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Simultaneous quantitative LC–ESI-MS/MS analyses of salicylic acid and jasmonic acid in crude extracts of *Cucumis sativus* under biotic stress

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Abstract

Salicylic acid (SA) and jasmonic acid (JA) are plant hormones involved in basal resistance against plant pathogens and also in induced resistance. The aim of this study is to develop a fast and sensitive method to determine simultaneously the levels of both these hormones. The present paper proposes a method that includes hormone extraction with MeOH–H_2O–HOAc (90:9:1, v/v), evaporation of the extracts, and injection into the liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) system in multiple reaction monitoring (MRM). Endogenous SA and JA levels in noninfested control cucumber cotyledons were 30.96 and 0.73 ng g^{-1} fresh weight, respectively. In roots, the levels were 8.31 and 15.82 ng g^{-1} FW, respectively. In plants treated with the biological control agent *Trichoderma asperellum* strain T-34, the levels of SA and JA did not differ from control plants. *Rhizoctonia solani*-diseased cucumber plants showed higher levels of SA and JA compared to noninfested controls (up to 2 and 13-fold higher, respectively). Detection limits for SA and JA were 0.45 and 0.47 ng g^{-1} fresh weight, respectively. The results of our research include the development of a method that is both fast and highly sensitive in the simultaneous quantitation of SA and JA from crude cucumber plant extracts, avoiding any purification and derivatization steps.

Keywords: *Cucumis sativus*; Cucumber; Jasmonic acid; Salicylic acid; LC–ESI-MS/MS; Plant defense; Quantitation; *Rhizoctonia solani*; *Trichoderma* spp.

1. Introduction

Plants have evolved a number of inducible defense mechanisms to respond to both biotic and abiotic stress. Local or systemic resistance is triggered in the majority of plants by pathogen attack, lesions produced by insect feeding, and other kinds of physical damage, as well as certain chemical treatment and the presence of some biological control agents such as nonpathogenic rhizobacteria (Harman et al., 2004). Systemic acquired resistance (SAR) produced by pathogen attack is based on salicylic acid (SA) signaling and leads to pathogenesis-related proteins (PR) and phytoalexin synthesis, which may confer protection against later attacks (Sticher et al., 1997). A similar response is produced when the plant is attacked by a necrotrophic pathogen and/or after insect wounding. In this case, molecular signaling is based on jasmonic acid (JA) and ethylene (Pieterse and Van Loon, 1999). Another kind of JA-dependent response is the so-called induced systemic resistance (ISR), which is produced when the roots are colonized by certain nonpathogenic rhizobacteria. In this latter case, PR and phytoalexins do not accumulate until later pathogen attack, when the plant response is magnified (Van Loon et al., 1998; Pozo et al., 2004). While many SA and JA responses show mutual antagonism, some genes are induced by both compounds, revealing complexities in the network of defense pathways (Delaney, 2004). Thus, the plant hormones JA and SA are major regulators of plant response to pathogen attack. However, there is

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little information about the effect of fungal biological control agents such as *Trichoderma* spp. on these plant hormones (Shoresh et al., 2005). A sensitive and reliable method to quantitate simultaneously such hormones in plant extracts would thus be of special interest for a better understanding of plant defense mechanisms.

Under physiological conditions the plant regulators SA and JA, like all plant hormones, are present at very low concentrations against a background of a wide range of more abundant primary and secondary metabolites. Therefore, analytical methods to quantitate these hormones simultaneously must be extremely selective and sensitive (Chiwocha et al., 2003). Different types of methods have been described to estimate SA and JA in plants. Liquid chromatography (LC), with fluorescence detection has been used successfully for the quantitation of both SA (Meuwly and Metraux, 1993) and JA (Anderson, 1985), but these procedures include complex purification steps in order to separate the desired compounds from a high number of other interfering molecules present in the plant sample as well as a derivatization step (in the case of JA). Gas chromatography coupled to mass spectrometry (GC/MS) has also been applied for the quantitation of SA (Scott and Yamamoto, 1994) and JA (Mueller and Brodschelm, 1994) after derivatization of these molecules. Even though the GC/MS quantitation step shows a high sensitivity for these compounds, these methods rely on elaborate purification and concentration steps. Usually, ion-exchange columns or hydrophobic columns are used to purify partially and to clean the plant samples (Mueller and Brodschelm, 1994; Scott and Yamamoto, 1994; Muller et al., 2002). These time-consuming steps severely limit the number of samples that can be processed in a day. A new GC/MS method for the simultaneous quantitation of SA and JA based on the collection of derivatized and volatilized compounds on polymeric adsorbent (Super Q) as the only purification step has been described (Engelberth et al., 2003) but still relies on both purification and derivatization steps. LC coupled to mass spectrometry (LC/MS) is better suited for the analysis of nonvolatile polar compounds in their natural form (Glassbrook and Ryals, 2001). The selectivity and sensitivity of this method relies on the application of multiple reaction monitoring (MRM), in which each ionized compound gives a distinct precursor-to-product ion transition that is diagnostic for the presence of that particular compound in an extract. Also, the need for complete resolution of compounds prior to analysis is bypassed because peaks containing co-eluting compounds can be resolved by monitoring for specific precursor-to-product ion transitions (Chiwocha et al., 2003). LC–MS has been applied for the quantitation of JA in plant samples (Tamogami and Kodama, 1998) but to our knowledge only one paper describes simultaneous quantitation of both SA and JA by using this technique (Wilbert et al., 1998).

The aim of this study was to develop a method to determine simultaneously SA and JA levels in cucumber cotyledons and roots in a way that would combine speed with the highest accuracy and with low limits of detection. In order to achieve these objectives an LC–MS/MS method is proposed which shows noticeable chromatographic ameliorations with respect to other existing methods. These procedures were tested to evaluate the effect of plant infestation with the necrotrophic pathogen *Rhizoctonia solani* and the biological control agent *Trichoderma asperellum* strain T-34 on the endogenous plant SA and JA levels.

2. Results

2.1. Liquid chromatography–mass spectrometry (LC–MS/MS)

Basic pH conditions as described by Wilbert et al. (1998) gave a poor peak shape for both compounds and SA eluted showing peak tailing. The 5 mM NH_4OAc pH 5/MeCN gradient method produced well-shaped peaks for SA at 2.7 min with good sensitivity. Nevertheless, JA at 7 min presented a high decrease in sensitivity in comparison with the other LC conditions. Finally, acid pH conditions (0.05% HOAc in H_2O/MeCN gradient) with a Discovery C_{18} 150 × 2.1 mm, 5 μm (Supelco, Bellefonte, USA) gave the best results in terms of sensitivity and peak shape. This column has the main advantage of low bleeding at the end of the MeCN gradient compared with the other columns. Retention times in these conditions were 4.0 min for SA and 6.6 min for JA. These conditions allowed the injection of a relatively high number of samples in a short time (up to 100 per day). Fig. 1 shows the overlaid trace chromatograms of cucumber extracts from control plants and Rs-inoculated plants for SA and JA.

The spectra generated for both compounds in negative ion detection gave the deprotonated molecule $[M - H]^-$ (m/z 137 for SA and m/z 209 for JA). The product ion scan spectrum of m/z 137 gave the ion $[M - H - COO]^-$ (m/z 93) for SA. For JA, the product ion scan of m/z 209 gave the $[M - H - COO]^-$ (m/z 165) and the m/z 59. It should be noted that the MRM 209/59 transition gave a signal 10 times greater than the 209/165 at the described MS/MS conditions (CE-25). Quantitation of SA and JA in cucumber plantlets was done by injection of extracted and spiked samples in the LC–ESI(−)-MS/MS system in MRM mode (137/93 for SA and 209/59 for JA). Identification of SA and JA was done on the basis of retention time and presence of peak in the MRM trace compared with those of the standards. When possible, product ion scan experiments were done to confirm positively the presence of both acids in the samples. As can be seen in Fig. 2 an interference that almost coelutes with JA is present in the 209/165 MRM trace in the cucumber extracts and does not allow this trace to be used for quantification. Using these MRM conditions background noise was minimized and sensitivity was very high.

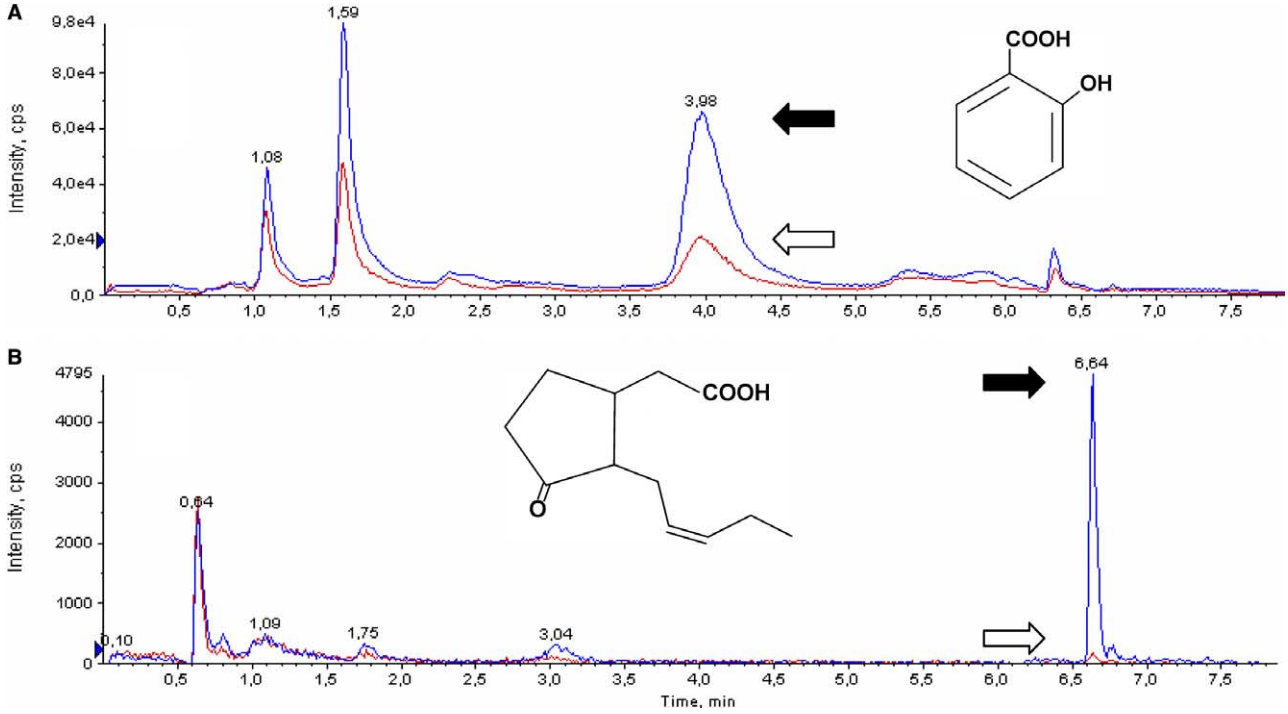

Fig. 1. Overlaid trace chromatograms in MRM mode of 137/93 for salicylic acid in cotyledon samples (A) and 209/59 for jasmonic acid in root samples (B). Empty arrows show the peak corresponding to the control sample. Filled arrows show the peak of the *Rhizoctonia solani*-inoculated sample.

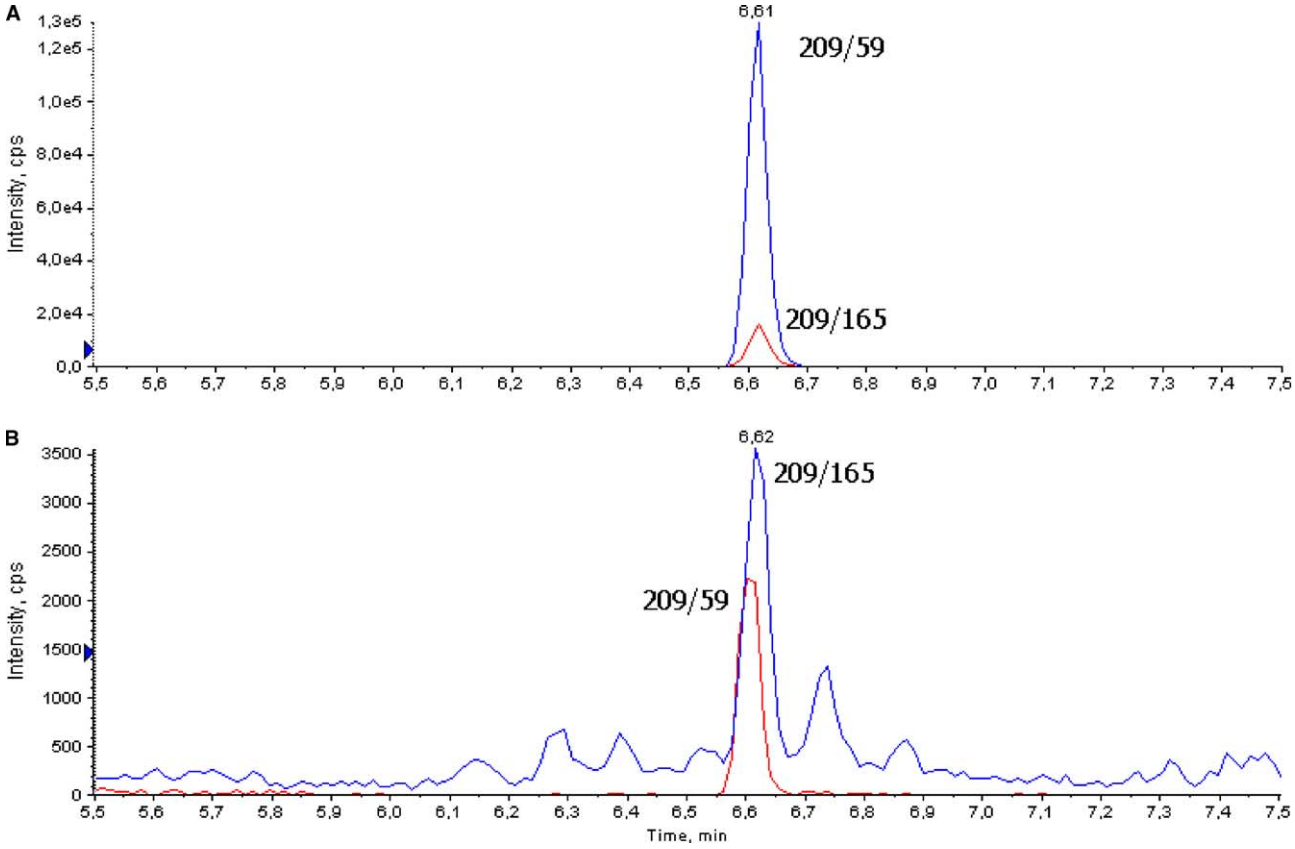

Fig. 2. Trace chromatograms in MRM mode of 209/59 overlaid onto those of 209/165 for a 1 ng μl^{-1} jasmonic acid standard (A) and for a sample of cucumber extract (B).

2.2. Quality parameters of the LC–MS/MS method

In the optimum LC–MS/MS conditions described above, standards of SA and JA (200 and 4 ppb, respectively) were quantified ($n = 10$) on three different days using a standard addition calibration curve (from 50 to 1000 and from 1 to 20 ppb, respectively). Good correlation coefficients ($r \geqslant 0.999$) were obtained in the concentration range studied. The results for reproducibility were a relative standard deviation (RSD) of 1.5% and 3.5% for run-to-run precision, and 2% and 3.5% for day-to-day precision on SA and JA concentrations, respectively. The method also showed good precision with regard to retention time (0.4% and 0.1% for run-to-run and 0.7% and 0.1% in day-to-day for SA and JA, respectively). Detection limit (LOD) based on a signal-to-noise ratio of 3:1 was calculated through the standard addition curves, giving a value of 0.45 and 0.47 ng g^{-1} of SA and JA, respectively, in fresh weight of plant.

2.3. Quantitation of SA and JA in cucumber cotyledons and roots

Incidence of *R. solani* disease at 7 days after seeding was 100% in *R. solani*-treated plants (Rs treatment) and 0% in plants from both control and *T. asperellum* isolate T-34 treatments. Disease severity was 4.0 ± 0.1 (mean ± s.e.) in Rs-infected plants.

Cotyledons from diseased cucumber plants (grown in Rs-inoculated substrate) showed significant higher levels of endogenous SA and JA (up to 2- and 13-fold higher, respectively) than healthy plants (those grown in T-34-inoculated or control substrates) 72 h after seeding (Fig. 3(A) and (C)). In the same way, roots from diseased cucumber plants showed significant higher levels of endogenous SA and JA (up to 2 and 2.5-fold higher, respectively) than healthy plants 72 h after seeding (Fig. 3(B) and (D)). It is noticeable that SA quantities in cotyledons of Rs-treated plants were approximately 4-fold higher than in roots, whereas JA quantities were 4-fold higher in roots than in cotyledons and up to 20-fold higher in roots in the case of healthy plants.

Extractions from lyophilized samples yielded half as many SA levels as those from fresh samples. JA yields were not affected by lyophilization (data not shown).

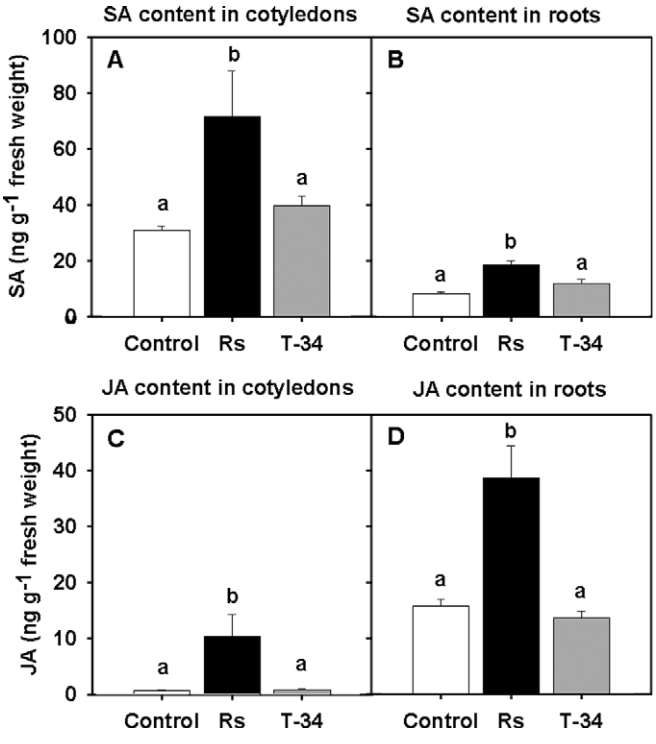

Fig. 3. Salicylic acid (SA) content in cotyledons (A) and roots (B), and jasmonic acid (JA) content in cotyledons (C) and roots (D) of cucumber plants 72 h after seeding. Treatments consisted in different growth media inoculation: Control, noninoculated; Rs, *Rhizoctonia solani*-inoculated; T-34, *Trichoderma asperellum* T-34-inoculated. Values represent the mean ± s.e. ($n = 5$). Different letters indicate statistically significant differences between treatments in a Duncan multiple range test ($P < 0.05$).

3. Discussion

In this paper we present a rapid, sensitive LC–MS/MS method for simultaneous quantitation of SA and JA, avoiding any purification and derivatization steps. The method shows good results in terms of detection limits, repeatability, and linearity. The method presents better LOD for SA than that obtained by Meuwly and Metraux (1993) in cucumber leaves of 4 ng g^{-1} of fresh weight (10-fold more sensitive), and slightly better LOD for JA than that obtained by Rakwal et al. (2002) of 1 pg. Our method showed better chromatographic profiles than those obtained following the method described by Wilbert et al. (1998) which is the only other existing method for simultaneous quantitative LC–MS/MS analysis of JA and SA. Another advantage of the method we describe is its speed and simplicity, allowing the analysis of high numbers of samples.

The SA levels found in healthy cucumber cotyledons and roots in the present article are similar to those obtained in other studies in the same plant species (Molders et al., 1996; Shoresh et al., 2005). Moreover basal SA levels were higher in the cotyledons than in the roots, which is in accordance with other studies (Molders et al., 1996; Shoresh et al., 2005), while JA levels behaved in the opposite way.

Our results show that both SA and JA endogenous levels rise locally (roots) and systemically (cotyledons) in *R. solani*-diseased cucumber plants. The rise in SA after pathogen attack has also been described in some works since the first reports (Malamy et al., 1990; Metraux et al., 1990). Free cucumber SA levels rose both locally and systemically after Tobacco Necrosis Virus inoculation (Molders et al., 1996), while potato inoculation with *Phytophthora infestans* led only to local increases of free SA in the infection site (Coquoz et al., 1995). The local and

systemic rise of free JA in *Arabidopsis* after *Alternaria brassicola* inoculation has also been reported (Penninckx et al., 1996). The increases in SA and JA observed in the diseased cucumber plants in comparison to healthy plants are noticeable (from 2 to 13-fold), since a 59% increase in endogenous SA concentration is sufficient to induce the accumulation of PR protein in tobacco leaves (Yalpani et al., 1991).

The higher increases found for JA in cotyledons, in comparison to those obtained for SA, suggest a major implication of JA in this plant pathogen interaction. This is consistent with the general observation that necrotrophic pathogens' basal resistance is based on JA-related mechanisms (Thomma et al., 1998) while biotrophic or hemibiotrophic basal resistance is based on SA-related mechanisms (Delaney et al., 1994; Kachroo et al., 2000).

The fact that 72 h after seeding SA levels did not rise in T-34-inoculated cucumber plants agrees with the observation made on the same plant species between 0 and 96 h post inoculation with *T. asperellum* strain T203 (Shoresh et al., 2005). However, in the cited article the authors indirectly prove that JA may be playing a role in the induction of resistance by the T203, while in our work we could not observe, at the moment of the analyses, any increase of JA due to the application of T-34.

4. Experimental

4.1. Plant material

Fifteen cucumber seeds (*Cucumis sativus* L. 'Negrito') were placed in each of five 400 ml plastic pots containing peat from Klasmann (Palleter, Spain) amended with 4 g l^{-1} $CaCO_3$. Growth media had been previously inoculated with 2 g l^{-1} of a *R. solani* AG-4 isolate soil inoculum (Rs treatment) prepared according to Ko and Hora (1971) or 5×10^5 cfu ml^{-1} of T-34 isolate from *T. asperellum* (T-34 treatment) (Trillas and Cotxarrera, 2003). We also included a noninoculated treatment (control treatment). Pots were kept in a growth chamber at 25 ± 1 °C under a 16 h photoperiod (200 μE m^{-2} s^{-1}). Plants were fertilized twice a day with 50 ml of the following solution: 0.5 g l^{-1} Peter's foliar feed 27-15-12 from Scotts (Heerlen, The Netherlands) complemented with 0.22 g l^{-1} $CaCl_2$ and 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$.

4.2. Disease evaluation

Seven days after seeding, plants showing crown root damage or damping-off, which are typical symptoms produced by *R. solani*, were considered as diseased plants. Disease incidence was calculated as the percentage of diseased plants over the total number of plants from each pot. Disease severity was assessed using the following scale: 1, healthy plant; 2, light wounds; 3, severe wounds; 4, post emergency damping-off; 5, pre emergency damping-off.

4.3. Chemicals

Standards of salicylic acid >99% (Fluka, Buchs, Switzerland) and (±)-jasmonic acid >97% (Sigma–Aldrich, Steinheim, Germany) were prepared at a concentration of 500 mg l^{-1} in MeOH. The working SA and JA solutions of 1000 and 20 μg l^{-1}, respectively, were made by diluting the standard solutions with the initial LC mobile phase (0.05% HOAc in H_2O–MeCN, 85:15, v/v). MeOH of HPLC grade was purchased from Panreac (Montcada i Reixac, Spain), MeCN of HPLC grade from Sigma–Aldrich (Steinheim, Germany), HOAc from Merck (Darmstadt, Germany), and ultrapure H_2O (Milli-Q) was obtained from Millipore System (Bedford, USA).

4.4. Sample preparation

Seventy-two hours after seeding, the cucumber plantlets were washed under running tap water for 5 min and dried gently. Roots and cotyledons (excised from the shoots) from all the plantlets in each pot were pooled separately and quick-frozen in liquid N_2. Frozen samples were then ground under liquid N_2 with mortar and pestle. An amount of 250 mg of the resulting powder was extracted with 750 μl MeOH–H_2O–HOAc (90:9:1, v/v/v) and centrifuged for 1 min at 10,000 rpm. The supernatant was collected and the extraction was repeated. Pooled supernatants were dried under N_2, resuspended in 200 μl of 0.05% HOAc in H_2O–MeCN (85:15, v/v), and finally filtered with a Millex-HV 0.45 μm filter from Millipore (Bedford, USA). Alternatively, frozen samples were lyophilized and ground with agate mortar and pestle; in this case only 45 mg of the resulting powder was used for the extraction. Quantitation was done by the standard addition method by spiking control plant samples with SA and JA solutions (ranging from 50 to 1000 ng ml^{-1} and from 1 to 20 ng ml^{-1}, respectively), and extracting as described above.

4.5. Liquid chromatography

Analyses were carried out using an Agilent 1100 (Waldrom, Germany) quaternary pump equipped with an autosampler. A Supelco Discovery C_{18} (Supelco, Bellefonte, USA) column (2.1 × 150 mm, 5 μm) was used at ambient temperature and the injected volume was 10 μl. The elution gradient was carried out with binary solvent system consisting of 0.05% HOAc in H_2O (solvent A) and MeCN (solvent B) at a constant flow-rate of 600 μl min^{-1} and a split 1/3. A linear gradient profile with the following proportions (v/v) of solvent B was applied (*t* (min), %B): (0, 15), (3, 15), (5, 100), (6, 100), (7, 15), (8, 15) with 5 min for re-equilibration.

4.6. Mass spectrometry

MS and MS/MS experiments were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex,

Concord, Ont., Canada). All the analyses were performed using the Turbo Ionspray source in negative ion mode with the following settings: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 4 (arbitrary units). For SA analysis we used the following parameters: declustering potential (DP) −30 V, focusing potential (FP) −150 V, entrance potential (EP) −10 V, collision energy (CE) −20 V (137/93), CXP −23 V. For JA analysis the following parameters were used: declustering potential (DP) −37 V, focusing potential (FP) −180 V, entrance potential (EP) −10 V, collision energy (CE) −25 V (209/59), CXP −23 V. Drying gas (N_2) was heated to 400 °C and introduced at a flow-rate of 5000 $cm^3 min^{-1}$ All the MS and MS/MS parameters were optimized in infusion experiments using individual standard solutions of SA and JA at a concentration of 1 ng μl^{-1} diluted in mobile phase A/B (1:1, v/v). These solutions were infused at a flow-rate of 10 µl min^{-1} into the mass spectrometer using a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). Full scan data acquisition was performed scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In product ion scan experiments, MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the second analyzer of the instrument. In negative mode, the spectrum for SA and JA gave the deprotonated molecule $[M - H]^-$. Quantitation was performed by injection of samples in MRM mode, because many compounds could present the same nominal molecular mass. Thus, the combination of the parent mass and unique fragment ions was used to monitor selectively SA and JA in crude plant extracts. MRM acquisition was done by monitoring the 137/93 and 209/59 transitions for SA and JA, respectively; with a dwell time of 1000 ms for each transition.

4.7. Other LC methods used for comparison

Different LC conditions were tested in order to obtain the highest sensitivity and system performance. Basic pH conditions as described by Wilbert et al. (1998) were first tested using a mobile phase 1% NH_3/5 mM NH_4OAc in H_2O in a XTerra MS C_{18} 50 × 2.1 mm, 3.5 µm (Waters, Milford, MA, USA) column. A Gemini 250 × 4.6 mm, 5 µm (Phenomenex, Torrance, CA, USA) column was also tested with a 5 mM NH_4OAc pH 5/MeCN gradient (using a flow-rate of 1.2 ml min^{-1}).

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