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TECHNOLOGY REVIEW

Signaling Substances Used in Plant Defense: HPLC-MS/MS Analysis of Jasmonates

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Introduction

Plants must be capable of responding to climatic fluctuations, diurnal rhythms, available supplies of water and nutrients, and insect attacks and infestations. To ensure such responses, plants need a network of regulating substances called phytohormones. These substances enable plants to respond to both biotic and abiotic stresses by initiating a cascade of orchestrated actions, and to trigger development-specific processes. In this article, we will discuss a highly sensitive analytical method for quantitative determination of phytohormones. The main representatives of the plant hormones are jasmonic acid (JA), cytokines, auxins, abscisic acid, salicylic acid, gibberellins, and strigolactones.

The phytohormone JA plays a pivotal role as a wound-signaling substance in the defense against chewing insects. In the leaf, chewing or sucking insects (**Figure 1**) induce the activation of JA biosynthesis and thereby the accumulation of JA. All JA derivatives, produced by methylation, glycosylation, hydroxylation, or esterification with amino acids are subsumed under the term "jasmonates." The amino acid ester derivative of isoleucine, jasmonoyl-isoleucine (JA-Ile), plays a key role in this. JA-Ile represents the biologically active form of the phytohormone.[1] It triggers a number of immune responses, like the production of locally toxic or digestion-inhibiting substances. After insect attack, plants can synthetize proteinase inhibitors that impair the activity of digestive proteinases in the gut of the feeding insect.[1] Volatile substances, such as methyl jasmonate, are emitted to attract predators (*e.g.*, chewing enemies, predatory insects) of the plant pests.[2] Moreover, the jasmonates work like mobile alarm signals that put the not-infested plant parts of an infested plant into a heightened state of defensive readiness.[3]

It is important to keep this defensive state narrowly limited in time because this energy-intensive process can diminish plant growth and its adaptability to other stress conditions. Regulating the local accumulation of JA and the biologically active JA-Ile signal molecule demands strict control of the synthesis, transport, and degradation of the substances. Hence, the characterization of the substrate and breakdown products of JA metabolism and their quantification in wounded, stressed, or infested plant tissues is the focus of current research.

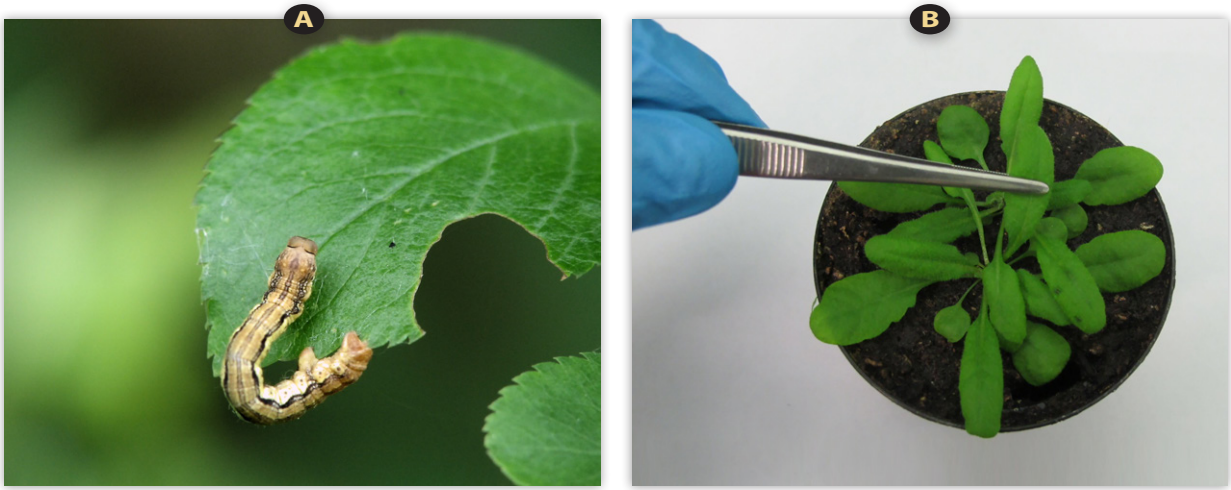

FIGURE 1. Damage to the leaf caused by chewing or sucking insects **(A)** triggers the synthesis of the wound signaling substance, jasmonic acid. Mechanical wounding of the leaves **(B)** can simulate insect herbivory. This stimulus likewise leads to an accumulation of jasmonic acid in the leaves.

(**Image credits: (A)** © Michael Martini, Fotolia.com; and **(B)** Department for Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Sciences, Georg-August University of Goettingen, Germany.)

HPLC-MS/MS Analysis of Jasmonates

Extremely sensitive analyses are required to investigate the wound response because phytohormones in plant tissue are only present in minute concentrations (<10 nmol/g fresh weight [FW]). Besides this, the plant matrix constitutes a highly complex mixture of substances which can encumber detection and quantification of the sparsely abundant analytes. High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is well-suited for the challenging analytical task of separating the metabolites of the leaf extract through a column according to their chemical structure. In the case of the reversed-phase chromatography process used in this study, the substances are bound by hydrophobic interactions to the stationary phase of the column and eluted using an ascending gradient of organic solvent. Next, the eluted compounds are detected by mass spectrometry. The specific retention time in which a substance is eluted and its mass-to-charge ratio (*m/z*) captured by the mass spectrometer are used to identify a compound. A tandem mass spectrometer is operated in the multiple-reaction monitoring (MRM) mode for this purpose. The *m/z* of the intact deprotonated molecule ($[M\text{-}H]^-$), in conjunction with the *m/z* of a specific fragment of this ion, is used to unequivocally identify the chemical compound.

In HPLC-MS applications, ultrapure water or ultrapure eluents (LC-MS grade) are indispensable materials given that the sensitivity and reliability of the method are critically dependent on the purity of the eluents.

Quantification of Jasmonates and Precursors

The shock-frozen sample material was extracted in a two-phase partitioning system using a mixture of tert-butyl methyl ether, methanol, and water.[4] For quantification, defined quantities of the corresponding deuterated phytohormone standards were added. After extraction, the organic phase was used for analysis. This was performed on an HPLC/nanoESI-MS/MS system consisting of an Agilent 1100 HPLC system connected to an Applied Biosystems/SCIEX 3200 QTRAP mass spectrometer featuring hybrid triple quadrupole/linear ion trap capabilities.[5] During the process, nano-electrospray ionization (nanoESI) was ensured by a chip-based ion source (TriVersa NanoMate; Advion) (**Figure 2**).

The reversed-phase HPLC separation was performed using a C_{18} column (EC 50/2 Nucleodur C_{18} gravity 1.8 µm; 50 × 2.1 mm, particle size 1.8 µm; Machery and Nagel) and a binary solvent gradient of acetonitrile (Fisher Scientific) and ultrapure water from the arium® pro VF TOC system (Sartorius) each with 0.1% acetic acid. The phytohormone species were detected in the negative ESI mode by multiple-reaction monitoring. For quantification, calibration curves were plotted based on the intensity ratios (*m/z*) of the unlabeled substances to the corresponding deuterium-labeled substances vs. the molar quantities of the unlabeled substances.

FIGURE 2. HPLC/nanoESI-MS/MS system for jasmonate analytics.

An Applied Biosystems/SCIEX 3200 QTRAP featuring hybrid triple quadrupole/linear ion trap capabilities was used for the mass spectrometry analysis. NanoESI was ensured by the TriVersa NanoMate, a chip-based ion source. The Agilent HPLC system is not shown.

(**Image credit:** Department for Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Sciences, Georg-August University of Goettingen, Germany.)

Production of Ultrapure Water

The arium pro VF TOC system (**Figure 3**) was used to produce ultrapure water for HPLC/nanoESI-MS/MS analysis. The unit removes any impurities still present in pretreated potable water. Ultrapure water production requires continuous recirculation of the water through the system. The additionally required constant water flow is accomplished by a pump system equipped with a pressure control unit. The conductivity of the water is measured at both the feed water inlet as well as at the product water outlet. The TOC (total organic carbon) content is monitored by a special TOC monitor. The arium pro VF TOC system used in the experiments conducted in this study works with two different cartridges. They are filled with a special activated carbon adsorber and mixed-bed ion exchange resins which are capable of supplying ultrapure water with a TOC content as low as <2 ppb. Furthermore, a UV lamp is integrated in the unit which emits wavelengths of 185 nm and 254 nm to exert an oxidizing and germicidal action. Additionally, an ultrafiltration module is installed in the arium pro VF TOC system and operated as a crossflow filter. The ultrafiltration membrane used retains colloids, microorganisms, endotoxins, as well as RNA and DNA. A 0.2 µm final filter is installed at the water outlet to remove particulate matter and bacteria from the ultrapure water produced during batching. The process of device-specific water purification is presented in **Figure 4**.

Ultrapure water with a TOC content <5 ppb and a conductivity of 18.2 MΩ×cm (compensated to 25°C) was used for the HPLC/nanoESI-MS/MS analysis.

FIGURE 3. The arium pro VF TOC ultrapure water system. (**Image credit:** Sartorius)

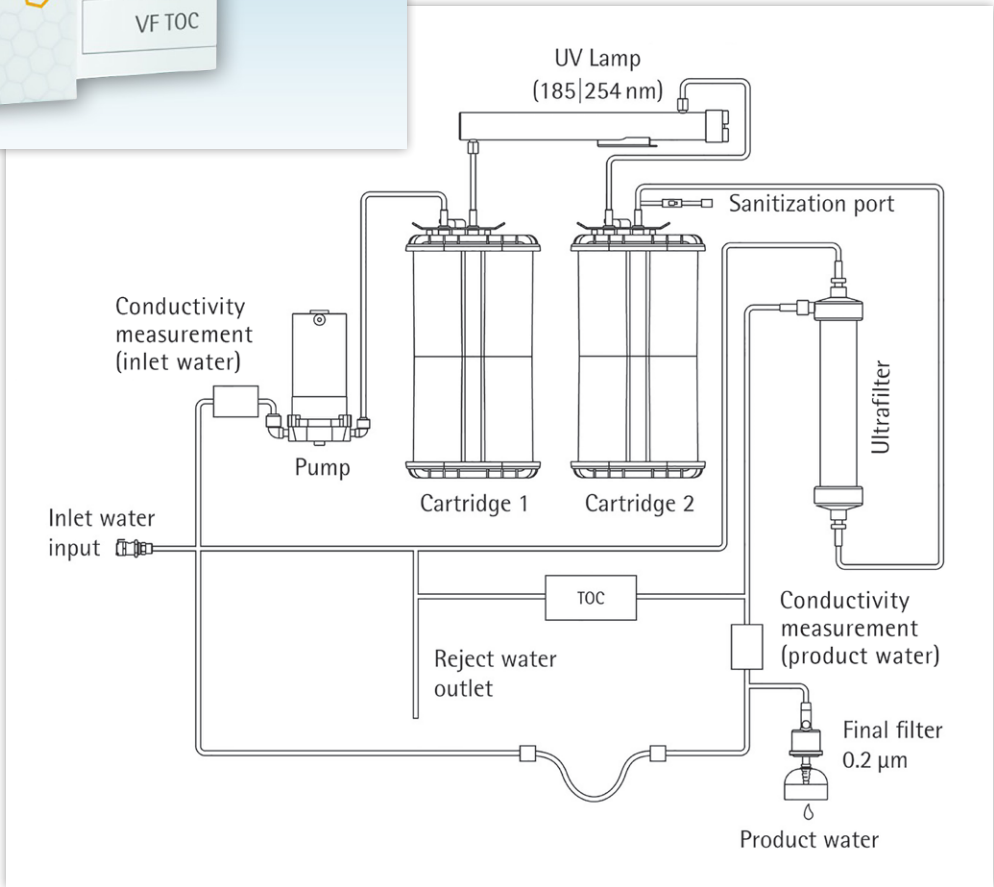

FIGURE 4. Schematic depiction of the arium pro VF TOC ultrapure water system. For enhanced clarity, the valves and their controls are not illustrated.

Results

The wound response of plants has been intensively investigated in many plant species, like tomato (*Lycopersicum esculentum*), tobacco (*Nicotiana tabacum*) and mouse ear cress (*Arabidopsis thaliana*). For our analyses, we used the plant model of *A. thaliana*. Rosettes of *A. thaliana* were wounded with saw-toothed forceps over the midrib of the leaf (**Figure 1B**) and harvested 30 minutes or 120 minutes post-wounding, respectively. The rosettes of non-wounded plants served as controls.

In addition to JA and the biologically active derivative, JA-Ile, their biosynthesis precursors 12-oxo-phytodienoic acid (OPDA) and 3-oxo-2-(2-pentenyl)-cyclopentane-1-tetranoic acid (OPC-4) were also included in the analysis (**Figure 5**). Quantitative detection of these metabolites in the plant tissue by HPLC/nanoESI-MS/MS analysis requires the addition of a defined amount of deuterated substances of an identical or very similar chemical structure (D_5-OPDA, D_6-JA, and D_3-JA-Leu) as internal standards.

For this purpose, before starting extraction, the standard substances were added in quantities of 30 ng (D_5-OPDA) or 10 ng (D_6-JA and D_3-JA-Leu) to 200 mg plant material.

The signals of the analytes detected by HPLC/nanoESI-MS/MS analysis (OPDA, OPC-4, JA, and JA-Ile) and the corresponding deuterated standard substances are depicted in **Figure 6** as extracted ion chromatograms (EIC). OPC-4, JA-Ile were not detectable in the non-wounded controls. Only the respective deuterated standards and minor quantities of OPDA were detected (**Figure 6A**). By contrast, clear signals from the endogenous jasmonates (JA and JA-Ile/Leu) and weaker signals from the precursors (OPC-4 and OPDA) were measured in the wounded plants (**Figure 6B**).

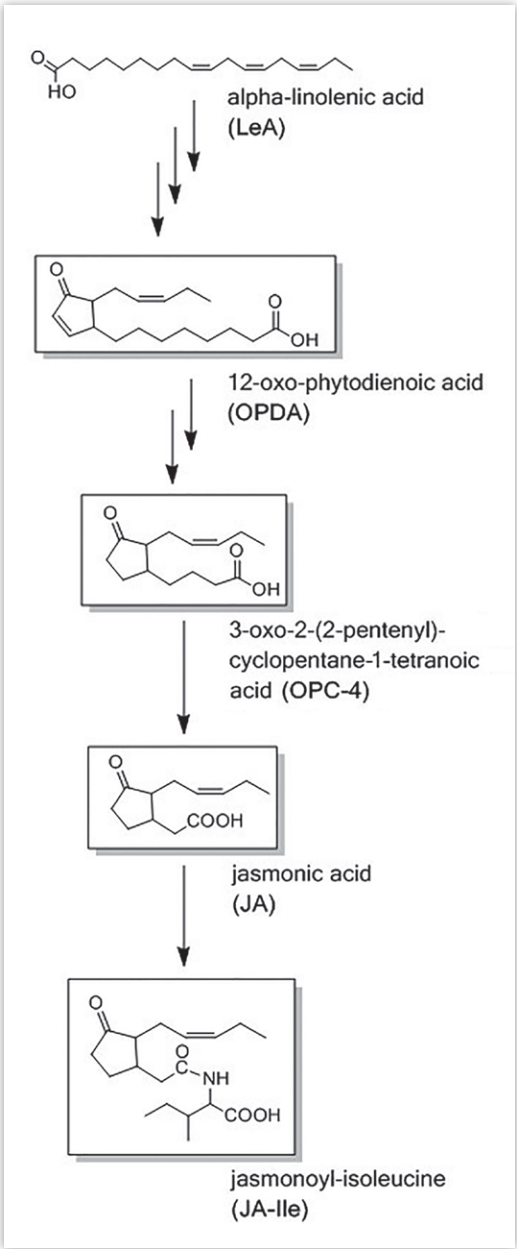

FIGURE 5. Biosynthesis of the JA and the active derivative JA-Ile via the precursors, alpha-linolenic acid (LeA), OPDA, and OPC-4.

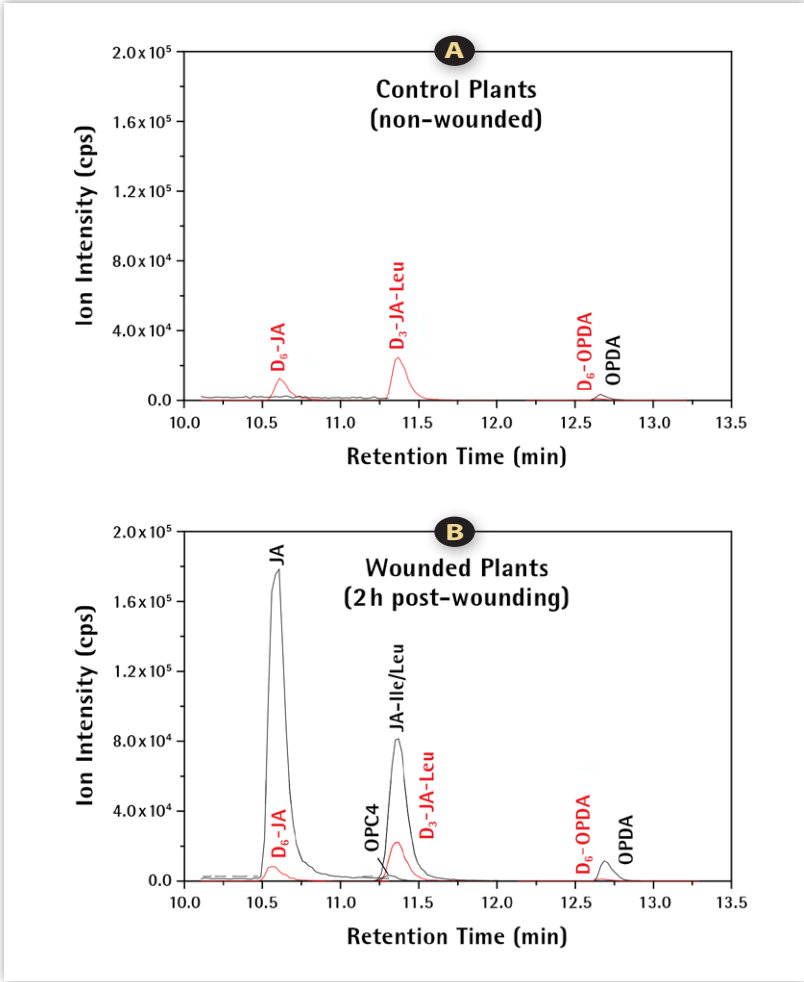

FIGURE 6. EICs from the HPLC/nanoESI-MS/MS analysis (OPDA, OPC-4, JA, and JA-Ile/Leu) and the corresponding deuterated standard substances (D_5-OPDA, D_6-JA, and D_3-JA-Leu). **(A)** Non-wounded controls. **(B)** Wounded plants.

HPLC/nanoESI-MS/MS analysis permits exact quantification of JAs and their precursors (OPDA and OPC-4) in the leaf extracts through the use of the respective deuterated standards. Given that a wounding stimulus in plants is perceived and processed within minutes[3], the OPDA, OPC-4, JA, and JA-Ile/Leu accumulated intensely in the *A. thaliana* rosettes as early as 30 minutes post-wounding (**Figure 7**). In this experiment, the concentration of JA was now 2.2 nmol/g FW. JA-Ile/Leu was present in concentrations of 0.8 nmol/g FW. The concentration of these two jasmonates increased over the next 90 minutes negligibly to 2.5 and 1.2 nmol/g FW, respectively. The precursors OPDA and OPC-4 also accumulated in parallel to this time curve (**Figure 7**). By the method of HPLC/nanoESI-MS/MS, analyses presented here using the ultrapure water produced by the arium pro VF TOC system, the detection limit of the jasmonates and their precursors in the leaf tissue of *A. thaliana* was 1 pmol/g FW for OPDA and JA-Ile/Leu, and 15 pmol/g FW for OPC-4 and JA.

Discussion

The method of HPLC-coupled tandem mass spectrometry described here allows the quantitative analysis of phytohormones in the picomolar concentration range. The high sensitivity and specificity required of the method also places special demands on the quality of the employed solvents. During chromatographic separation, the respectively used gradient of the solvents is responsible for the stepwise elution of the analytes in the column. In the mass spectrometer source, the solvents are then evaporated by a gas flow, and the analytes, transferred as free ions to the gas phase. In the case of positive ionization, the analytes are ideally present in protonated form ($[M+H]^+$), whereas in negative ionization, they are deprotonated ($[M-H]^-$). Nevertheless, the uptake of foreign ions (*e.g.*, Na^+, NH_4^+, Cl^-, CH_3COO^-) can also produce quasi-molecular ions during ESI. The electrical field can likewise transport these ions into the mass spectrometer which then generate detector signals. That is why problems arise when ion impurities are present in the solvent leading to a number of potential adducts of the analyte. In particular, sodium cations play a meaningful role in adduct formation. In competition with $[M+H]^+$ and/or $[M-H]^-$, they form, among others, $[M+Na]^+$ or $[M+Na+\text{acetic acid-2H}]^-$ adducts. When the adduct formation is intense, a correspondingly strong reduction takes place in the quantity of protonated and/or deprotonated ions (which are generally used for analysis). This adduct formation can substantially diminish the sensitivity of a method and negatively impact the analyte detection limit. No matter how high the purity (*e.g.* LC-MS grade), longer storage of water in glass bottles can cause sodium cations to be released from the glass. This can also generate an increased adduct formation during analysis. Thanks to the use of the arium pro VF TOC system, this problem was avoided because freshly treated ultrapure water was continually available for the HPLC/nanoESI-MS/MS analyses. Preliminary experiments (April 23, 2013) showed that arium pro VF TOC ultrapure water had a significantly lower TOC content—averaging 3.82 ppb—than did LC-MS-grade water out of freshly opened bottles from commercial vendors, averaging 45.5 ppb TOC. These findings were also confirmed by the analyses of Tarun *et al.*[6], who proved that commercially purchased bottled water for HPLC analysis had higher levels of organic impurities as compared to freshly processed ultrapure water.

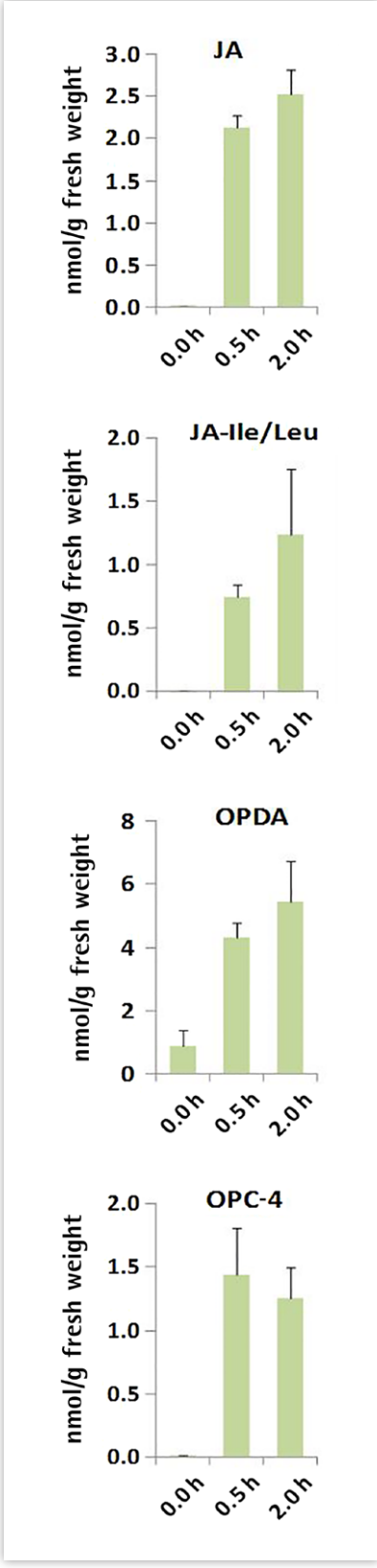

FIGURE 7. Quantitative analysis of JA, JA-Ile/Leu, and the precursors OPDA and OPC-4, in leaf extracts of wounded plants, and the corresponding *A. thaliana* controls. The rosettes of the plants to be analyzed were harvested (non-wounded) at the beginning of the experiment, as well as 30 and 120 minutes post-wounding, respectively, and then shock-frozen in liquid nitrogen immediately afterward. Following extraction, the analytes were quantified by analysis using HPLC/nanoESI-MS/MS.

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