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# Development of an Ultrahigh-Performance Liquid Chromatography – Electrospray Ionization–Tandem Mass Spectrometry Method for the Simultaneous Determination of Salicylic Acid, Jasmonic Acid, and Abscisic Acid in Rose Leaves

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## ABSTRACT

This paper describes a method to detect and quantitate the endogenous plant hormones (±)-2-cis-4-trans-abscisic acid, (-)-jasmonic acid, and salicylic acid by means of ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) in hybrid rose leaf matrices. Deuterium-labeled [<sup>2</sup>H<sub>6</sub>] (+)-2-cis-4-trans-abscisic acid, [<sup>2</sup>H<sub>6</sub>] (±)-jasmonic acid, and [<sup>2</sup>H<sub>4</sub>]-salicylic acid were used as internal standards. Rose samples (10 mg) were extracted with methanol/water/acetic acid (10:89:1) and subsequently purified on an Oasis MCX 1 cm<sup>3</sup> Vac SPE cartridge. Performance characteristics were validated according to Commission Decision 2002/657/EC. Recovery, repeatability, and within-laboratory reproducibility were acceptable for all phytohormones tested at three different concentrations. The decision limit and detection capability for (±)-2-cis-4-trans-abscisic acid, (-)-jasmonic acid, and salicylic acid were 0.0075 and 0.015 µg/g, 0.00015 and 0.00030 µg/g, and 0.0089 and 0.018 µg/g, respectively. Matrix effects (signal suppression or enhancement) appeared to be high for all substances considered, implying the need for quantitation based on matrix-matched calibration curves.

**KEYWORDS:** mass spectrometry, multiple reaction monitoring, SPE, Rosa, phytohormones

## INTRODUCTION

Physiological processes in plants, e.g., apical dominance, bud formation, cell division, enlargement and differentiation of cells, flowering, seed germination and dormancy, senescence, etc., are all regulated by phytohormones.<sup>1–3</sup> Also, in abiotic and biotic stress responses, endogenous plant hormones play a key role. This allows the plant to mediate host responses upon pathogen attack, insect herbivory, and drought/cold/heat stress. Salicylic acid (1) is associated with the resistance response against biotrophic pathogens, while jasmonic acid (2) is required in defense responses toward necrotrophic pathogens.<sup>4,5</sup> Compound 1 acts antagonistically toward compound 2 signaling and conversely. In addition, timing and amplitude of hormone signals are essential to determine the final pathological phenotype related to

the efficiency of the plant response.<sup>6,7</sup>

Pathogenicity in plants is a complex process involving repertoires of effector proteins. Functions of these effector proteins include the modification of basal phytohormone levels while the disease develops. Sequencing data of plant pathogens reveal information on mechanisms of pathogenicity as, for example, in the hemibiotrophic bacterial pathogen, *Pseudomonas syringae* pv. tomato DC3000, which delivers ~30 effector proteins into the plant cell during foliar infection.<sup>8,9</sup> These proteins redundantly modify host signaling pathways, of which the suppression or modification of plant hormone responses is one example.<sup>10,11</sup>

Although the abiotic stress hormone abscisic acid (3) is well-known for its role in response to drought stress and maintenance of seed dormancy, compound 3 can also influence the plant pathogen interaction.<sup>12-16</sup> Dependent upon the characteristics of the infecting pathogen, antagonistic inter-actions between abscisic acid and jasmonate/ethylene<sup>17</sup> or salicylic acid signaling pathways were found. The increasing evidence that phytohormones play an important role in stress responses leads to an increasing need to measure changes in endogenous concentrations of these hormones at different stages of the infection process. Moreover, the crosstalk between biotic and abiotic stress pathways has received more attention. Therefore, studies have been designed to unravel how plants prioritize stress responses in multiple stress conditions and under given conditions.<sup>18</sup> Increasing evidence shows that perturbation of one hormone pathway leads to profound effects on synthesis and accumulation of other plant hormones.<sup>19</sup> Therefore, a single analytical run for detection and quantitation of different phytohormones allows focus on this multiple phytohormonal crosstalk and is time-saving and cost-effective. Examples of conventional methods for measuring plant hormones are enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography with diode-array and fluorometric detection (LC-DAD/FLD),<sup>20</sup> and gas chromatography-mass spectrometry (GC-MS).

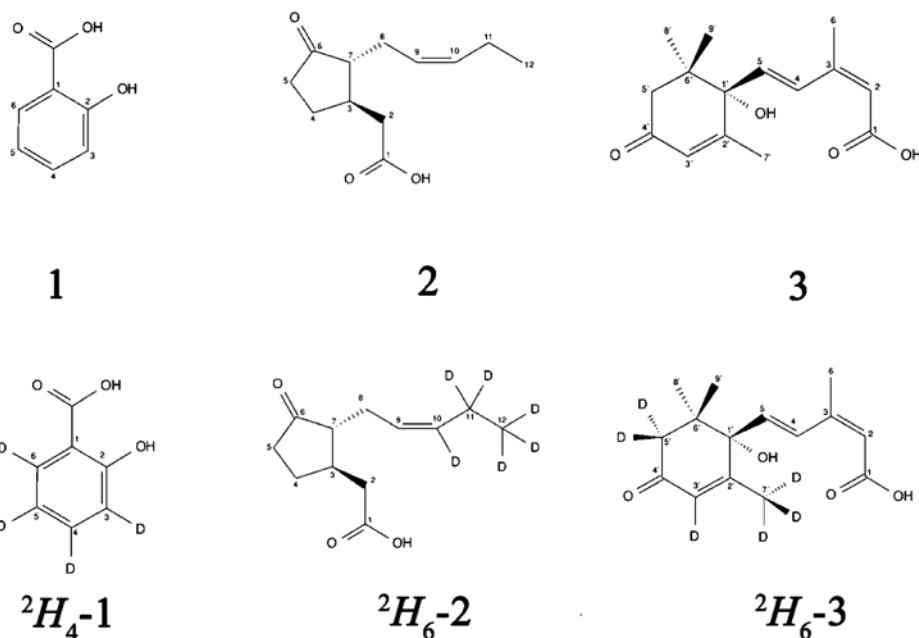


Figure 1. Chemical structures of salicylic acid (1), (-)-jasmonic acid (2), (±)-2-cis-4-trans-abscisic acid (3), and deuterium-labeled internal standards [<sup>2</sup>H<sub>4</sub>]-salicylic acid (<sup>2</sup>H<sub>4</sub>-1), [<sup>2</sup>H<sub>6</sub>]-(-)-jasmonic acid (<sup>2</sup>H<sub>6</sub>-2), and [<sup>2</sup>H<sub>6</sub>]-(+)-2-cis-4-trans-abscisic acid (<sup>2</sup>H<sub>6</sub>-3).

Sensitivity of these analytical approaches is often not sufficient to provide hormone content information on specific plant tissue in small samples. GC-MS is routinely applied for the multiplex analysis of various hormones in plant tissues.<sup>19</sup> However, the hydrophilic groups need to be derivatized to obtain the high analyte volatility for proper GC separation. Besides this relatively time-consuming step, another disadvantage is the need for at least 300 mg of fresh plant material to reach the dynamic range of the mass spectrometer even with the modern GC-MS approaches.<sup>21</sup> An elegant way to overcome the limitations of GC-MS is the use of reversed-phase high-performance liquid chromatography (RP-HPLC)- electrospray ionization (ESI)-MS. This technique offers a high separation efficiency and selectivity and minimal ESI matrix effects, and it ensures the sensitive detection of analytes without the need for derivatization.<sup>22,23</sup> Sensitivity is improved by tandem mass spectrometry (MS/MS) with triple quadrupole instruments operating in multiple reaction monitoring (MRM) mode by fast duty cycles and reduced chemical noise.<sup>24</sup> A rapid analysis of multiple hormones is possible through the relatively short dwell times typical for modern mass spectrometers.<sup>25</sup> In addition, the application of ultrahigh-performance liquid chromatography (UHPLC)<sup>26</sup> as well as solid-phase extraction (SPE)-based enrichment<sup>27</sup> may lead to the further improvement of sensitivity of phytohormone analysis.

The aim of this study was to develop a selective and sensitive analytical method for the simultaneous quantitation of (±)-2-cis-4-trans-abscisic acid (3), (-)-jasmonic acid (2), and salicylic acid (1) in rose leaves based on SPE purification and UHPLC- MS/MS analysis. [<sup>2</sup>H<sub>4</sub>]-salicylic acid (<sup>2</sup>H<sub>4</sub>-1), [<sup>2</sup>H<sub>6</sub>]-(-)-jasmonic acid (<sup>2</sup>H<sub>6</sub>-2), and [<sup>2</sup>H<sub>6</sub>]-(+)-2-cis-4-trans-abscisic acid (<sup>2</sup>H<sub>6</sub>-3) were used as internal standards (Figure 1). The method developed was validated according to Commission Decision 2002/657/EC.<sup>28</sup>

## MATERIALS AND METHODS

**Reagents and Chemicals.** Analytical-reagent-grade chemicals were used. Water was HPLC-grade, generated by a Milli-Q gradient purification system (Millipore, Billerica, MA). Methanol (MeOH, LC-MS absolute) and acetic acid (99%, ULC-MS) were supplied by Biosolve B.V. (Biosolve B.V., Valkenswaard, Netherlands). Phytohormones 1, 2, and 3 and the deuterium-labeled internal standards <sup>2</sup>H<sub>4</sub>-1, <sup>2</sup>H<sub>6</sub>-2, and <sup>2</sup>H<sub>6</sub>-3 were all purchased from OIChemIm (OIChemIm, Olomouc, Czech Republic).

**Stock and Working Solution Preparation.** Stock standard solutions (1 mg/mL) of compounds 1, 2, and 3 were prepared using methanol/water (75:25, v/v). Working standard solutions were prepared by dilution of the stock solution to obtain concentrations ranging between 0 and 60.0 µg/g for compound 3 and between 0 and 20.0 µg/g for compounds 1 and 2.

**Rose Leaf Samples and Sample Preparation.** Rose plants were grown in the greenhouse of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium) and were subjected to standard cultural practices. The rose genotype used was a F1 hybrid of 'Yesterday' x Rosa wichurana. For the method development and validation, young (just unfolded) leaves were used. The collected rose leaves were free from any damage and were immediately frozen in liquid nitrogen to prevent enzymatic and thermal degradation of the phytohormones. They were stored at -80 °C until analysis. Prior to extraction, the leaf material was homogenized by removing the biggest veins and grinding with liquid nitrogen, using a mortar and pestle. In total, 20 mg of each homogenized sample was transferred to a 10 mL glass tube.

Extraction buffer containing the internal standard (IS) was prepared with final concentrations of 20 ng/mL <sup>2</sup>H<sub>4</sub>-1, 1 ng/mL <sup>2</sup>H<sub>6</sub>-2, and 400 ng/mL <sup>2</sup>H<sub>6</sub>-3.

Table 1. Optimized ESI-MS/MS Conditions with Indication of the Precursor Ion (m/z), Cone Voltage (V), Product Ions (m/z), Collision Energy (eV), and Chromatographic Retention Time (min) of Salicylic Acid (1), (-)-Jasmonic Acid (2), ( $\pm$ )-2-cis-4-trans-Abscisic Acid (3), and Deuterium-Labeled Internal Standards [ $^2\text{H}_4$ ]-Salicylic Acid ( $^2\text{H}_4$ -1), [ $^2\text{H}_6$ ]-(-)-Jasmonic Acid ( $^2\text{H}_6$ -2), and [ $^2\text{H}_6$ ]-(+)-2-cis-4-trans-Abscisic Acid ( $^2\text{H}_6$ -3)

compound	precursor ion (m/z)	cone voltage (V)	product ions (m/z)	collision energy (eV)	retention time (min)
1	137.00	40	64.95/92.96 <sup>a</sup>	22/14	4.86
2	209.15	25	58.77 <sup>a</sup> /165.00	12/17	5.65
3	263.02	25	153.19 <sup>a</sup> /219.00	17/17	5.28
$^2\text{H}_4$ -1	140.74	35	96.84	17	4.84
$^2\text{H}_6$ -2	215.11	30	58.77	12	5.63
$^2\text{H}_6$ -3	269.12	35	159.10	30	5.26

<sup>a</sup>Product ion used in the screening program.

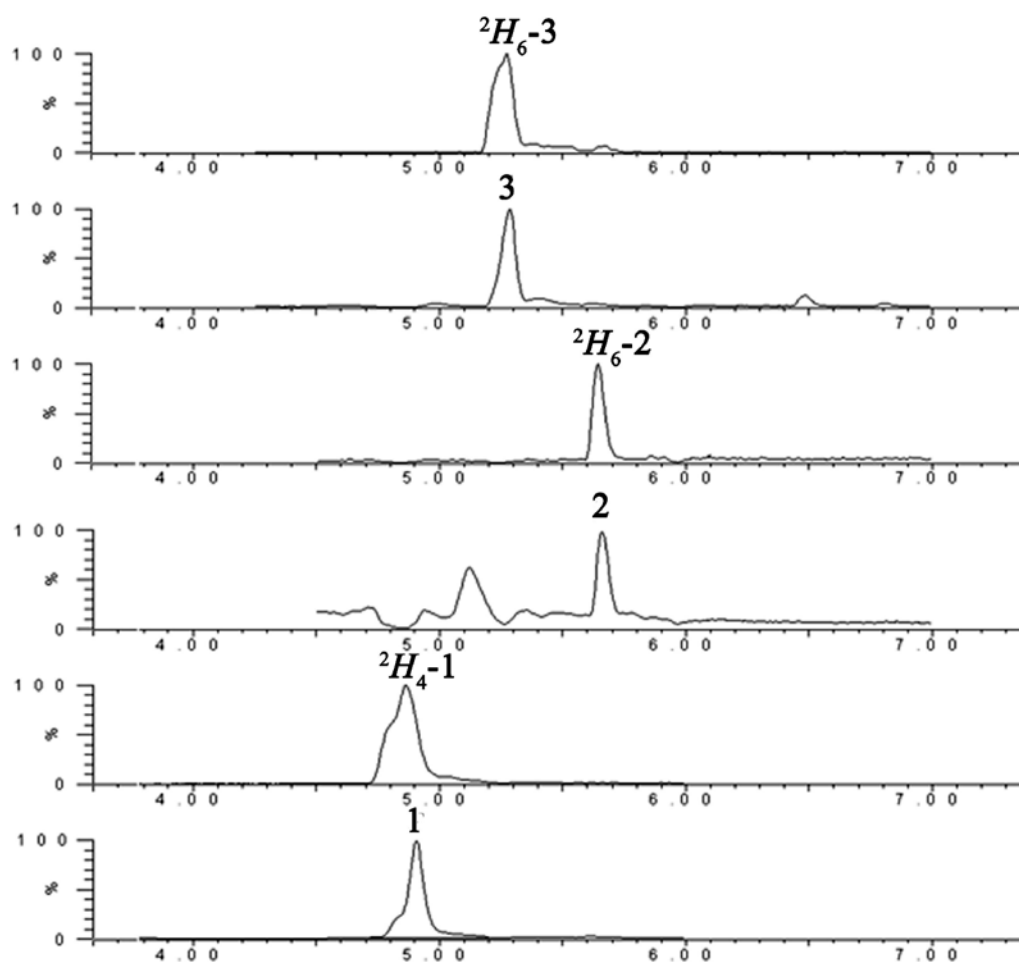


Figure 2. Representative total ion chromatograms (TIC) of the rose leaf matrices analyzed using the method developed (the concentrations of the peaks in the chromatogram correspond to endogenous concentrations, with the addition of 1.25  $\mu\text{g/g}$  of compounds 1 and 2 and 3.75  $\mu\text{g/g}$  of compound 3, with the intensities of the signals at  $4.26 \times 10^6$ ,  $1.11 \times 10^6$ , and  $1.36 \times 10^6$ , respectively).

Extraction was performed using 2 mL of methanol/water/acetic acid (10:89:1) for 16 h at 4 °C in the dark on an orbital shaker rotating at 210 rpm. The extract was filtered through a 0.22 µm Millex-GV filter (Millipore, Billerica, MA). Before loading 1 mL of this eluate onto Oasis MCX 1 cm<sup>3</sup> SPE cartridges (Waters, Milford, MA), these cartridges were first conditioned with 1 mL of methanol (100%), followed by 1 mL of water for equilibration. The acidic hormones bound onto the column were washed with 0.5 mL of H<sub>2</sub>O and then eluted with a total of 1 mL of methanol (100%), applied in two consecutive steps of 0.5 mL each. Subsequently, the total eluate was evaporated to dryness using nitrogen gas at 35 °C, and the residues were redissolved in 0.5 mL of methanol/water (75:25, v/v). This extract was transferred to a microvial and stored at 4 °C prior to injection (10 µL) into the UHPLC–MS/MS system.

**UHPLC–MS/MS Analyses.** An Acquity UHPLC system coupled to a Xevo TQ-S triple quadrupole MS detector (Waters, Milford, MA) was used for analytical determination of the target compounds in the leaf samples. Chromatographic separation was achieved using a 100 × 2.1 mm inner diameter, 1.7 µm, Acquity UHPLC BEH C<sub>18</sub> column (Waters, Milford, MA) at 30 °C. The mobile phase consisted of 0.3% acetic acid in water (A) and 0.3% acetic acid in methanol (B). The flow rate was set at 0.3 mL/min. The gradient was adapted from a previous report<sup>29</sup> as follows: 0 min, 2% B; 1.0–2.5 min, 2–40% B; 2.5–4.0 min, 40–50% B; 4.0–5.0 min, 50–80% B, held for 2.0 min; 7.0–7.1 min, 80–100% B, held for 0.9 min; and 8.0–11.0 min, 100– 2% B.

The mass spectrometer operated in the ESI negative mode with MRM. Desolvation gas was set at 1000 L/h and a temperature of 600 °C. The MRM settings in the MS/MS function with corresponding cone voltage and collision energy were optimized for each compound (Table 1). Data were acquired and processed using MassLynx, version 4.1 (Waters, Milford, MA). A standard mix at a concentration of 100 µg/g was prepared and injected into the LC–MS/MS system prior to each experiment to check the system sensitivity and reproducibility.

**Method Validation.** Because of the absence of specific guidelines for the analysis of phytohormones in plant tissue, the Commission Decision 2002/657/EC was used as a guideline for the validation of the extraction and detection method developed for compounds 1, 2, and 3. Appropriate deuterium-labeled internal standards, <sup>2</sup>H<sub>4</sub>-1, <sup>2</sup>H<sub>6</sub>-2, and <sup>2</sup>H<sub>6</sub>-3, were used to correct the data obtained for the extraction of the corresponding phytohormones from rose leaf tissue. Matrix-matched calibration curves making use of these deuterium-labeled internal standards were used for quantitation. The validation parameters considered were specificity, linearity (R<sup>2</sup>), matrix effect by signal suppression/enhancement (SSE), apparent recovery (R<sub>A</sub>), repeatability through the relative standard deviation (RSD<sub>r</sub>), intra-laboratory reproducibility [also through calculation of the relative standard deviation (RSD<sub>R</sub>)], and the limits of decision (LOD) and quantitation (LOQ).

## RESULTS AND DISCUSSION

**Method Development.** By infusion of pure standards into the UHPLC mass spectrometer, the analyte-dependent mass spectrometric parameters were optimized for both the precursor ion and product ions (Table 1). The shortest analysis time with good resolution and peak shapes was observed using the Acquity UHPLC BEH C<sub>18</sub> column. The effect of the column temperature on analyte separation within the range of 25–45 °C was investigated, with the most optimal results obtained at 30 °C. Representative chromatograms for compounds 1, 2, and 3 in rose leaves are shown in Figure 2. The retention times of compounds 1, 2, and 3 were 4.86, 5.65, and 5.28 min, respectively.

After optimization of the LC–MS/MS conditions, a suitable extraction procedure for the three phytohormones 1, 2, and 3 from rose leaves was developed. Although the cleanup of raw extracts can theoretically be kept to a minimum when applying analytical methods as sensitive and specific as UHPLC–MS/MS, the “dilute-and-shoot” principle was not tested in this study. Given the complexity of

the plant material considered and the yet to be proven long-term suitability of such methods without cleanup step(s) in terms of minimally polluting the MS equipment, we chose to test a SPE cleanup method. When searching for suitable SPE columns, several factors should be taken into account: the sample matrix, analyte-specific physicochemical properties, and the nature of the bonded phase. Besides enrichment, SPE-based procedures also provide a reduction in sample complexity and help to minimize the matrix effect by SSE. To select an appropriate SPE column capable of retaining the analytes of interest, which are characterized by different hydrophobicities and  $pK_a$  values, a screening experiment with five commercially available solid-phase materials was conducted using a standard mixture of the three hormones 1, 2, and 3. In this experiment, Oasis mixed-mode ion-exchange chemistry MAX (strong anion exchanger), WAX (weak anion exchanger), MCX (strong cation exchanger), WCX (weak cation exchanger), and Oasis HLB (universal polymeric reversed-phase sorbent) were evaluated. For the three hormones 1, 2, and 3, the average recovery percentage of three repetitions was determined. This experiment showed that the anion-exchange cartridges MAX and WAX can be used for the purification of compounds 2 and 3 but not for compound 1 because the latter is not retained by the stationary phase (data not shown). In contrast, cation-exchange cartridges MCX and WCX and the generic cartridge HLB retained all three phytohormones examined. Balcke et al.<sup>30</sup> developed a purification method similar to the method presented in this paper. They tested 11 types of SPE devices and found strong cation-exchange solid-phase material to give the best recovery results. Our findings are in agreement with these results and the choice of the MCX cartridge as the best SPE cartridge for the purification of the phytohormones 1, 2, and 3 from plant material. The method currently presented offers a more effective extraction protocol when compared to the method by Balcke et al.<sup>30</sup> The amount of leaf material used was reduced to 20 mg and combined with an easier analysis procedure. Furthermore, the method was tested on a woody plant. It is known that interference of secondary metabolites makes woody plants more recalcitrant.

At present, extraction with organic solvents is the most widely used method for plant hormones. Many procedures and solvents have been developed and used for plant hormone extraction.<sup>29,30</sup> The polarity of the extraction solvent is chosen to closely match that of the target compounds; thus, the ratio of organic solvent/water is defined according to the polarity of hormones. Nonpolar solvents, such as ether, are rarely used to extract plant hormones. Instead, methanol is the preferred solvent, allowing for efficient plant cell penetration during extraction.<sup>31,32</sup> Because methanol produced the best recoveries in preliminary experiments (data not shown), experiments were performed using methanol alone<sup>30</sup> and with different proportions of ultrapure water [methanol/water, 75:25;<sup>20</sup> methanol/water/formic acid, 75:20:5;<sup>29</sup> and methanol/water/ acetic acid, 10:89:1<sup>33</sup>]. The results obtained are summarized in Table 2.

Table 2. Percentage of Recovery with Different Solvents and Solvent Combinations Used for Extraction of Salicylic Acid (1), (-)-Jasmonic Acid (2), and ( $\pm$ )-2-cis-4-trans-Abscisic Acid (3)

solvent	phytohormone recovery (%)		
	1	2	3
100% methanol	29	100	93
75:25% methanol/water	46	72	100
75:20:5% methanol/water/formic acid	42	78	78
10:89:1% methanol/water/acetic acid	100	68	98

The extraction solvent methanol/water/acetic acid (10:89:1) resulted in the highest recovery for compound 1 and a good recovery for compounds 2 and 3, whereas the other extraction solvents led to a significantly lower recovery of compound 1. For the optimization of the extraction time, three situations were tested: 1, 2, and 16 h. These data indicated that 1 h of extraction was sufficient for the recovery of compound 3 (100%), but the recovery of compounds 1 and 2 was only 70%. An extraction



of 2 h yielded a recovery of 100% for the phytohormones 2 and 3 and 83% for phytohormone 1. After 16 h of extraction, the recovery of compound 1 was 100%. On the basis of these data and logistical issues, the analyzes were carried out after 16 h of extraction.

**Method Validation. Specificity.** When pure phytohormone standards were injected separately, the obtained UHPLC–MS/ MS signals clearly did not interfere with the traces of the other phytohormones, baseline separation of all analytes could be achieved, and no crosstalk between different mass ranges of the MRM experiment was observed.

Table 3. Overview of the Percentage of Apparent Recovery ( $R_A$ ), Repeatability by Calculation of the Relative Standard Deviation ( $RSD_r$ ), and Interday Precision over 3 Days also Calculated as the Relative Standard Deviation ( $RSD_R$ ) at the Three Concentrations Used for Validation, LOD ( $\mu\text{g/g}$ ), LOQ ( $\mu\text{g/g}$ ), and SSE (%) for Salicylic Acid (1), (-)-Jasmonic Acid (2), ( $\pm$ )-2-cis-4-trans-Abscisic Acid (3)

phytohormone ( $\mu\text{g/g}$ )	concentrations	low concentration			medium concentration			high concentration			LOD ( $\mu\text{g/g}$ )	LOQ ( $\mu\text{g/g}$ )	SSE (%)
		$R_A$ (%)	$RSD_r$ (%)	$RSD_R$ (%)	$R_A$ (%)	$RSD_r$ (%)	$RSD_R$ (%)	$R_A$ (%)	$RSD_r$ (%)	$RSD_R$ (%)			
1 <sup>a</sup>	1.25, 5, and 10	109	11	11	103	7	6	100	3	13	0.0089	0.017	+130
2	1.25, 5, and 10	104	3	8	106	3	4	103	4	6	0.00015	0.00030	+202
3	3.75, 15, and 30	105	3	11	103	2	6	100	2	4	0.0075	0.015	+140

<sup>a</sup>n = 4 for first day of validation. For the second and third days, n = 6.

**Linearity.** Linearity was evaluated by preparing seven-point calibration curves in the matrix for the different phytohormones (in triplicate). The “blank” samples were fortified with concentrations ranging from 0.00 to 60.0  $\mu\text{g/g}$  for compound 3 and from 0.00 to 20.0  $\mu\text{g/g}$  for compounds 1 and 2. Correlation coefficients ( $R^2$ ) obtained for these compounds were all >0.99, which indicated good linearity within the considered concentration ranges.

**Matrix Effect.** SSE is common in LC–ESI–MS/MS because of competition of charge carriers between analytes and co-eluting compounds during the ionization process. Matrix effect (SSE), ionization suppression or enhancement, in liquid chromatography/electrospray ionization mass spectrometry (LC/ESI–MS) is caused by matrix components co-eluting with the analytes. The matrix effect is also highly variable from sample to sample, making it difficult to compensate. To determine possible matrix effects, calibration curves of spiked extracts (i.e., matrix-matched calibration curves) were compared to the corresponding calibration curves of the pure standards. These effects were expressed in terms of SSE and calculated as follows:  $\text{SSE} (\%) = 100 \times (\text{slope of the matrix-matched standard} / \text{slope of the pure standard})$ . The values of SSE shown in Table 3 indicate that all phytohormones considered suffered from signal enhancement ( $\geq 100\%$ ), with the most profound enhancement observed for compound 2 (202% SSE). The observed SSE emphasized the need to quantitate these analytes by means of matrix-matched calibration curves.

**Recovery, Precision, LOD, and LOQ.** Table 3 gives an overview of the percentage of apparent recovery ( $R_A$ ), intraday precision ( $RSD_r$ ), interday precision over three non-consecutive days ( $RSD_R$ ), LOD ( $\mu\text{g/g}$ ), and LOQ ( $\mu\text{g/g}$ ) for each phytohormone.  $R_A$  percentages were within an acceptable range (80–110%). Intralaboratory reproducibility and repeatability were all considered acceptable according to Commission Decision 2002/657/EC (“performance criteria”). For the phytohormones, the LOD and LOQ were theoretically calculated on the basis of calibration curves in the matrix:  $\text{LOD} = 3(\text{seb}/\text{slope})$  and  $\text{LOQ} = 6(\text{seb}/\text{slope})$ , with seb being the standard deviation on the intercept of the calibration curve with the y axis and slope being the slope of the calibration curve (Table 3). The LOD and LOQ for compounds 1, 2, and 3 were 0.00015 and 0.015  $\mu\text{g/g}$ , 0.0075 and 0.018  $\mu\text{g/g}$ , and 0.0089 and 0.00030  $\mu\text{g/g}$ , respectively. In our method, LOD and LOQ values show that hormones can be analyzed at

endogenous concentrations. One can conclude that the values for the LOD and LOQ obtained with the multi-phytohormones method developed in this study were below typical endogenous concentrations.<sup>34</sup>

**Analysis of Leaf Samples.** In this study, concentrations of compounds 1, 2, and 3 measured in leaves were  $0.68 \pm 0.014$ ,  $0.17 \pm 0.009$ , and  $0.65 \pm 0.032$   $\mu\text{g/g}$ , respectively ( $n = 3$ ). The resulting values agreed with the ranges found in previous studies.

Knowledge of the compound 3 concentration in leaves is of particular interest to understand plant responses to abiotic stresses. Compound 3 is involved in the adaptability toward drought, salinity, cold, and other environmental stresses. A recent study<sup>35</sup> in roses under dark/light conditions and moderate/high humidity indicated compound 3 leaf quantitation levels in rose leaves ranging from ca. 1.00 to ca. 5.00  $\mu\text{g/g}$  using UHPLC–ESI–MS/MS. The data obtained by our method for compound 3 (0.4–0.9  $\mu\text{g/g}$ ) showed comparable results to those by Giday et al.,<sup>36</sup> but no data were shown in the latter study on the concentrations of compounds 1 and 2 in rose leaves.

Plant matrix complexity complicates the analysis of phytohormones. Accurate quantitation of amounts of these compounds depends upon a robust method. We have developed a highly specific protocol for simultaneous determination of compounds 1, 2, and 3 in rose leaf material. The method is a rapid, sensitive, and accurate way to simultaneously determine the amount of compounds 1, 2, and 3 in rose leaves based on SPE purification and UHPLC separation, coupled with MS detection in MRM mode. The procedure described allows for quantitation of endogenous plant hormones in plants, without the need of a derivatization step. The compounds studied are representative of three groups of acidic plant hormones with several important biological properties. This method offers the possibility to incorporate other plant hormones and related metabolites into one single analysis. The simultaneous determination of phytohormones could support scientific efforts in plant functional genomics and hormone signal transduction. Using matrix-matched calibration curves for quantitation, we worked with a woody plant species. We assume that the method is also applicable and improves the analysis of phytohormones proposed earlier<sup>30</sup> in other plant material (e.g., tomato, rice, etc.) as well.

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## ABBREVIATIONS USED

MRM, multiple reaction monitoring; SPE, solid-phase extraction; SSE, signal suppression/enhancement

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