mass spectrometer (Quattro LC, Micromass). Drying gas, as well as nebulizing gas, was nitrogen generated from pressurized air in an NG-7 nitrogen generator (Aquila, Etten-Leur, The Netherlands). The nebulizer gas flow was set to 80 L/h and the desolvation gas flow to 800–900 L/h. For operation in the tandem MS (MS/MS) mode, the collision gas was 99.995% pure argon (Carburos Metálicos, Valencia, Spain) with a pressure of  $2 \times 10^{-3}$  mbar in the collision cell. The desolvation gas temperature was 350 °C, the source temperature 120 °C, and the capillary voltage –3 kV. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The cone voltage and collision energies depended on the compound under investigation and are summarized in **Table 1**. Masslynx NT version 4.0 (Micromass) software was used to process the chromatograms.

## RESULTS AND DISCUSSION

The complexity of any plant matrix makes phytohormone analyses particularly difficult. An accurate quantification of trace amounts of these compounds requires robust methods. By applying the selectivity of reversed-phase chromatography coupled to tandem mass spectrometry, we have assessed the concentration of ABA, IAA, and JA in different plant tissues despite a plethora of coeluting components.

**Table 1.** Optimized MS/MS Conditions for the Analysis of Selected Phytohormones

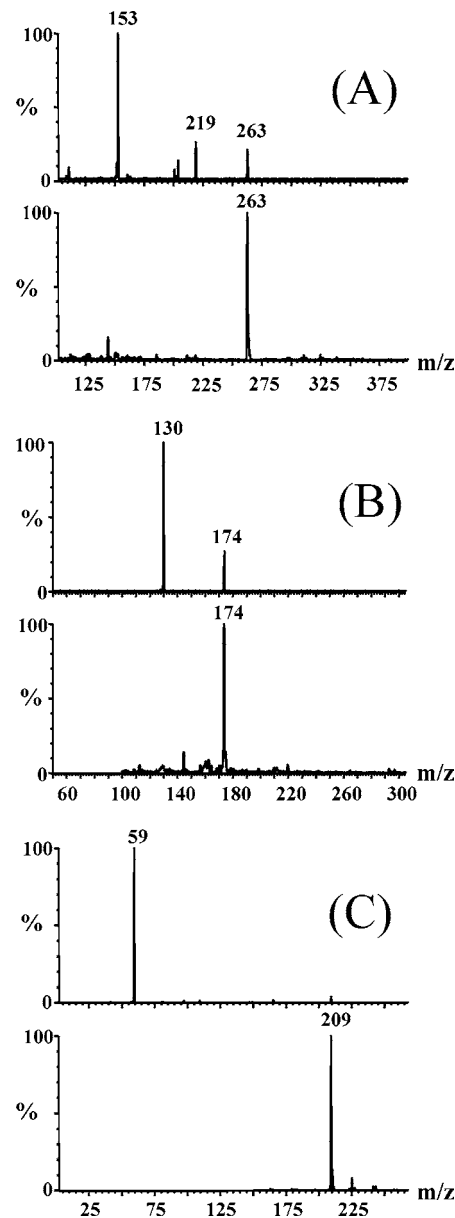
compd	cone voltage (V)	collision energy (eV)	precursor ion ( <i>m/z</i> )	diagnostic transition ( <i>m/z</i> )
ABA	25	12	263	263 → 153
[ <sup>2</sup> H <sub>6</sub> ]ABA	25	12	269	269 → 159
IAA	20	10	174	174 → 130
[ <sup>2</sup> H <sub>2</sub> ]IAA	20	10	176	176 → 132
JA	40	15	209	209 → 59
[ <sup>2</sup> H <sub>6</sub> ]JA	40	15	215	215 → 59

The procedure described is highly specific for ABA, IAA, and JA due to the tandem mass spectrometry. The phytohormones are first separated by their hydrophobic properties on a C18 reversed-phase column, then selected by their *m/z* ratio in the first quadrupole of the mass spectrometer, and finally selected as a fragment ion after collisional activation. An internal standard for each analyte allows automatic correction for losses during sample preparation and chromatography as well as serves as the internal standard for quantification during MRM mass spectrometry analysis.

**Selection of Precursor and Product Ions.** The appropriate precursor-to-product ion transition for each compound was determined by infusion of standard solutions (1 or 3 μg/mL, in methanol/0.01% CH<sub>3</sub>COOH in water, 20:80) of the phytohormones and deuterium-labeled compounds. The full-scan MS and MS/MS spectra of the three unlabeled hormones are shown in **Figure 2**. The main product ions obtained after the molecular fragmentation were used as diagnostic product ions. Similar results (with the expected shift of the ions) were obtained with MS/MS spectra of the deuterated compounds (data not shown). **Table 1** summarizes the MS/MS conditions and the diagnostic transitions chosen for each unlabeled compound and deuterium-labeled analogue that were used to quantify the plant hormones in the different plant tissues used in this work.

**Optimization of LC Parameters.** To obtain a fast and robust method that could be used for frequent analysis of hormones in plant tissues, the chromatographic conditions were optimized by using gradient elution. Two gradients were tried: Gradient A started with a proportion of methanol/0.01% CH<sub>3</sub>COOH in water of 10:90 (v/v) that linearly increased to 60:40 (v/v) in 10 min. In the following 4 min, the gradient increased to 80:20 (v/v). Isocratic conditions of 80:20 were then retained during the last 2 min of the run. Gradient B started with a proportion of acetonitrile/0.01% CH<sub>3</sub>COOH in water of 10:90 (v/v) that linearly increased to 40:60 (v/v) in 7 min. Isocratic conditions of 40:60 were then retained for 7 min. Gradient A was chosen as the optimum mobile phase for separation as the matrix effect was considerably reduced and the resolution of the different plant hormones improved. A 5 min solvent delay was imposed to avoid overloading of the mass spectrometer interface with fast-eluting contaminants. In preliminary experiments 0.01% HCOOH in water was used to acidify the mobile phase, but later it was changed to CH<sub>3</sub>COOH to reduce the background noise in the IAA transition. **Figure 3** show the retention time of the three phytohormones when gradient A was used.

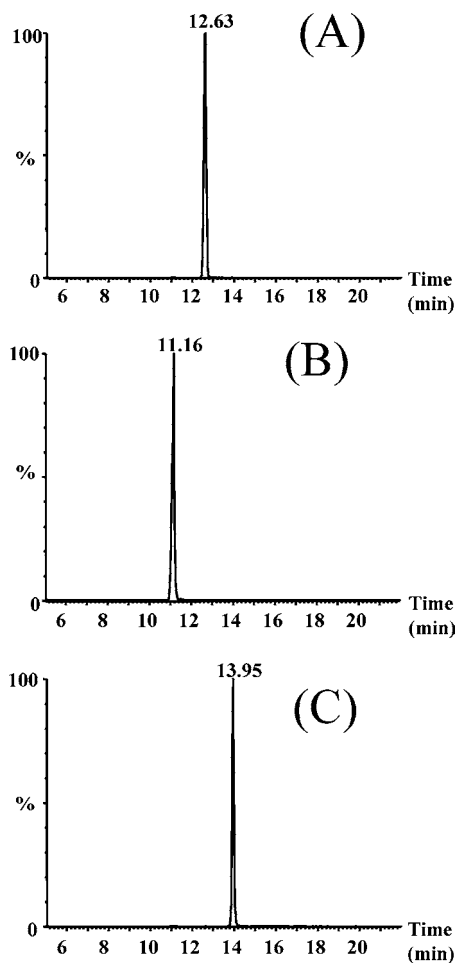
**Extraction Procedures.** Following previous work (12), a rapid approach to extract phytohormones from plant tissues was first tested: Plant tissue was homogenized with either ultrapure water or a mixture of methanol/water (80:20, v/v) and the extract centrifuged. Water was previously shown to be as effective as methanol as an extractant for ABA (12). When these two methods were compared for IAA and JA determination, water was as good as methanol in terms of absolute levels and



**Figure 2.** Fragmentation patterns for the phytohormones studied: (A) precursor (*m/z* 263, lower part) and product (*m/z* 153, upper part) ions of ABA standard analyzed in negative ion mode; (B) precursor (*m/z* 174, lower part) and product (*m/z* 130, upper part) ions of IAA standard analyzed in negative ion mode; (C) precursor (*m/z* 209, lower part) and product (*m/z* 59, upper part) ions of JA standard analyzed in negative ion mode.

normalized concentrations (considering the ratio among the endogenous compounds and the deuterated standards) for both phytohormones (data not shown). Therefore, we conclude that water can be an effective solvent for the initial extraction.

From this point, the purification procedures were reduced to a minimum in both cases and, as described in the Materials and Methods, only a partition against diethyl ether was performed. The use of a partition against organic solvent has the advantage of its simplicity. Acidic phytohormones will be recovered in the organic phase, while the large amounts of sugars and amino acids present in plant samples will remain in the aqueous phase. However, the limitation of this step is that most sugar hormone conjugates (such as ABA–glucose ester) will likely also be excluded (tossed away with the water phase). An alternative method for purification could be the use of solid-phase extraction. However, this will require the use of optimized



**Figure 3.** LC-ES tandem MS chromatograms of a standard mixture containing 40 ng/mL ABA, IAA, and JA under MRM conditions: (A) ABA,  $m/z$  263  $\rightarrow$   $m/z$  153 transition; (B) IAA,  $m/z$  174  $\rightarrow$   $m/z$  130 transition; (C) JA,  $m/z$  209  $\rightarrow$   $m/z$  59 transition. The numbers printed above the peaks indicate the retention times.

solid-phase extraction procedures to achieve a good limit of quantification in distinct plant matrixes (for example, in our hands, the use of C18-based cartridges was not efficient for phytohormone determination in photosynthetic tissues, data not shown). Then a similar problem is faced: the pursued selectivity would exclude a broad range of analytes. Therefore, depending on the approach, this point should be deeply considered.

**Method Validation and Analysis of Plant Samples.** The method proposed had to be validated to provide evidence that when correctly applied produces results that are fit for hormone determination in plant extract.

As an initial question, it should be noted that plant tissue manipulation should be reduced to a minimum. In this sense, if plant samples have to be lyophilized, the technical conditions of this preliminary procedure should be totally confirmed and optimized to the kind of tissue used. Otherwise, this procedure could cause decomposition changes in metabolites, loss of semivolatiles hormones, etc.

The linearity of this procedure was assayed by analyzing the calibration curves. These curves were obtained by using solutions containing increasing amounts of each plant hormone and a fixed amount of the corresponding deuterium-labeled internal standard. The amounts of internal standards used in here were identical to those added for spiking of plant samples (see the Materials and Methods). The calibration curves (data not shown) indicated a linear behavior in the concentration ranges

chosen ( $R^2$  values usually close to 0.999 and always higher than 0.989) and typically varied from 1 to 200 ppb.

The accuracy of the method was determined by using the "standard addition method" due to the impossibility of finding a blank sample (any plant matrix contains a certain amount of plant hormones). Endogenous amounts of each phytohormone were determined in citrus leaves, and then the same samples were spiked at two different levels (5 and 10 times the endogenous amount of each phytohormone). The percentages of recovery were calculated ( $n = 4$ ), and they were always higher than 96.5% for ABA, 99.2% for JA, and 99.4% for IAA considering the two levels of spiking.

Precision was calculated by a series of 10 measurements obtained from the same homogeneous sample. The average and the relative standard deviation (RSD) of these determinations are shown in **Table 2**. Four different plant matrixes were employed, citrus leaves, barley seedlings, barley immature embryos, and papaya roots. The precision for the different determinations was appropriate considering the low concentration of the studied compounds found in the plant samples. In this way, the RSD for ABA determinations was always lower than 2.67, that for JA determinations lower than 3.24, and that for IAA determinations lower than 3.46. These results also demonstrate that the method is sensitive enough to quantify the phytohormones in different plant matrixes. To illustrate with an example the kind of chromatograms typically obtained, **Figure 4** shows the results of quantifying IAA in citrus leaves (panel B), barley seedlings (panel C), and barley immature grains (panel D).

To study the stability of the different plant hormones in each of the plant extracts shown in **Table 2**, two approaches were taken. First, HPLC-injected plant extracts were maintained at room temperature for 12 h and re-injected. This experiment was performed 10 times with each of the plant extracts, and the areas obtained for the different compounds before and after the storage time were virtually identical. Second, analyzed plant extracts were frozen and kept at  $-20^\circ\text{C}$  for two months. After this time, the extracts were re-injected in the HPLC system. In this case, the percentages of recovery were on average 93% for ABA, 90% for JA, and 85% for IAA.

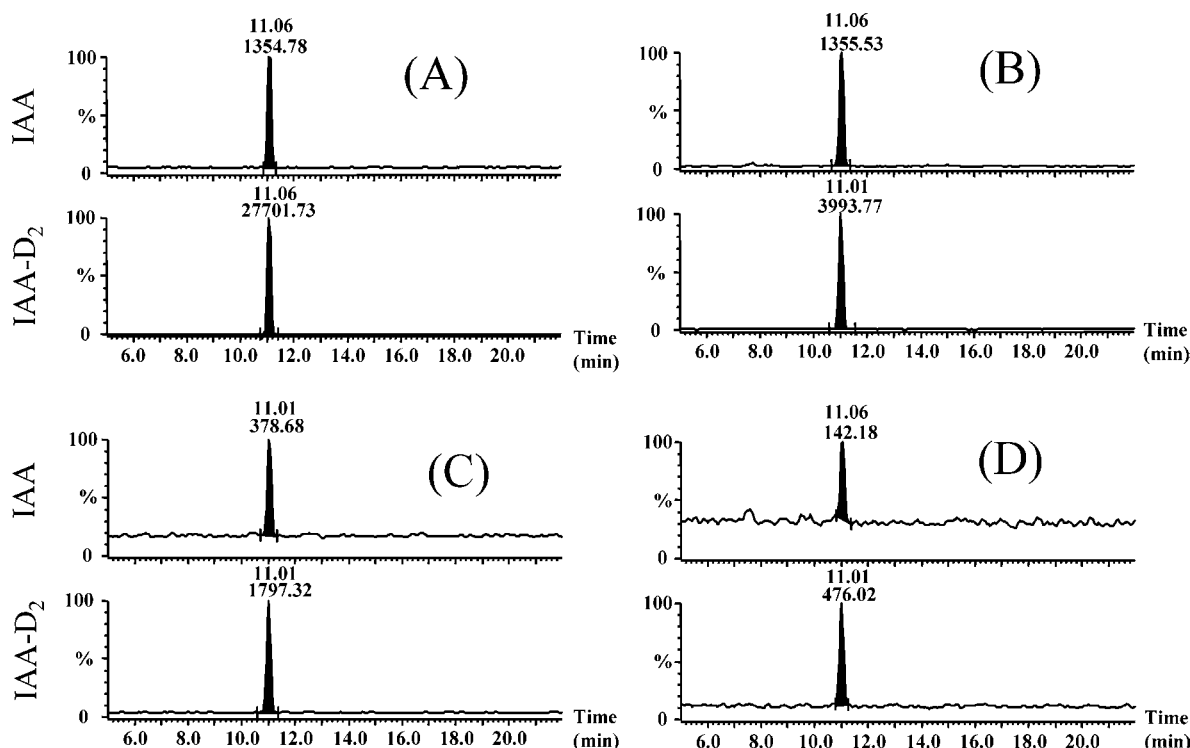
The limit of quantification (LOQ), defined as the lowest concentration that the analytical procedure can reliably differentiate from background levels, was considered to be the signal that was 5 times the background noise of a plant extract chromatogram. Following this definition, LOQs for the three plant hormones in the four tissues studied are shown in **Table 2**. Data indicate that, for the three compounds, the LOQs were, in general, low enough to permit the reduction of the initial amount of plant tissue if required. It is interesting to note that the LOQ calculated in this study for ABA in citrus leaves (68.40 pmol of ABA/g) was lower than that reported in ref 12 (83.33 pmol of ABA/g, when the same criterion is taken,  $S/N = 5$ ). Taking into consideration the fact that the instrument and the MS conditions used in both studies were identical, the slight differences observed could be due to the use of methanol instead of acetonitrile in the mobile phase. Other published methods achieved better LOQs than those presented here for IAA. For example, the method reported in ref 22 used LC-MS/MS to quantify IAA and several IAA conjugates. In this method excellent LOQs in *Arabidopsis* were obtained by methylation of the sample and detection in positive ESI mode.

**Method Comparison.** The main strength of the method reported here is the combination of a simple extraction with a sensitive and accurate method for phytohormone determination.

**Table 2.** Endogenous Amounts of Phytohormones, RSDs, and LOQs for the Different Plant Tissues<sup>a</sup>

plant tissue	ABA			IAA			JA		
	amount (pmol/g)	RSD	LOQ	amount (pmol/g)	RSD	LOQ	amount (pmol/g)	RSD	LOQ
citrus leaves (DW)	380.22	1.34	68.40	143.67	3.46	74.81	50.28	3.24	7.23
barley seedlings (FW)	71.09	2.67	54.88	517.24	1.22	114.40	107.65	2.22	12.93
barley embryos (FW)	456.30	1.66	65.08	994.22	2.22	133.65	104.78	1.82	17.05
papaya roots (DW)	43.80	1.85	12.66	211.26	2.98	114.86	964.78	2.47	31.90

<sup>a</sup> Calculations for endogenous amounts and RSDs were based on 10 independent extractions.



**Figure 4.** LC-ES tandem MS chromatograms [IAA ( $m/z$  174  $\rightarrow$   $m/z$  130 transition) and  $d_2$ -IAA ( $m/z$  176  $\rightarrow$   $m/z$  132 transition)] of an IAA standard solution and plant samples. The traces represent (A) 10 ng/mL IAA standard, (B) citrus leaves, (C) barley seedlings, and (D) barley immature grains. The upper and lower numbers printed above the peaks indicate retention time and area values, respectively.

By using the proposed method, a single person could easily extract 48 samples in approximately 5 h with minimum laboratory equipment, the rate-limiting steps being sample weighing and homogenization. This means that the primary limitation in the number of samples analyzed relies on the HPLC-MS/MS system. Considering the total running time per sample, up to 65 samples per day could be processed. This represents a slight improvement over some of the most efficient published methods for phytohormone determination (36, 48, and 60 samples per day in refs 13, 20, and 21, respectively). An additional strength of this method is that it has been tested in various plant tissues with very different matrixes (barley seedlings and citrus leaves containing photosynthetic pigments, barley half-seeds containing high amounts of starch, and papaya roots). Previous published methods on phytohormone determination by using LC-MS only tested their analytical advantages in a specific plant tissue (12, 13).

On the other hand, one of the weaknesses of the reported method is the relatively limited number of plant hormones analyzed compared with that of a previous method based on LC-MS/MS (13). However, the actual work includes JA determination (simultaneously with ABA and IAA) that had not been reported before.

It is also worth mentioning that, despite the advantages of the method described in this work, the separation of stereoisomers for the compounds studied is not possible. For example, it is known that the biological activities of two optical isomers, (3*R*,7*R*)-JA and (3*R*,7*S*)-JA, are different (26). Therefore, depending on the scientific approaches, other techniques would still be necessary.

**Conclusion.** We have described a rapid, sensitive, and accurate method to determine three phytohormones in different plant tissues. The compounds studied are representatives of three different groups of acidic plant hormones (auxins, abscisic acid, and jasmonates) with very distinct chemical and biological properties. The procedure described allows quantifying the plant hormones in their natural states without any derivatization step prior to analysis. The results show that, if deuterium-labeled analogues are included as internal standards, ES-MS/MS coupled with liquid chromatography separation allows accurate and sensitive quantification of these small plant molecules by MRM. This kind of method opens the possibility of incorporating other plant hormones and related metabolites into a single analysis (see also refs 13 and 27).

With all the important efforts made nowadays by the scientific community in precision agriculture, plant functional genomics

and hormone signal transduction will be highly complemented with these multiple residue analyses.

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#### LITERATURE CITED

- Gómez-Cadenas, A.; Mehouchi, J.; Tadeo, F. R.; Primo-Millo, E.; Talon, M. Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus *Planta* **2000**, *210*, 636–643.
- Kessler, S.; Sinha, N. Shaping up: the genetic control of leaf shape. *Curr. Opin. Plant Biol.* **2004**, *7*, 65–72.
- Gómez-Cadenas, A.; Tadeo, F. R.; Primo-Millo, E.; Talon, M. Involvement of abscisic acid and ethylene in the responses of citrus seedlings to salt shock. *Physiol. Plant.* **1998**, *103*, 475–484.
- O'Donnell, P. J.; Schmelz, E.; Block, A.; Miersch, O.; Wasternack, C.; Jones, J. B.; Klee, H. J. Multiple hormones act sequentially to mediate a susceptible tomato pathogen defense response. *Plant Physiol.* **2003**, *133*, 1181–1189.
- Ho, T.-H. D.; Gomez-Cadenas, A.; Zentella, R.; Casaretto, J. Crosstalks between abscisic acid and gibberellins in barley aleurone layers. *J. Plant Growth Regul.* **2003**, *22*, 185–194.
- Vogler, H.; Kuhlemeier, C. Simple hormones but complex signaling. *Curr. Opin. Plant Biol.* **2003**, *6*, 51–56.
- Zegzouti, H.; Jones, B.; Marty, C.; Lelievre, J. M.; Latche, A.; Pech, J. C.; Bouzayen, M. ER5, a tomato cDNA encoding an ethylene-responsive LEA-like protein: characterization and expression in response to drought, ABA and wounding. *Plant Mol. Biol.* **1997**, *35*, 847–854.
- Wasternack, C.; Parthier, B. Jasmonate signalled plant gene expression. *Trends Plant Sci.* **1997**, *2*, 302–307.
- Gómez-Cadenas, A.; Tadeo, F. R.; Talon, M.; Primo-Millo, E. Leaf abscission induced by ethylene in water-stressed intact seedlings of cleopatra mandarin requires previous abscisic acid accumulation in roots. *Plant Physiol.* **1996**, *112*, 401–408.
- Fernandez, B.; Centeno, M. L.; Feito, I.; Sanchez-Tames, R.; Rodriguez, A. Simultaneous analysis of cytokinins, auxins and abscisic-acid by combined immunoaffinity chromatography, high-performance liquid-chromatography and immunoassay. *Phytochem. Anal.* **1995**, *6*, 49–54.
- Beale, M. H. Immunological methods in plant hormone research. In *Biochemistry and Molecular Biology of Plant Hormones*; Hooykaas, P. J. J., Hall, M. A., Libbenga, K. R., Eds.; Elsevier Science: New York, 1999; pp 61–88.
- Gómez-Cadenas, A.; Pozo, O. J.; García-Augustin, P.; Sancho, J. V. Direct analysis of abscisic acid in crude plant extracts by liquid chromatography-electrospray/tandem mass spectrometry. *Phytochem. Anal.* **2002**, *13*, 228–234.
- Chiwocha, S. D. S.; Abrams, S. R.; Ambrose, S. J.; Cutler, A. J.; Loewen, M.; Ross, A. R. S.; Kermode, A. R. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodynamicity of lettuce (*Lactuca sativa* L.) seeds. *Plant J.* **2003**, *35*, 405–417.
- Liu, X.; Ma, L.; Lin, Y. W.; Lu, Y. T. Determination of abscisic acid by capillary electrophoresis with laser-induced fluorescence detection. *J. Chromatogr., A* **2003**, *1021*, 209–213.
- Glassbrook, N.; Beecher, C.; Ryals, J. Metabolic profiling on the right path. *Nat. Biotechnol.* **2000**, *18*, 1142–1143.
- Birkemeyer, C.; Kolasa, A.; Kopka, J. Comprehensive chemical derivatization for gas chromatography-mass spectrometry-based multi-targeted profiling of major phytohormones. *J. Chromatogr., A* **2003**, *993*, 89–102.
- Ribnicky, D. M.; Cooke, T. J.; Cohen, J. D. A microtechnique for the analysis of free and conjugated indole-3-acetic acid in milligram amounts of plant tissue using a benchtop gas chromatograph mass spectrometer. *Planta* **1998**, *204*, 1–7.
- Neil, S. J.; Horgan, R.; Heald, J. K. Determination of the levels of abscisic acid-glucose ester in plants. *Planta* **1983**, *157*, 371–375.
- Ueda, J.; Miyamoto, K.; Kamisaka, S. Separation of a new type of plant growth regulator, jasmonates, by chromatographic procedures. *J. Chromatogr., A* **1994**, *658*, 129–142.
- Schmelz, E. A.; Engelberth, J.; Tumlinson, J. H.; Block, A.; Alborn, H. T. The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. *Plant J.* **2004**, *39*, 790–808.
- Müller, A.; Düchting, P.; Weiler, E. W. A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to *Arabidopsis thaliana*. *Planta* **2002**, *216*, 44–56.
- Kowalczyk, M.; Sandberg, G. Quantitative analysis of indole-3-acetic acid metabolites in *Arabidopsis*. *Plant Physiol.* **2001**, *127*, 1845–1853.
- Zhou, R.; Squires, T. M.; Ambrose, S. J.; Abrams, S. R.; Ross, A. R. S.; Cutler, A. J. Rapid extraction of abscisic acid and its metabolites for liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2003**, *1010*, 75–85.
- Tamogami, S.; Kodama, O. Quantification of amino acid conjugates of jasmonic acid in rice leaves by high-performance liquid chromatography turboionspray tandem mass spectrometry. *J. Chromatogr., A* **1998**, *822*, 310–315.
- Niessen, W. M. A.; Tinke, A. P. Liquid-chromatography mass-spectrometry—general-principles and instrumentation. *J. Chromatogr., A* **1995**, *703*, 7–57.
- Holbrook, L.; Tung, P.; Ward, K.; Reid, D. M.; Abrams, S.; Lamb, N.; Quail, J. W.; Moloney, M. M. Importance of the chiral centers of jasmonic acid in the responses of plants—Activities and antagonism between natural and synthetic analogs. *Plant Physiol.* **1997**, *114*, 419–428.
- Prinsen, E.; Van Dongen, W.; Esmans, E. L.; Van Onckelen, H. A. Micro and capillary liquid chromatography tandem mass spectrometry: a new dimension in phytohormone research. *J. Chromatogr., A* **1998**, *826*, 25–37.
- Miersch, O. Synthesis of (±)-(10-h-2,11-(h-2)2,12-(h-2)(3) jasmonic acid. *Z. Naturforsch.* **1991**, *46b*, 1727–1729.

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