



# Validated Method for Strigolactone Quantification by Ultra High-Performance Liquid Chromatography - Electrospray Ionisation Tandem Mass Spectrometry Using Novel Deuterium Labelled Standards

Stephanie Boutet-Mercey, François Perreau, Amélie Roux, Guillaume Clavé, Jean-Paul Pillot, Isabelle Schmitz-Afonso, David Touboul, Gregory G. Mouille, Catherine Rameau, François-Didier Boyer

## ► To cite this version:

Stephanie Boutet-Mercey, François Perreau, Amélie Roux, Guillaume Clavé, Jean-Paul Pillot, et al.. Validated Method for Strigolactone Quantification by Ultra High-Performance Liquid Chromatography - Electrospray Ionisation Tandem Mass Spectrometry Using Novel Deuterium Labelled Standards. *Phytochemical Analysis*, 2018, 29 (1), pp.59-68. 10.1002/pca.2714 . hal-02324805

**HAL Id: hal-02324805**

**<https://hal.science/hal-02324805>**

Submitted on 23 Mar 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - ShareAlike 4.0 International License

**Title: Validated Method for Strigolactone Quantification by Ultra High-performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry Using Novel Deuterium Labelled Standards**

**Short title: Strigolactone Quantification Using Novel Deuterium Labelled Standards**

Stéphanie Boutet-Mercey,<sup>a</sup> François Perreau,<sup>a</sup> Amélie Roux,<sup>b</sup> Guillaume Clavé,<sup>b</sup> Jean-Paul Pillot,<sup>a</sup> Isabelle Schmitz-Afonso,<sup>b,c</sup> David Touboul,<sup>b</sup> Grégory Mouille,<sup>a</sup> Catherine Rameau<sup>a</sup> and François-Didier Boyer<sup>a,b\*</sup>

\*Correspondence to: François-Didier Boyer, <sup>a</sup>Institut Jean-Pierre Bourgin, INRA, AgroParisTech, CNRS, Université Paris-Saclay, RD10, F-78026 Versailles, France

or <sup>b</sup>Institut de Chimie des Substances Naturelles, CNRS UPR2301, Univ. Paris-Sud, Université Paris-Saclay, 1 av. de la Terrasse, F-91198 Gif-sur-Yvette, France

E-mail: [francois.boyer@inra.fr](mailto:francois.boyer@inra.fr), [francois-didier.boyer@cnrs.fr](mailto:francois-didier.boyer@cnrs.fr)

Telephone: 33 1 69 82 30 17

Fax: 33 1 69 07 72 47

<sup>a</sup>Institut Jean-Pierre Bourgin, INRA, AgroParisTech, CNRS, Université Paris-Saclay, RD10, F-78026 Versailles, France

<sup>b</sup>Institut de Chimie des Substances Naturelles, CNRS UPR2301, Univ. Paris-Sud, Université Paris-Saclay, 1 av. de la Terrasse, F-91198 Gif-sur-Yvette, France

<sup>c</sup>Normandie Univ, COBRA, UMR 6014 and Univ Rouen, INSA Rouen, CNRS, FR3038, IRCOF, 1 Rue Tesnière, F-76821 Mont St Aignan, France

**Short Abstract:** Strigolactone (SL) plant hormones control plant architecture. They are difficult to analyze because they occur in very small concentrations especially in comparison with other plant hormones. A method was developed for determining endogenous fabacyl acetate and orobanchyl acetate in plant tissue based on novel deuterium labelled standards and orobanchol using a synthetic SL GR24 as internal standard.

**Abstract:**

**Introduction** – Strigolactones (SLs) are important plant hormones. They are difficult to analyze because they occur in very small concentrations especially in comparison with other plant hormones and other substances can interfere with their detection.

**Objective** – Developing a procedure for the extraction, purification and quantification of SLs from plant roots.

**Methodology** – Samples were prepared by extraction of plant root tissues with ethyl acetate. Then the extracts were further purified with silica column chromatography. The natural SLs in the final extracts were quantified using novel deuterium labelled SLs. The results of the methodology were compared with those of the Yoneyama et al. procedure.

**Results** – This procedure required about 1-g root samples to detect and quantify simultaneously the SLs (orobanchyl acetate and fabacyl acetate) concentration with high reliability.

**Conclusion** – A method was developed for determining endogenous fabacyl acetate and orobanchyl acetate in plant tissue based on novel deuterium labelled standards. A method of orobanchol quantification using a synthetic SL GR24 as internal standard was proposed.

**Keywords:** Strigolactones, Plant hormones, Deuterium-labelled strigolactones, Pea, Ultra high-performance liquid chromatography-electrospray ionization tandem mass spectrometry

## Introduction

Plant hormones are key signalling biomolecules acting at lower concentrations ( $\mu\text{M}$  to  $\text{pM}$ ) to regulate numerous aspects of plant growth and development. In the past decades, structurally diverse plant hormones including auxins, cytokinins, abscisic acid, gibberellins, ethylene, brassinosteroids, jasmonates and salicylic acid have been identified (Davies 2010). Strigolactones (SLs), terpenoid-derived compounds are the most recent class of hormones identified in plants initially for their role in the control of shoot branching (Gomez-Roldan, et al. 2008, Lopez-Obando, et al. 2015, Umehara, et al. 2008). In addition, these compounds have long been known for their roles as allelochemicals in symbiotic and parasitic interactions (Xie, et al. 2010) in the rhizosphere. SLs are widely distributed in the plant kingdom. To date, more than 20 natural SLs (Lopez-Obando, et al. 2015, Xie 2016) have been isolated from plants including non-vascular plants, the moss *Physcomitrella patens* and liverworts and Charales (Lopez-Obando, et al. 2015). The structural core of the SLs is a tricyclic lactone (ABC part) connected via an enol ether bridge to an  $\alpha,\beta$ -unsaturated furanone moiety (D ring) (Figure 1). These compounds have one or two methyl groups on the A ring and one or more hydroxyl or acetoxyl groups in the A/B rings but always with the same C-D moiety. Carlactone, biosynthesized from all-*trans*- $\beta$ -carotene, is the common precursor of SLs (Lopez-Obando, et al. 2015) (Figure 1). SLs are highly hydrolysable carotenoid derivatives rapidly leading to inactive derivatives (formyl ABC tricyclic lactone and hydroxymethylbutenolide) in an aqueous medium. In order to study the functions of plant hormones, it is important to determine the concentrations of endogenous plant hormones (Novak, et al. 2017). Plant hormones are generally difficult to analyze because they occur in very small concentrations and abundant matrices can interfere with their detection. SL concentrations in root exudates or *in planta* have been measured at extremely low levels (Sato, et al. 2005) in comparison with the other plant hormones (Davies 2010) and they may be unstable during the purification process due to their sensitivity to hydrolysis in an aqueous medium (Boyer, et al. 2012). From cotton seedlings, strigol and strigyl acetate production are about 15 and 2 pg/plant/day (Sato, et al. 2005). An analytical method for plant hormones requires an efficient extraction procedure and a highly sensitive and selective analytical system. Advances in liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) have facilitated the rapid identification of novel SLs (Sato, et al. 2003). SLs have

been detected in various plants (Xie 2016). It has been discovered that the production of SLs are strongly enhanced in low phosphorus (P) culture conditions (Yoneyama, et al. 2012) allowing their detection. Plants have developed this strategy to enhance the host recognition by symbiotic arbuscular mycorrhizal fungi from which plants benefit for their P nutrition.

Determining the accurate concentration of SLs in different parts of plants is extremely challenging. To date, SLs have been mostly quantified from different plants in root exudates (pea (Braun, et al. 2012, Foo and Davies 2011, Foo, et al. 2013, Pavan, et al. 2016), red clover, lettuce, wheat, marigold, chinese milk vetch, alfalfa, tomato (Yoneyama, et al. 2012)) and in some cases in root tissues (pea (Foo and Davies 2011, Foo, et al. 2013), tomato (Visentin, et al. 2016), sorghum (Yoneyama, et al. 2016)) through direct analysis after concentration or through the use of an intermediate purification step with solid-phase extraction (SPE). In all cases, the authors used methods derived from the developed procedure by Yoneyama and coworkers (Sato, et al. 2003). But the latter method suffers from the absence of labelled standards that has hindered precise quantifications of SLs. Their method was based on the standard addition procedure taking into account the matrix effects due to the complex crude extracts without extensive validation in terms of linearity, trueness and precision as examples. Few isotope-labelled standards of low quality, i.e., only partially labelled (3a,4,4,5,5,6'-<sup>2</sup>H<sub>6</sub>-5-deoxystrigol) (Ueno, et al. 2010) or those containing a single <sup>2</sup>H or two <sup>13</sup>C (6'-<sup>2</sup>H-orobanchol, 6'-<sup>2</sup>H-orobanchyl acetate, 6'-<sup>2</sup>H-fabacyl acetate, <sup>13</sup>C<sub>2</sub>-strigol) (Foo and Davies 2011, Proust, et al. 2011), or those available through laborious multi-step syntheses adapted from the syntheses of non-labelled SLs (Zwanenburg, et al. 2016) and limited to labelled 5-deoxystrigols (7'-<sup>2</sup>H<sub>3</sub>-5-deoxystrigol, 7'-<sup>2</sup>H<sub>3</sub>, <sup>13</sup>C-5-deoxystrigol, 7'-<sup>13</sup>C-5-deoxystrigol) and 2'-epimers (Cheng, et al. 2015) have been described.

We are especially interested in the SLs in pea, our plant model to study shoot branching (Boyer, et al. 2012, Gomez-Roldan, et al. 2008). Five SLs have been characterized in pea (Xie 2016) (Figure 1), belonging to the orobanchol-type SL with an  $\alpha$ -oriented C ring. Only orobanchol (**1**), orobanchyl acetate (**2**) and fabacyl acetate (**4**) have already been quantified in root exudates (Braun, et al. 2012, de Saint Germain, et al. 2016, Pavan, et al. 2016) and root tissues (Foo and Davies 2011, Foo, et al. 2013). In (Braun, et al. 2012, de Saint Germain, et al. 2016, Foo and Davies 2011, Foo, et al. 2013) the pea SLs were quantified using 6'-<sup>2</sup>H-orobanchol, 6'-<sup>2</sup>H-orobanchyl acetate and 6'-<sup>2</sup>H-fabacyl acetate. In (Pavan, et al. 2016) the quantifications were performed using <sup>13</sup>C<sub>2</sub>-strigol as an internal standard. In both cases, the

SL concentration levels in low P culture conditions were found similar (0.1 to 10,000 pg.g FW<sup>-1</sup> (fresh weight) depending on each natural SL).

We describe here the synthesis of deuterium labelled orobanchyl and fabacyl acetates, according to fast and efficient procedures leading to standards following the requirements for MS quantification. These are standards with 3 deuteriums to avoid overlapping the isotopic patterns between the endogenous SLs and the labelled internal standards. In addition, we have developed a procedure for the extraction and purification of **1**, **2**, **4** from different pea genotypes. We propose a quantification method for orobanchol (**1**), for which no 3-deuterium labelled standard was available, using the synthetic SL GR24 (Figure 1) as internal standard. Quantification of orobanchyl acetate (**2**) and fabacyl acetate (**4**) were performed using our corresponding deuterium labelled SLs. SL levels were measured and the results compared with those of the published procedure (Foo, et al. 2013). Our protocol provided a practical and validated method to study the levels of different endogenous SL plant hormones.

## Experimental

### Reagents and instruments for the SL standards preparation

Analytical thin-layer chromatography (TLC) was performed on plates precoated with silica gel layers. Compounds were visualized by illumination with a short wavelength UV lamp (i.e.,  $\lambda = 254$  nm), Nuclear magnetic resonance spectra (<sup>1</sup>H; <sup>13</sup>C) were recorded respectively at [300; 75] MHz on a Bruker DPX 300 spectrometer. For the <sup>1</sup>H spectra, data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet), coupling constant in Hz and integration. IR spectra are reported in reciprocal centimeters (cm<sup>-1</sup>). Mass spectra (MS) and high-resolution mass spectra (HRMS) were acquired by electrospray ionization (ESI) coupled to a time-of-flight analyzer (Waters LCT Premier XE). Several chromatographic systems were used for the analytical experiments and the purification steps. Analytical UHPLC was performed on an Acquity Waters UPLC system equipped with a PDA and a mass spectrometer detector. Semi-preparative HPLC was performed on a Waters system equipped with 600E pump system, a Waters 2767 sample manager, injector and collector and a waters PDA 2996 UV/Vis detector. (±)-Orobanchol was obtained from OlChemIm Ltd (Olomouc, Czech Republic), <sup>2</sup>H<sub>6</sub>-acetic anhydride, <sup>2</sup>H<sub>3</sub>-iodomethane and the other reagents were purchased from Sigma-Aldrich Co (Saint Quentin Fallavier, France). (±)-GR24 (**7**) was prepared according to the (Magnus, et

al. 1992) procedure. (±)-Orobanchyl acetate (**2**), (±)-fabacol (**3**) and (±)-fabacyl acetate (**4**) were synthesized from (±)-orobanchol by acetylation with acetic anhydride in pyridine and according to the (Xie, et al. 2009) procedure, respectively.

## Deuterium labelled SL preparation and characterization

### Preparation of (±)-7'-<sup>2</sup>H<sub>3</sub>-GR24 (**10**)

Tricyclic lactone **9** (Mangnus, et al. 1992) (112 mg, 0.55 mmol, 1 equiv), 6-<sup>2</sup>H<sub>3</sub>-5-Bromo-3-methyl-2(5H)-furanone (**8**) (see Supporting Information) (100 mg, 0.55 mmol, 1 equiv.), and anhydrous K<sub>2</sub>CO<sub>3</sub> (153 mg, 1.11 mmol, 2 equiv) were dissolved in anhydrous acetone (5 mL). The reaction mixture was stirred for 18 h and concentrated under reduced pressure. The residue was dissolved in EtOAc (10 mL), filtered, evaporated under reduced pressure, and purified by chromatography on a silica gel (EtOAc/heptane 0/10 to 3/7 v/v) to give the desired product **10** (30 mg, 18%) and its 2'-epimer (37 mg, 22%) as white amorphous solids. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.48 (m, 1H, *H*Ar), 7.45 (s, 1H, *H*6'), 7.19-7.34 (m, 3H, *H*Ar), 6.95 (d, 1H, *J* = 1.3 Hz, *H*3'), 6.15 (d, 1H, *J* = 1.3 Hz, *H*2'), 5.92 (d, 1H, *J* = 7.7 Hz, *H*8b), 3.92 (m, 1H, *H*3a), 3.41 (dd, 1H, *J* = 17.0 Hz, *J* = 9.4 Hz, *H*4α), 3.08 (dd, 1H, *J* = 17.0 Hz, *J* = 3.4 Hz, *H*4β). <sup>13</sup>C NMR (75.5 MHz, CDCl<sub>3</sub>) δ: 171.4 (Cq, C2), 170.4 (Cq, C5'), 151.2 (CH, C6'), 142.8 (CH, C8a), 141.2 (CH, C3'), 139.0 (CH, C4a), 136.1 (Cq, C4'), 130.2 (CH, CAr), 127.7 (CH, CAr), 126.6 (CH, CAr), 125.3 (CH, CAr), 113.5 (Cq, C3), 100.8 (CH, C2'), 86.1 (CH, C8b), 39.0 (CH, C3a), 37.5 (CH<sub>2</sub>, C4), 9.7 (Cq, C7'). IR ν (film, cm<sup>-1</sup>): 1786, 1733, 1675. HRMS (ESI): Calculated for C<sub>17</sub>H<sub>12</sub>D<sub>3</sub>O<sub>5</sub> [M + H]<sup>+</sup>: *m/z* 302.1108. Found: *m/z* 302.1115.

### Preparation of (±)-<sup>2</sup>H<sub>3</sub>-orobanchyl acetate (**11**)

To a solution of (±)-orobanchol **1** (1 mg, 2.89 mmol) in pyridine (0.2 mL), was added <sup>2</sup>H<sub>6</sub>-acetic anhydride (0.2 mL). The mixture was stirred overnight at room temperature. After several coevaporations with toluene, the residue was purified by preparative chromatography on silica gel with 40% EtOAc in heptane (v/v) to give <sup>2</sup>H<sub>3</sub>-orobanchyl acetate (**11**) (1.09 mg, 96%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.46 (d, 1H, *J* = 2.9 Hz, *H*6'), 6.94 (t, 1H, *J* = 1.5 Hz, *H*3'), 6.15 (t, 1H, *J* = 1.5 Hz, *H*2'), 5.74 (s, 1H, *H*4), 5.61 (d, 1H, *J* = 7.8 Hz, *H*8b), 3.45 (ddd, 1H, *J* = 7.3 Hz, *J* = 2.9 Hz, *J* = 2 Hz, *H*3a), 2.03 (t, 3H, *J* = 1.5 Hz, *H*7'), 1.96 (m, 2H, *H*5), 1.70 (m, 2H, *H*6), 1.43 (m, 2H, *H*7), 1.16 (s, 3H, *H*9), 1.14 (s, 3H, *H*10). <sup>13</sup>C NMR (75.5

MHz, CDCl<sub>3</sub>)  $\delta$ : 171.8 (Cq, C1'), 170.7 (Cq, C5'), 170.1 (Cq, C2), 150.4 (CH, C6'), 140.9 (CH, C4a), 136.2 (Cq, C4'), 136.2 (Cq, C8a), 110.6 (Cq, C3), 99.7 (CH, C2'), 85.5 (CH, C8b), 82.9 (CH, C4), 45.3 (CH, C3a), 38.7 (CH<sub>2</sub>, C7), 28.0 (Cq, C8), 27.9 (CH<sub>3</sub>, C9), 27.5 (CH<sub>3</sub>, C10), 23.7 (CH<sub>2</sub>, C5), 21.7 (Cq, C4c), 18.8 (CH<sub>2</sub>, C6), 10.7 (CH<sub>3</sub>, C7'). C4b was not found in the <sup>13</sup>C NMR spectrum. IR  $\nu$  (film, cm<sup>-1</sup>): 1800, 1750, 1675, 1255, 1010, 795. HRMS (ESI): Calculated for C<sub>21</sub>H<sub>21</sub>D<sub>3</sub>O<sub>7</sub>Na [M + Na]<sup>+</sup>:  $m/z$  414.1608. Found:  $m/z$  414.1610.

### Preparation of (±)-<sup>2</sup>H<sub>3</sub>-fabacyl acetate (**12**)

(±)-Orobanchol (**1**) (1 mg, 2.9  $\mu$ mol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (200  $\mu$ L), thereafter *m*-CPBA (3 mg, 17  $\mu$ mol) was added and the resulting reaction mixture was periodically stirred using a vortex every 4 h. The reaction was followed by RP-UHPLC (system A) until complete conversion after 12 h. The crude extract was purified by preparative TLC with a mixture of heptane/ EtOAc (4/6, v/v) giving epoxyorobanchol as white solid.  $R_f$  (heptane/EtOAc, 4/6, v/v) 0.30. HRMS (ESI):  $m/z$  calc. for C<sub>19</sub>H<sub>23</sub>O<sub>7</sub> [M + H]<sup>+</sup>: 363.1444, found :  $m/z$  363.1449. The crude epoxyorobanchol was dissolved in a mixture of pyridine/<sup>2</sup>H<sub>6</sub>- acetic anhydride (1/1, v/v, 400  $\mu$ L) and the resulting reaction mixture was periodically stirred using a vortex every 2 h. The reaction was checked for completion by RP-UHPLC [BEH C18 column, 12.1 mm  $\times$  50 mm, particle size 1.7  $\mu$ m) with 0.1% formic acid in CH<sub>3</sub>CN and 0.1% formic acid in water (pH 2.8) as eluents [5% CH<sub>3</sub>CN, followed by linear gradient from 5 to 100% of CH<sub>3</sub>CN (10 min) at a flow rate of 0.6 mL/min] until complete conversion after 6 h. The solvent was evaporated to dryness and the resulting residue was purified by semi-preparative RP-HPLC and then lyophilized giving (±)-<sup>3</sup>H<sub>2</sub>-fabacyl acetate (**12**) as a white amorphous powder (700  $\mu$ g, 2.2  $\mu$ mol, 60% over the two steps). HRMS (ESI):  $m/z$  calc. for C<sub>21</sub>H<sub>21</sub>D<sub>3</sub>O<sub>8</sub>Na [M + Na]<sup>+</sup>:  $m/z$  430.1557, found:  $m/z$  430.1553.

### Plant materials and sample preparation

**Pea hydroponic culture:** The *Pisum sativum* L. mutant lines used were *rms2-1* and *rms4-1* derived from wild type (WT) Torsdag (Beveridge, et al. 1994, Beveridge, et al. 1996). The *rms1-2T* (Torsdag) mutant line was obtained by backcrossing the *rms1-2* allele from the line WL5147 derived from Weitor (Beveridge, et al. 1997) into the WT line Torsdag four times. The basal nutrient solution (5 mM nitrate, 0.25 mM phosphate) was prepared by adding, in water (1,000 L), the following macronutrients: KH<sub>2</sub>PO<sub>4</sub> (0.25 mM), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.25 mM), KNO<sub>3</sub> (4 mM), Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O (0.5 mM), NaCl (0.2 mM), Sequestrene<sup>®</sup> (10 g) (Fe-EDTA



solution),  $(\text{NH}_4)_2\text{MoO}_4$  (0.04  $\mu\text{M}$ ),  $\text{H}_3\text{BO}_4$  (24.3  $\mu\text{M}$ ),  $\text{ZnSO}_4$  (3.48  $\mu\text{M}$ ) and  $\text{CuSO}_4$  (1  $\mu\text{M}$ ). The nutrient solution containing low phosphate concentration (5 mM nitrate, 0.05 mM phosphate), was prepared by adding, in water (1,000 L), the following macronutrients:  $\text{KH}_2\text{PO}_4$  (0.05 mM),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.25 mM),  $\text{K}_2\text{SO}_4$  (0.1 mM),  $\text{KNO}_3$  (4 mM),  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (0.5 mM),  $\text{NaCl}$  (0.2 mM), Sequestrene<sup>®</sup> (10 g) (Fe-EDTA solution),  $(\text{NH}_4)_2\text{MoO}_4$  (0.04  $\mu\text{M}$ ),  $\text{H}_3\text{BO}_4$  (24.3  $\mu\text{M}$ ),  $\text{ZnSO}_4$  (3.48  $\mu\text{M}$ ) and  $\text{CuSO}_4$  (1  $\mu\text{M}$ ). Pea seeds were germinated in wet sand for 6 days. Germinated seeds were placed in premade holes in the lid (20 plants/lid, 20 mm diameter) of a hydroponic PVC opaque pot containing the hydroponic culture solution (16 L, pH 5.9). The plants were grown in a growth chamber at 22 °C/16 °C with a 16/8-h photoperiod at 350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The seedlings were grown hydroponically in basal nutrient medium (5 mM nitrate, 0.25 mM phosphate) for 9 days. Plants (*rms1*, *rms2*, *rms4*, WT) (4 pots) were then subjected for 10 days to a P-deficient nutrient medium (5 mM nitrate, 0.05 mM phosphate) except for one pot (WT) used as control to a basal nutrient medium (5 mM nitrate, 0.25 mM phosphate). Thereby the roots of 4 plants were pooled together to reach a sufficient amount to limit differences between plants.

### Extraction and purification of natural SLs

The plant material was immediately frozen in liquid nitrogen after harvest and stored at –80 °C. It was then ground in liquid nitrogen using mortar and pestle to obtain a fine powder. EtOAc (3 mL) containing stable isotope-labelled internal standards ( $^2\text{H}_3$ -GR24 (**10**) (10 ng),  $^2\text{H}_3$ -fabacyl acetate (**12**) (10 ng) and  $^2\text{H}_3$ -orobanchyl acetate (**11**) (1 ng)) were added to a 1 g-aliquot of the fresh plant material for extraction: it was vigorously shaken for 30 s, sonicated for 1 min at 25 Hz, and shaken for 2 hours at 4 °C. After centrifugation at 1,000g (10 min, 4 °C), the supernatant (2.5 mL) was transferred into a glass tube. The pellet was re-extracted with EtOAc (3 mL), vigorously shaken for 30 s, sonicated (1 min; 25 Hz) and shaken overnight at 4 °C. Following centrifugation, the two supernatants were pooled and dried. The dry extract was dissolved in heptane/EtOAc (9/1 v/v, 2 mL) and percolated through a Upti clean Si-S 500 mg/3 mL Solid-phase extraction (SPE) column (Interchim, Montluçon, France) previously activated with heptane (2 mL) and pre-equilibrated with heptane/EtOAc (9/1 v/v, 2 mL). The SPE column was washed with heptane/EtOAc (75/25 v/v, 2 mL). The first fraction containing GR24 (**7**), fabacyl acetate (**4**) and orobanchyl acetate (**3**) was eluted with heptane/EtOAc (45/55 v/v, 2 mL). After another wash with heptane/EtOAc (40/60 v/v, 2 mL), the second fraction containing orobanchol was obtained by elution with heptane/EtOAc

(17/83 v/v, 2 mL). Each fraction was evaporated to dryness, kept at  $-20\text{ }^{\circ}\text{C}$  and finally dissolved in 100  $\mu\text{L}$  of acetonitrile before LC-MS/MS analysis (Figure 3).

### **RP-UHPLC setup and procedure**

Separation was performed on a BEH  $\text{C}_{18}$  column ( $2.1 \times 100\text{ mm}$ , particle size  $1.7\text{ }\mu\text{m}$ , Waters, Milford, MA, USA) at  $40\text{ }^{\circ}\text{C}$ , using an ACQUITY UPLC I-class system (Waters, Milford, MA, USA), equipped with an ACQUITY Sample Manager keeping the samples at  $4^{\circ}\text{C}$  and ACQUITY Binary Solvent Manager with a flow rate of  $0.4\text{ mL/min}$ , for  $1\text{ }\mu\text{L}$  injected. The mobile phase consisted of water (A) and acetonitrile (B). Elution was as follows: 5% B for 2 min, the gradient elution increased linearly to 50% B in 8 min, followed by a further linear increase to 100% B in 3 min, then 100% B for 3 min and the final gradient linear elution decreased to 5% B for 3 min.

### **Mass spectrometry conditions**

SLs were detected online using a Waters Xevo TQ-S equipped with an electrospray (ESI) source and operated in positive ion mode. The source parameters for full scan MS and MRM mode were as follows: ion capillary voltage  $1.5\text{ kV}$ , nebulizing gas 7 bar, source temperature  $120\text{ }^{\circ}\text{C}$ , drying gas  $800\text{ L/h}$ , drying temperature  $500\text{ }^{\circ}\text{C}$ , and cone gas  $250\text{ L/h}$ , optimized by infusion of GR24 solution. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode for quantification. For each compound, quantification was based on the most intense MRM transition while the second most intense one was used for confirmation. All MRM conditions (precursor and ion products, cone voltage and collision energy) were optimized on each standard solution (Table 1).

Data were analyzed using MassLynx 4.1 software.

### **Preparation of standard solutions**

The pure reference substances were dissolved at  $1\text{ mg/mL}$  in acetone. Dilutions were prepared from this solution in order to establish a calibration curve in the concentration range of  $0.2 - 56.0\text{ }\mu\text{g/L}$  in acetonitrile for the different SLs (GR24 (**7**) from  $4.0$  to  $56.0\text{ }\mu\text{g/L}$ , orobanchol (**1**) from  $0.2$  to  $3.0\text{ }\mu\text{g/L}$ , fabacyl acetate (**4**) from  $2.0$  to  $30.0\text{ }\mu\text{g/L}$  and orobanchyl acetate (**2**) from  $0.5$  to  $7.5\text{ }\mu\text{g/L}$ ).

## Method evaluation

The effectiveness of this analytical method was evaluated in terms of matrix effects, SPE recovery, sensitivity and linearity. Intra-and inter-day relative standard deviations (RSDs) of peak area ratio were calculated to determine the reproducibility of the method. Intra-day variation was determined by analyzing standard mixtures solution stored at 4 °C for 4 times within 1 day. Regarding inter-day variability, the standard mixtures solutions stored at –20 °C were analyzed 5 times for 6 months. The extraction recovery yields under SPE conditions were investigated on each SL standard, using blank matrix sample (*rmsI* root extract) spiked with standards. Sensitivity and linearity were evaluated *via* the limit of detection (LOD) and the correlation coefficients ( $R^2$ ) of the calibration curves, respectively.

The UHPLC-ESI-MS/MS method described earlier was used to determine the response factors of GR24 (**7**), orobanchol (**1**), orobanchyl acetate (**2**) and fabacyl acetate (**4**).

The quantitative determination of **2**, **4** and **7** was performed with an internal deuterium-labelled stock solution at a concentration of 100 µg/L for **2** and 1000 µg/L for **4** and **7**. 10 µL of this solution was added to each plant material before extraction.

For the quantification of orobanchol (**1**), a calibration curve (quantity of **1** = f (Peak area of **1**/Peak area of GR24)) was built using a series of calibration solutions of orobanchol (**1**) (0.2 µg/L to 3.0 µg/L) containing the same concentration of reference standard GR24 (**7**) (50.0 µg/L). GR24 (**7**) (50.0 µg/L) was used as internal standard in the biological samples. The quantity of **1** in the sample was evaluated according to the calibration curve.

All values were expressed as the means ± standard deviation (SD). Due to a small sample size, the nonparametric method of Kruskal-Wallis was used with Dunnett's test for comparison to the control.

## Results and Discussion

### Preparation of deuterium labelled SLs

Three new labelled SLs, 7'- $^2\text{H}_3$ -GR24 (**10**), ( $\pm$ )- $^2\text{H}_3$ -orobanchyl acetate (**11**) and of ( $\pm$ )- $^2\text{H}_3$ -fabacyl acetate (**12**) were prepared by organic synthesis with deuterium labelled on the D ring for GR24 (**10**), on an acetate group for the orobanchyl acetate (**11**) and the fabacyl acetate (**12**), respectively (Figure 2). The deuterium labelled GR24 (**10**) synthesis involved the coupling between the deuterium labelled D-ring **8** and the ring ABC **9** obtained by the procedure of (Mangnus, et al. 1992). Deuterium labelled bromo butenolide **8** was synthesized in 4 steps in a 25% overall yield (see Supporting Information in comparison with 4 steps and in a 15% overall yield for the synthesis of (Cheng, et al. 2015)). The coupling between the enol **13** and the bromo butenolide **12** was accomplished in acetone using an excess of potassium carbonate to give the awaited labelled GR24 (**14**) and its 2'-epimer in moderate yield after separation by silica gel chromatography.

The synthesis of the deuterium labelled orobanchyl acetate and fabacyl acetate using the procedure described for GR24 (**10**) was not convenient due to the laborious multi-step sequences (Zwanenburg, et al. 2016) to access to the ABC precursor before coupling with the labelled butenolide **8**. Alternatively, the deuterium labelling was performed by acetylation of the commercially available orobanchol (**1**) using a mixture  $^2\text{H}_6$ -acetic anhydride/pyridine to furnish the labelled compound (**11**) in 96% yield. In the same manner, after epoxidation of **1** by *m*-CPBA as described by (Xie, et al. 2009), the resultant fabacol (**3**) was acetylated to give the deuterium labelled **12** in a 60% overall yield after HPLC purification. Replacing acetic anhydride with ( $^2\text{H}_6$ )-acetic anhydride provided a simple method for deuterated methyl group introduction. The ESI-MS spectra of **10-12** showed that their deuterium abundances were sufficient enough (up to 95%) for these compounds to be used as internal standards for quantification without overlapping with natural SLs in each mass spectrum.

### Optimization of MRM transitions

Standard solutions of the 3 endogenous SLs (orobanchol (**1**), orobanchyl acetate (**2**), fabacyl acetate (**4**)), synthetic GR24 (**7**) and their deuterium-labelled analogues (**10**, **11**, **12**) were used to identify the appropriate precursor-to-product ion transitions and to ensure selectivity of the quantification method. First, appropriate precursor ions were selected and conditions optimized in full scan MS, limiting in-source fragmentation of the compounds. All the compounds were analyzed in both positive and negative electrospray ( $\text{ESI}^+$  and  $\text{ESI}^-$  respectively) ionization modes, but no intense enough signal was obtained in  $\text{ESI}^-$  as in (Sato, et al. 2003). The protonated and sodium-adduct species ( $[\text{M}+\text{H}]^+$  and  $[\text{M}+\text{Na}]^+$ , respectively)

of SLs were the most prominent ions in ESI<sup>+</sup> MS full scan spectra (except for **2** and its deuterium-labelled analogue **11** which only produced [M+Na]<sup>+</sup>). Atmospheric pressure chemical ionization mode was tested, but even when the MS spectrum contained mostly [M+H]<sup>+</sup>, the sensitivity was significantly lower than that in the ESI mode (–40%), so ESI<sup>+</sup> was demonstrated as the most convenient mode. Mobile phases containing more acid or more sodium salt were tested but none gave a spectrum with only a single species as either [M+H]<sup>+</sup> or [M+Na]<sup>+</sup>, as previously obtained (Sato, et al. 2003). Both [M+H]<sup>+</sup> and [M+Na]<sup>+</sup> were therefore chosen as precursor ions for MRM. The MRM transitions were chosen in ESI mode as giving the maximal signal. Most of the product ions were selected as the most specific regarding the structure of the compounds (e.g., often losses of acetate group (–60 u non-labelled analyte, –63 u deuterium-labelled analyte) or D ring (–97 u)) (Figure 4A). Twenty-four transitions for quantification (Q) and confirmation (C) were found (Table 1), among them only seven were already known in the literature (Foo and Davies 2011, Gomez-Roldan, et al. 2008, Kohlen, et al. 2011, Pavan, et al. 2016, Sato, et al. 2003) and seventeen were newly found. MS/MS conditions were optimized to produce maximal signals for each compound (Table 1).

### Optimization of UHPLC-conditions

Separations were optimized using a solution containing a mixture of the three endogenous SLs and GR24 plus their isotopically-labelled standards by reversed-phase ultra-performance liquid chromatography on a UHPLC BEH C<sub>18</sub> column, followed by analysis by ESI-MS/MS. In order to separate individual hormones, reduce the analysis time and avoid the co-elution with other compounds, the following gradient elution using an increasing acetonitrile content was chosen. The four SLs were successfully separated. The separation window time was less than 3 min and the total analysis was performed in 19 min. Typical total ion current chromatograms of **1**, **2**, **4** and **7** are shown in Figure 4. Our time analysis is intermediate between that of (Gomez-Roldan, et al. 2008) (50-min analysis time using HPLC) and the very fast analysis developed by (Foo and Davies 2011) (7-min analysis time using UHPLC). The latter method avoids the use of acid in the mobile phase as it hampers the sensitivity of SLs in electrospray mass spectrometry (Sato, et al. 2003).

### Solid-phase extraction

In order to purify the root extracts, we first tested the SPE protocol used for purification of SLs in extracts from root exudates by (Kisugi, et al. 2013) which included silica solid phase extraction for purification with stepwise elution of 11 *n*-hexane/EtOAc (from 100/0 to 0/100 v/v) fractions. The process was however very time-consuming and required handling too many fractions. Only four of these fractions were worthwhile (e.g., containing SLs). Hence the protocol was simplified, the number of elution steps was reduced and the solvent composition modified. Hexane and heptane were compared as co-solvent of EtOAc for SPE elution. Heptane was finally selected because the signal of SLs was 20-30% higher in fractions using heptane than when using hexane. Some matrix could have been less soluble in heptane and therefore less in competition with SLs for ionization in the MS source. In addition, heptane is well known as being less toxic than hexane.

The SPE protocol was first reduced to its minimum with only 3 elution fractions (wash by 70/30, elution by 20/80 and elution of 100% EtOAc for the final step v/v) allowing the elution of all SLs in only one fraction (20/80), but matrix effect was high (until –80% for orobanchyl acetate (**2**)) and recoveries were very low (e.g., 40% for orobanchol (**1**)). Inversely, a 5-fraction elution (wash by 75/25, elution by 45/55, wash by 40/60, elution by 17/83 v/v and final elution by 100% EtOAc) gave better recoveries and a less matrix effect, while giving an acceptable number of fractions to handle. For example if the wash fraction was 70/30 the matrix effect of fabacyl acetate (**4**) was the worst  $\approx -77\%$  and the recovery of GR24 (**7**) or orobanchyl acetate (**2**) were calamitous with the elution fraction 50/50 because they presented in 2 consecutive fractions (50/50 elution fraction and the 40/60 second wash fraction). The composition of each elution solvent for each fraction was fine-tuned so that each SL was not spread among several fractions but in only one. GR24 (**7**), orobanchyl acetate (**2**) and fabacyl acetate (**4**) were found only in the 45/55 fraction and orobanchol (**1**) in the 17/83 fraction.

### Method performance

In this study, we took advantage of the pea *ramosus1* (*rms1*) mutant which is SL-deficient (Gomez-Roldan, et al. 2008), in order to obtain an actual blank matrix sample. To evaluate the linearity of the method, *rms1* root extract fraction 2 spiked with varying amounts of SL non-labelled standards (GR24 (**7**), orobanchyl acetate (**2**) and fabacyl acetate (**4**)) and fixed amounts of labelled standards ( $^2\text{H}_3$ -GR24 (**10**) 100  $\mu\text{g/L}$ ,  $^2\text{H}_3$ -fabacyl acetate (**12**) 100  $\mu\text{g/L}$  and  $^2\text{H}_3$ -orobanchyl acetate (**12**) 10  $\mu\text{g/L}$ ) were used to create isotopically-labelled internal calibration curves. Similarly, *rms1* root extract fraction 4 spiked with varying amounts of

standard orobanchol (**1**) and fixed amounts of its internal standard (GR24 (**7**) 100 µg/L) were used to create internal calibration curves. Four replicates were used for each level of concentration. Calibration curves were established plotting peak area ratio *versus* internal standard concentration.

Table 2 shows that calibration curves were linear in the concentration ranges selected for the selected hormones and the  $R^2$  values were in the range 0.988-0.999. The limits of detection (LODs) of UHPLC-MS/MS for the different plant hormones were in the range 0.14-1.24 µg/L and the limits of quantification (LOQs) ranged from 0.46 to 16.46 µg/L. Here the intercept method was used, based on the calibration curve, as recommended in (Kruve, et al. 2015): LOQ was calculated as 10 times the ratio of the standard deviation of the intercept divided by the slope of calibration curve. (Sato, et al. 2003) obtained a very close LOQ for LC-MS/MS of orobanchol (**1**) (1 µg/L) determined as a signal to noise ratio above 10. The reproducibility of the method was evaluated by analyzing the intra- and inter-day variations (Table 4). The RSDs of the peak area ratio were taken as the measure of the reproducibility. The intra-day variation was determined by analyzing the same standard mixtures four times within a day. The RSDs of the peak area ratio of intra-day were between 3.1 and 5.5%. For the inter-day variation, the solution was examined 5 times within six months. The RSDs of the peak area ratio inter-day were 3.1–8.2%. Accuracies were calculated as the differences between the true (spiked) and calculated values, relative to the true (spiked) value. These values (< 20%) show the reliability of this method in time.

Three kinds of solutions were used for recovery and matrix effect evaluations: (A) a 100 µL-standard solution of SLs at 100 µg/L for GR24 (**7**) and fabacyl acetate (**4**), 10 µg/L for orobanchyl acetate (**2**) and 1 µg/L for orobanchol (**1**), (B) a 100 µL-extract of *rms1* root where SLs were spiked at 100 µg/L for **7** and **4**, 10 µg/L for **2** and 1 µg/L for **1** and (C) a 100 µL-extract of *rms1* root previously spiked with SLs at 10 ng for **7** and **4**, 1 ng for **2** and 0.1 ng for **1**, before extraction. A and B therefore contained the same amount of SLs, and C could also contain the same amount under the assumption that the recovery of SLs from the sample treatment protocol was 100%. Recovery for a compound was evaluated as the peak area ratio of B/C and matrix effect was evaluated as the peak area ratio of B/A minus 1, and expressed in percentages. Every solution was prepared and analyzed in triplicate.

As estimated in Table 3, the recovery yields of the four plant hormones ranged from 57.3 to 77.0% which was considered reasonable for the SPE procedure. Losses could occur by resuspension, degradation, leak during percolation and wash on SPE cartridges, or too much retention on SPE phase. To test the degradation hypothesis of acetate strigolactones susceptible of turning into the corresponding alcohols, we extracted and analyzed *rms1* root spiked with orobanchyl acetate (**2**) and fabacyl acetate (**4**), searching for orobanchol (**1**) and fabacol (**3**). Fabacol (**3**) fragmentation conditions (MRM transitions 385>254; 363>97) were optimized using the signal produced during fabacyl acetate (**4**) in-source dissociation. We found no peaks for orobanchol (**1**) and fabacol (**3**) transitions, demonstrating that there was no degradation of fabacyl acetate (**4**) and orobanchyl acetate (**2**) into the corresponding alcohols. Stability of orobanchol (**1**) was also tested in all the solvents used during extraction and purification. After two days at 20 °C orobanchol (**1**) was stable in ethyl acetate, heptane and acetonitrile (data not shown), whereas a minor loss of 19% was observed in water, indicating that orobanchol degradation is negligible during sample treatment. So degradation was ruled out as a cause of losses. SPE percolation leak during percolation or wash steps were also ruled out since none of the SLs was detected in these fractions. One can therefore only suspect resuspension problems or too much SPE retention causing those below-100% recoveries. However these recoveries do not impact at all the accuracy of the method thanks to the use of the isotopically-labelled standard, e.g., except for orobanchol (**1**).

The matrix effect ranged within a large scale between negative values for GR24 (**7**) and orobanchol (**1**) (−33.6, −23.5%) and positive values for fabacyl acetate (**4**) and orobanchyl acetate (**2**) (+1.8, +62.1%), suggesting that the matrix promotes ionization of acetate-containing SLs. Likewise, the comparison of the matrix effect with or without SPE seems to confirm that the SPE step reduces the sample complexity with a significant value and allowed a gain of up to 40% in signal hence in sensitivity: the matrix effect ranged from −70 to −26% without SPE (data not shown). As well as the below 100% recoveries, the medium matrix effect obtained using SPE does not impact the accuracy of the method thanks to the use of isotopically-labelled standards.

For orobanchol (**1**) no isotope-labelled standard was available. GR24 (**7**), a synthetic SL not occurring in the sample but eluting in the proximity of **1** (Figure 4b) (retention time 9.54 min



versus 9.04 min) and possessing a similar interaction with the matrix (Table 3), was used as an internal standard.

Orobanchyl acetate (**2**), fabacyl acetate (**4**) and GR24 (**7**) were quantified using our deuterium labelled standards **11**, **12** and **10** respectively. The use of deuterium-labelled **10**, **11** and **12** as internal standards for the MS analysis appeared to be a more elegant method in order to avoid variations in sample preparation, injection and, in particular, ionization parameters. Nevertheless, the presence of two precursor ion forms for the SLs (protonated form and sodium-adduct form) makes the quantification more complex and less accurate, as the amount of only one form is not always representative of the amount of the initial molecule. The results depended on the balance between ion precursor forms, which is a function of the ionization environment, e.g., the coeluting matrix (data not shown). To overcome this variation, two quantification modes are compared, in terms of RSD and accuracy for **7** and **4**. The first quantification mode (Table 5A) classically took into account either a protonated form or a sodium-adduct. Results based on one form or another were close but different (4-6% of variation between of two values). The second quantification mode (Table 5B) using the sum of areas for both parent ion forms was demonstrated to be more accurate with less variation e.g., lower RSD values.

The characteristics of the method shown above make it valid and appropriate for the analysis of SLs in their natural state.

### **Application to root tissue of pea analysis**

To test its feasibility, this method was subsequently applied to the quantification of SLs in root tissues of pea stimulated by P starvation or not in WT and in the highly branched *ramosus* mutants (*rms1*, *rms2*, *rms4*) as done in (Foo, et al. 2013). The *RMS1* gene encodes the CAROTENOID CLEAVAGE DIOXYGENASE8 (CCD8) enzyme implicated in the SL biosynthesis. In the SL-deficient *rms1* mutant (*Pscdd8*) used in the present study, the *RMS1* gene is deleted (Gomez-Roldan, et al. 2008, Sorefan, et al. 2003). The response mutant (*rms4*) contains a lesion in the F-box protein (RMS4)(Johnson, et al. 2006), which interacts with the SL receptor (RMS3) after SL perception (de Saint Germain, et al. 2016). The *rms2* mutant also displays a high shoot branching and is affected in a shoot-to-root feedback signal which controls the expression of SL-biosynthesis genes (Beveridge, et al. 1994, Beveridge, et al. 1996, Foo, et al. 2005). As expected, no pea SL was detected in *rms1* root tissue with LOD

varying from 0.14 to 1.24 pg.g FW<sup>-1</sup> according to each SL (Table 2 for **1-2, 4**). This result confirmed the two reports that monitored SLs in *rms1* mutant plants. In our analyses, only orobanchol (**1**), orobanchyl acetate (**2**), fabacyl acetate (**4**) (Xie 2016) were detected in root tissue of *rms2*, *rms4* mutant plants and WT plants. Only the levels of the two major SLs fabacyl acetate (**4**), and orobanchyl acetate (**2**) were sufficient to be quantified even in root tissue from WT roots grown hydroponically and starved of P. The effect of P starvation was studied in WT and led to 18-fold and 11-fold increases for fabacyl acetate (**4**), and orobanchyl acetate (**2**) respectively in accordance with over a 10-fold increase found in (Foo, et al. 2013). Fabacyl acetate (**4**) was detected as the major SL with concentrations varying from  $3,194 \pm 0.146$  ng.g FW<sup>-1</sup> and  $3,559 \pm 0.410$  ng.g FW<sup>-1</sup> for *rms4* and *rms2* mutant plants, respectively, to  $8,640 \pm 0.480$  ng.g FW<sup>-1</sup> for WT plants. Orobanthyl acetate (**2**) was detected as a minor SL with concentrations varying from  $64 \pm 6$  pg.g FW<sup>-1</sup> and  $64 \pm 14$  pg.g FW<sup>-1</sup> for *rms4* and *rms2* mutant plants, respectively, to  $187 \pm 14$  pg.g FW<sup>-1</sup> for WT plants (Figure 5). In (Foo, et al. 2013), orobanchol (**1**) and fabacyl acetate (**4**) were measured as the major SLs. This discrepancy with our work in the major SL may be due to the culture conditions but also to the quantification method and the use of different deuterium labelled SL standards, however it is not due to degradation using our method, as previously demonstrated (see above). In Foo et al 2013, the SL concentration levels were found similar for *rms2*, *rms4* mutant plants and WT plants (500 pg.g FW<sup>-1</sup> for orobanchol (**1**) and fabacyl acetate (**4**) and 80 pg.g FW<sup>-1</sup> for orobanchyl acetate (**2**)). In our work, reduced SL levels were found for the *rms2* and *rms4* mutants in comparison to WT. This result is consistent with the reduced *RMS1* transcript level generally observed in the *rms2* pea stem but not for *rms4* where elevated *RMS1* transcript levels resulting for feedback regulation are observed. Further studies should investigate in the same tissues both transcript levels of SL biosynthesis genes and SL levels for a better understanding of regulation of SL levels at the plant level.

This study reported the development of newly labelled SL standards and validation of the UHPLC-ESI-MS/MS method for the analysis of SLs with high sensitivity and selectivity.

The effectiveness of extraction and purification using SPE was evaluated and the effect of the matrix quantified. This procedure required about 1-g root samples and, in comparison with the quantification from exudates, avoided laborious extractions or uses of large SPE cartridges and consumptions of large quantities of organic solvents. Matrix effect was limited to over -34% thanks to SPE, whereas recoveries ranged from 57 to 77%, with little impact on sensitivity. The use of isotopically-labelled standards allowed to overcome these sub-100%

recoveries and moderate matrix effect in order to obtain a satisfactory method accuracy (intra-day 6-19%, inter-day 5-14%). For orobanchol (**1**), we found a SL analog, GR24 (**7**), which could also lead to accurate results. It was the first time an analytical method was fully validated for SLs. Finally, using this UHPLC-ESI-MS/MS method to quantify plant hormones, it may be possible to quantify SLs in other complex plant samples to understand their implication in numerous biological events.

## Figures and Tables

Figure 1. Natural SLs detected in pea (Xie 2016), their biosynthetic precursor (+)-carlactone (**6**) and the synthetic SL GR24 (**7**).

Figure 2. Synthesis of the labelled standards **10-12**.

Figure 3. Protocol of extraction and purification of SLs.

Figure 4. (A) MS spectra of  $^2\text{H}_3$ -fabacyl acetate (**12**) and  $^2\text{H}_3$ -orobanchyl acetate (**11**), (B) Total ion current chromatogram from a UHPLC-MS/MS in MRM mode of a standard solution containing the four SLs non-labelled standards at 500  $\mu\text{g/L}$ .

Figure 5. Levels of fabacyl acetate (**4**) (A) and orobanchyl acetate (**2**) (B) detected in pea root tissues after 10-day phosphorus starvation. Nd = not detected. Mean  $\pm$  SD of 4 biological replicates. \*\*\* denotes significant differences with the WT starved control as determined using the Kruskal-Wallis test with the Dunnett type of contrast ( $p < 0.001$ ).

Table 1. LC-MS/MS parameters for compounds **7**, **10**, **1**, **4**, **12**, **2** and **11**: Retention time (RT), characteristic parent and product ions for MRM transitions, cone voltage (CV), collision energy (CE) and use of the transition (Q: quantification, C: confirmation).

Table 2. Response characteristics of the SL standards using UHPLC-ESI-MS/MS. Linearities with Y concentration of SL and x ratio of peaks area SL on internal standard (8 calibration levels, 4 replicates), LOQ and LOD of SLs in spiked blank matrix (*rmsl* pea extract).

Table 3. Recoveries calculated for the whole sample treatment process, and matrix effect for SLs. The value of matrix effect is positive when the presence of matrix promotes the signal and negative when it lowers the signal ( $n = 3$ ).

Table 4. Relative standard deviation (RSD) and precision of the quantification for repeatability (intra-day with  $n = 4$ ) and reproducibility (inter-day with  $n = 5$ ) in standard sample. nd = not determined.

Table 5. Relative standard deviation ( $n = 3$ ) and precision on two quantification modes for the compounds which have 2 ions in source (A) on each parent ion form  $[M + H]^+$  and  $[M + Na]^+$ , (B) on addition of area for the 2 parent ion forms.

### **Acknowledgements**

We thank Frank Pelissier from CNRS-ICSN for the preparative HPLC of standards. We thank Rosemary Beau for her comments on the manuscript. We are grateful to the Institut National de la Recherche Agronomique (INRA), the Agence Nationale de la Recherche (contract ANR-12-BSV6-004-01), la Région Ile de France, le department des Yvelines and the Stream COST Action FA1206 for financial support. The Institut Jean-Pierre Bourgin benefits from the support of the Labex Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS).

## References

- Beveridge CA, JJ Ross and IC Murfet. 1994. Branching mutant *rms2* in *Pisum sativum* - grafting studies and endogenous indole-3-acetic acid levels. *Plant Physiol* **104**: 953-959.
- Beveridge CA, JJ Ross and IC Murfet. 1996. Branching in pea - Action of genes *Rms3* and *Rms4*. *Plant Physiol* **110**: 859-865.
- Beveridge CA, GM Symons, IC Murfet, JJ Ross and C Rameau. 1997. The *rms1* mutant of pea has elevated indole-3-acetic acid levels and reduced root-sap zeatin riboside content but increased branching controlled by graft-transmissible signal(s). *Plant Physiol* **115**: 1251-1258.
- Boyer F-D, A de Saint Germain, J-P Pillot, J-B Pouvreau, VX Chen, S Ramos, A Stévenin, P Simier, P Delavault, J-M Beau and C Rameau. 2012. Structure-Activity Relationship Studies of Strigolactone-Related Molecules for Branching Inhibition in Garden Pea: Molecule Design for Shoot Branching. *Plant Physiol* **159**: 1524-1544.
- Braun N, A de Saint Germain, JP Pillot, S Boutet-Mercey, M Dalmais, I Antoniadi, X Li, A Maia-Grondard, C Le Signor, N Bouteiller, D Luo, A Bendahmane, C Turnbull and C Rameau. 2012. The pea TCP transcription factor PsBRC1 acts downstream of Strigolactones to control shoot branching. *Plant Physiol* **158**: 225-238.
- Cheng Y, WH Ding, Q Long, M Zhao, J Yang and XQ Li. 2015. Synthesis of stable isotopically labelled 3-methylfuran-2(5H)-one and the corresponding strigolactones. *J Labelled Compd Radiopharm* **58**: 355-360.
- Davies PJ. 2010. The Plant Hormones: Their Nature, Occurrence, and Functions. In *Plant Hormones: Biosynthesis, Signal Transduction, Action!* Davies PJ (Ed.) Springer Netherlands: Dordrecht; 1-15.
- de Saint Germain A, G Clave, M-A Badet-Denisot, J-P Pillot, D Cornu, J-P Le Caer, M Burger, F Pelissier, P Retailleau, C Turnbull, S Bonhomme, J Chory, C Rameau and F-D Boyer. 2016. An histidine covalent receptor and butenolide complex mediates strigolactone perception. *Nat Chem Biol* **12**: 787-794.
- Foo E, E Buillier, M Goussot, F Foucher, C Rameau and CA Beveridge. 2005. The branching gene RAMOSUS1 mediates interactions among two novel signals 464 and auxin in pea. *Plant Cell* **17**: 464-474.
- Foo E and NW Davies. 2011. Strigolactones promote nodulation in pea. *Planta* **234**: 1073-1081.

- Foo E, K Yoneyama, CJ Hugill, LJ Quittenden and JB Reid. 2013. Strigolactones and the Regulation of Pea Symbioses in Response to Nitrate and Phosphate Deficiency. *Mol Plant* **6**: 76-87.
- Gomez-Roldan V, S Fermas, PB Brewer, V Puech-Pages, EA Dun, J-P Pillot, F Letisse, R Matusova, S Danoun, J-C Portais, H Bouwmeester, G Becard, CA Beveridge, C Rameau and SF Rochange. 2008. Strigolactone inhibition of shoot branching. *Nature* **455**: 189-194.
- Johnson X, T Breich, EA Dun, M Goussot, K Haurogne, CA Beveridge and C Rameau. 2006. Branching genes are conserved across species. Genes controlling a novel signal in pea are coregulated by other long-distance signals. *Plant Physiol* **142**: 1014-1026.
- Kisugi T, X Xie, HI Kim, K Yoneyama, A Sado, K Akiyama, H Hayashi, K Uchida, T Yokota, T Nomura and K Yoneyama. 2013. Strigone, isolation and identification as a natural strigolactone from *Houttuynia cordata*. *Phytochemistry* **87**: 60-64.
- Kohlen W, T Charnikhova, Q Liu, R Bours, MA Domagalska, S Beguerie, F Verstappen, O Leyser, H Bouwmeester and C Ruyter-Spira. 2011. Strigolactones Are Transported through the Xylem and Play a Key Role in Shoot Architectural Response to Phosphate Deficiency in Nonarbuscular Mycorrhizal Host Arabidopsis. *Plant Physiol* **155**: 974-987.
- Krueve A, R Rebane, K Kipper, ML Oldekop, H Evard, K Herodes, P Ravio and I Leito. 2015. Tutorial review on validation of liquid chromatography-mass spectrometry methods: Part I. *Anal Chim Acta* **870**: 29-44.
- Lopez-Obando M, Y Ligerot, S Bonhomme, FD Boyer and C Rameau. 2015. Strigolactone biosynthesis and signaling in plant development. *Development* **142**: 3615-3619.
- Mangnus EM, FJ Dommerholt, RLP Dejong and B Zwanenburg. 1992. Improved Synthesis of Strigol Analog GR24 and Evaluation of the Biological-Activity of Its Diastereomers. *J Agric Food Chem* **40**: 1230-1235.
- Novak O, R Napier and K Ljung. 2017. Zooming In on Plant Hormone Analysis: Tissue- and Cell-Specific Approaches. *Annu Rev Plant Biol* **68**: 323-348.
- Pavan S, A Schiavulli, AR Marcotrigiano, N Bardaro, V Bracuto, F Ricciardi, T Charnikhova, C Lotti, H Bouwmeester and L Ricciardi. 2016. Characterization of Low-Strigolactone Germplasm in Pea (*Pisum sativum* L.) Resistant to Crenate Broomrape (*Orobanche crenata* Forsk.). *Mol Plant-Microbe Interact* **29**: 743-749.
- Proust H, B Hoffmann, X Xie, K Yoneyama, DG Schaefer, K Yoneyama, F Nogue and C Rameau. 2011. Strigolactones regulate protonema branching and act as a quorum sensing-like signal in the moss *Physcomitrella patens*. *Development* **138**: 1531-1539.
- Sato D, AA Awad, SH Chae, T Yokota, Y Sugimoto, Y Takeuchi and K Yoneyama. 2003. Analysis of strigolactones, germination stimulants for *Striga* and *Orobanche*, by high-performance liquid chromatography/tandem mass spectrometry. *J Agric Food Chem* **51**: 1162-1168.

- Sato D, AA Awad, Y Takeuchi and K Yoneyama. 2005. Confirmation and quantification of strigolactones, germination stimulants for root parasitic plants *Striga* and *Orobanche*, produced by cotton. *Biosci Biotechnol, Biochem* **69**: 98-102.
- Sorefan K, J Booker, K Haurogne, M Goussot, K Bainbridge, E Foo, S Chatfield, S Ward, C Beveridge, C Rameau and O Leyser. 2003. *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. *Genes & Development* **17**: 1469-1474.
- Ueno K, A Hanada, S Yamaguchi and T Asami. 2010. Preparation of multideuterated 5-deoxystrigol for use as an internal standard for quantitative LC/MS. *J Labelled Compd Radiopharm* **53**: 763-766.
- Umehara M, A Hanada, S Yoshida, K Akiyama, T Arite, N Takeda-Kamiya, H Magome, Y Kamiya, K Shirasu, K Yoneyama, J Kyojuka and S Yamaguchi. 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**: 195-200.
- Visentin I, M Vitali, M Ferrero, Y Zhang, C Ruyter-Spira, O Novák, M Strnad, C Lovisolo, A Schubert and F Cardinale. 2016. Low levels of strigolactones in roots as a component of the systemic signal of drought stress in tomato. *New Phytol* **212**: 954-963.
- Xie X. 2016. Structural diversity of strigolactones and their distribution in the plant kingdom. *J Pestic Sci* **41**: 175-180.
- Xie X, K Yoneyama and K Yoneyama. 2010. The Strigolactone Story. In *Annu Rev Phytopathol*, Vol 48. VanAlfen NK, Bruening G and Leach JE (Eds.); 93-117.
- Xie XN, K Yoneyama, Y Harada, N Fusegi, Y Yamada, S Ito, T Yokota, Y Takeuchi and K Yoneyama. 2009. Facetyl acetate, a germination stimulant for root parasitic plants from *Pisum sativum*. *Phytochemistry* **70**: 211-215.
- Yoneyama K, X Xie, HI Kim, T Kisugi, T Nomura, H Sekimoto and T Yokota. 2012. How do nitrogen and phosphorus deficiencies affect strigolactone production and exudation? *Planta* **235**: 1197-1207.
- Yoneyama K, X Xie, T Nomura and K Yoneyama. 2016. Extraction and Measurement of Strigolactones in Sorghum Roots. *Bio-protocol* **6**: e1763.
- Zwanenburg B, S Cavar Zeljkovic and T Pospisil. 2016. Synthesis of strigolactones, a strategic account. *Pest Manage Sci* **72**: 15-29.

Compounds	RT (min)	Diagnostic transition MRM	CV (V)	CE (eV)	Q/C
GR24 (7)	9.54	299 > 157 <sup>d</sup>	10	15	Q
		299 > 100	10	20	C
		321 > 224	10	15	Q
		321 > 196	10	20	C
<sup>2</sup> H <sub>3</sub> -GR24 (14)	9.40	302 > 157	10	15	Q
		302 > 100	10	20	C
		324 > 224	10	15	Q
		324 > 196	10	20	C
Orobanchol (1)	9.04	347 > 233 <sup>d,e</sup>	10	12	Q
		347 > 205 <sup>b,d</sup>	10	17	C
		369 > 272 <sup>a,c</sup>	10	17	C
		369 > 299	10	27	Q
Fabacyl acetate (4)	10.75	405 > 97 <sup>b,c</sup>	30	25	C
		405 > 231 <sup>b,e</sup>	30	15	Q
		427 > 219	30	20	Q
		427 > 242	30	20	C
<sup>2</sup> H <sub>3</sub> -Fabacyl acetate (16)	10.75	408 > 97	30	25	C
		408 > 231	30	15	Q
		430 > 222	30	20	Q
		430 > 242	30	20	C
Orobanchyl acetate (2)	11.13	411 > 254 <sup>c</sup>	30	15	Q
		411 > 239	30	25	C
<sup>2</sup> H <sub>3</sub> -Orobanchyl acetate					



(15)	11.13	414 > 254	30	15	Q
		414 > 239	30	25	C

Table 1: LC-MS/MS parameters for each compound: Retention time (RT), characteristic parent and product ions for MRM transitions, cone voltage (CV), collision energy (CE) and use of the transition (Q: quantification, C: confirmation). a. (Sato, et al. 2003) b. (Pavan, et al. 2016) c. (Gomez-Roldan, et al. 2008) d. (Kohlen, et al. 2011) e. (Foo and Davies 2011)

Analyte	Linear range $\mu\text{g/L}$	Regression data		LODs $\mu\text{g/L}$	LOQs $\mu\text{g/L}$
		Equation of calibration curve	$R^2$ Value		
GR24 (7)	4.0-56.0	$Y = 14.78x + 4.94$	0.988	4.94	16.46
Orobanchol (1)	0.2-3.0	$Y = 2,679.28x + 0.18$	0.996	0.18	0.61
Fabacyl acetate (4)	2.0-30.0	$Y = 29.36x + 1.25$	0.997	1.25	4.16
Orobanchyl acetate (2)	0.5-7.5	$Y = 104.25x + 0.14$	0.999	0.14	0.46

Table 2. Response characteristics of the SL standards using UHPLC-ESI-MS/MS. Linearities with Y concentration of SL and x ratio of peaks area SL on internal standard (8 calibration levels, 4 replicates), LOQ and LOD of SLs in spiked blank matrix (*rmsI* pea extract).

Analyte	Recovery (% , $\pm$ SD)	Matrix effect (%)
GR24 (7)	70.5 $\pm$ 4.6	– 33.6
Orobanchol (1)	57.3 $\pm$ 11.5	– 23.5
Fabacyl acetate (4)	76.1 $\pm$ 7.5	+ 1.8
Orobanchyl acetate (2)	77.0 $\pm$ 4.0	+ 62.1

Table 3. Recoveries calculated for the whole sample treatment process, and matrix effect for SLs. The value of matrix effect is positive when the presence of matrix promotes the signal and negative when it lowers the signal ( $n = 3$ ).

Analyte	Intra-day $n = 4$		Inter-day $n = 5$	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
GR24 (7)	5.5	5.9	8.2	4.8
Orobanchol (1)	8.5	12.0	nd	nd
Fabacyl acetate (4)	3.1	10.5	3.1	10.4
Orobanchyl acetate (2)	4.6	19.2	6.7	14.1

Table 4. Relative standard deviation (RSD) and precision of the quantification for repeatability (intra-day with  $n = 4$ ) and reproducibility (inter-day with  $n = 5$ ) in standard sample. nd = not determined.

A.

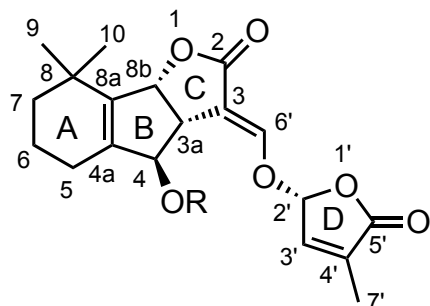
Analyte	RSD (%)	Accuracy (%)
GR24 (7) $[M + H]^+$	14.2	12.5
GR24 (7) $[M + Na]^+$	4.3	6.8
Fabacyl acetate (4) $[M + H]^+$	4.5	3.5
Fabacyl acetate (4) $[M + Na]^+$	5.4	8.5

B.

Analyte	RSD (%)	Accuracy (%)
GR24 (7) $[M + H]^+ + [M + Na]^+$	4.8	7.1
Fabacyl acetate (4) $[M + H]^+ + [M + Na]^+$	4.0	5.1

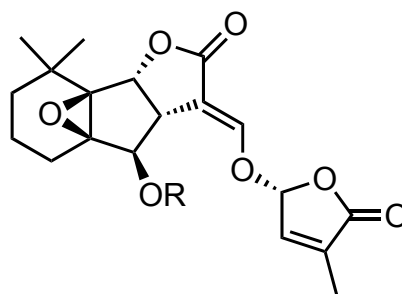
Table 5. Relative standard deviation ( $n = 3$ ) and precision on two quantification modes for the compounds which have 2 ions in source (A) on each parent ion form  $[M + H]^+$  and  $[M + Na]^+$ , (B) on addition of area for the 2 parent ion forms.

## Top of the figure



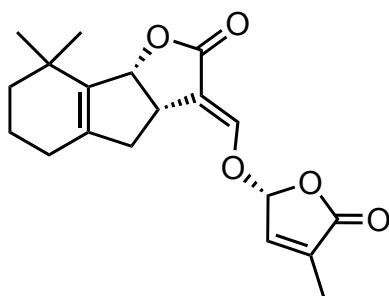
R = H: (-)-Orobanchol (**1**)

R = Ac: (-)-Orobanchyl acetate (**2**)

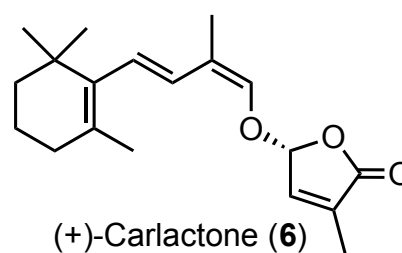


R = H: (-)-Fabacol (**3**)

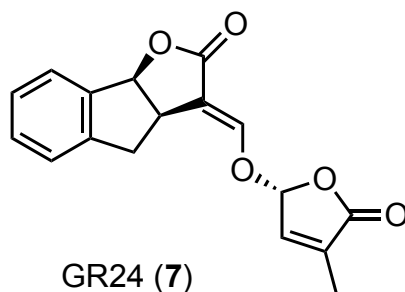
R = Ac: (-)-Fabacyl acetate (**4**)



(-)-4-Deoxyorobanchol (**5**)

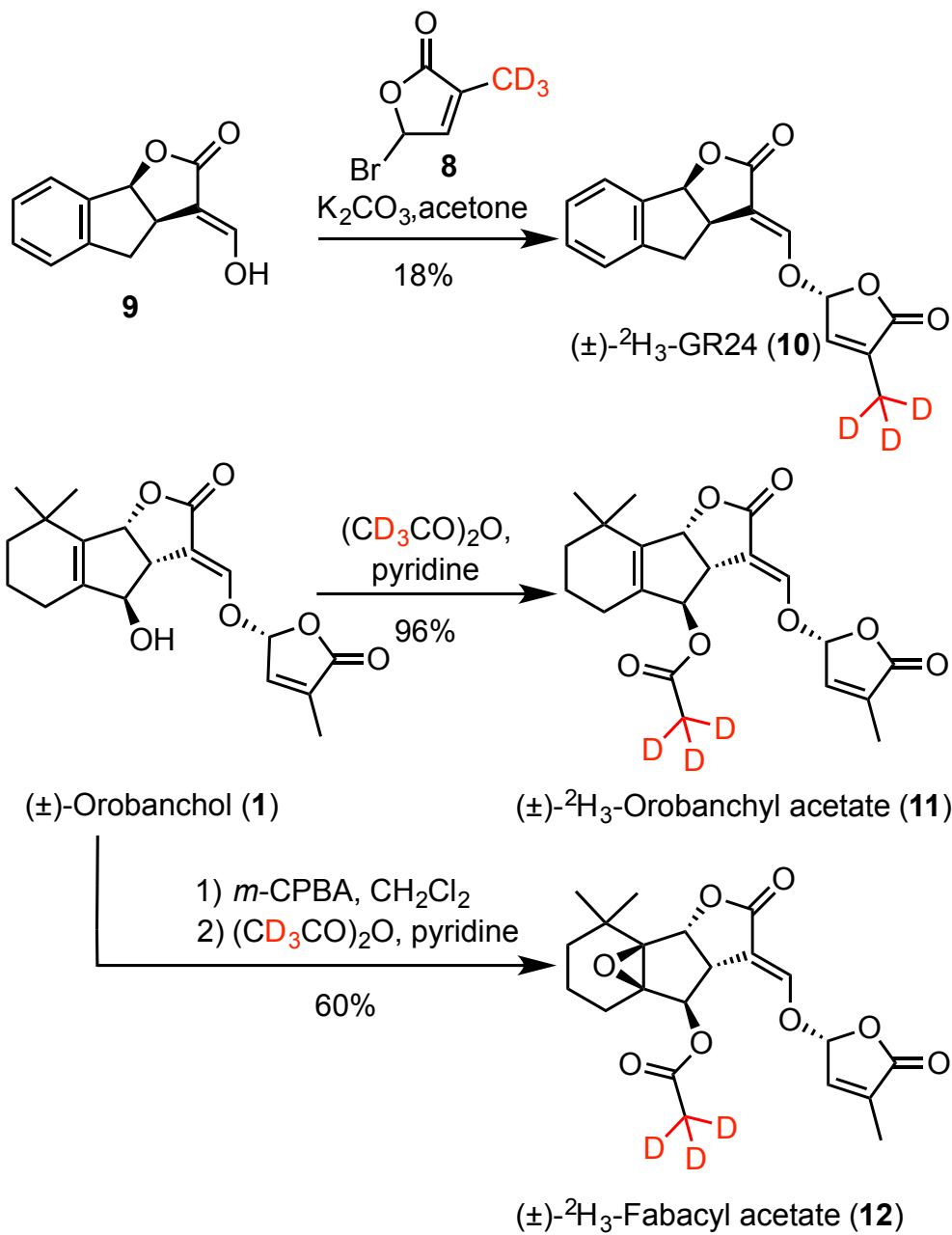


(+)-Carlactone (**6**)

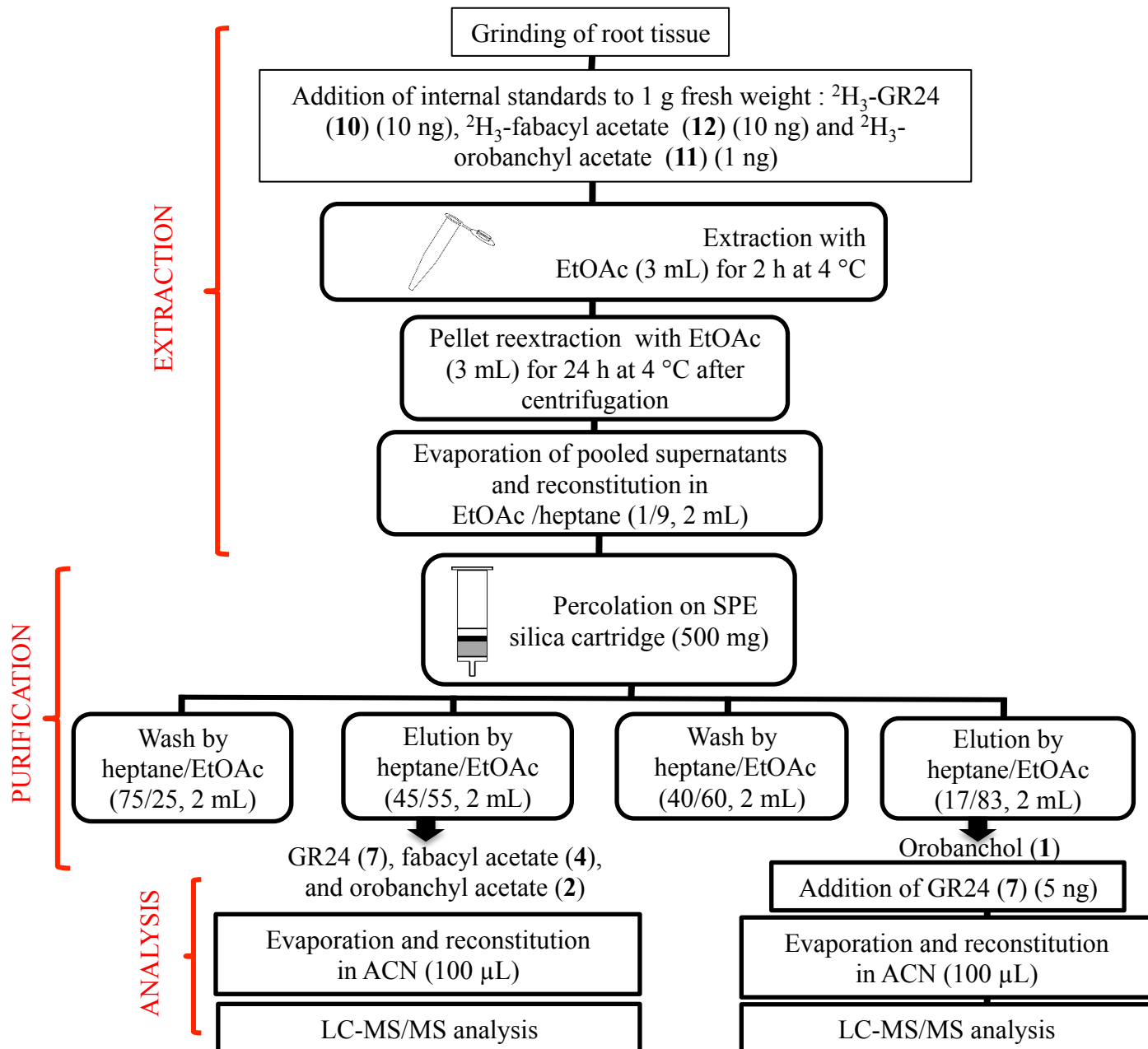


GR24 (**7**)

# Top of the figure

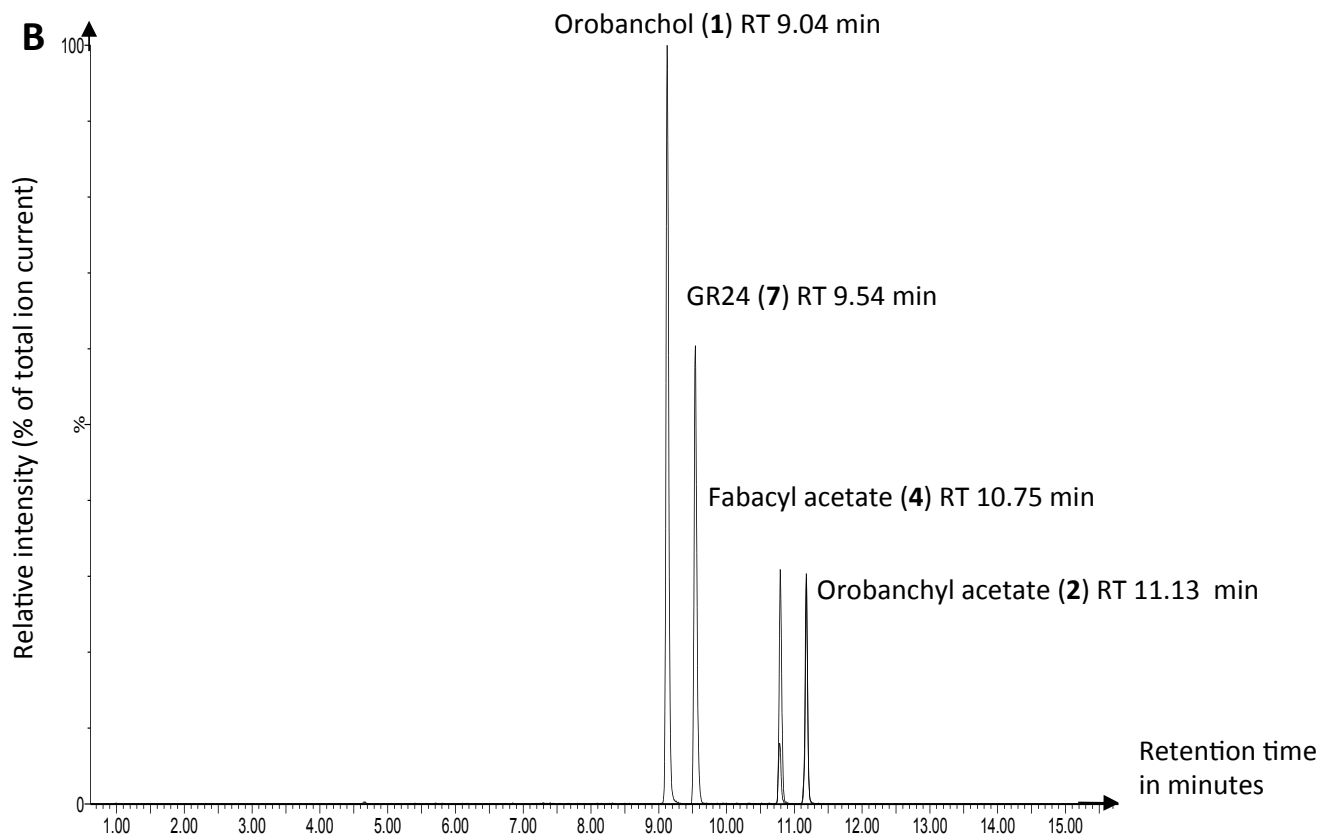
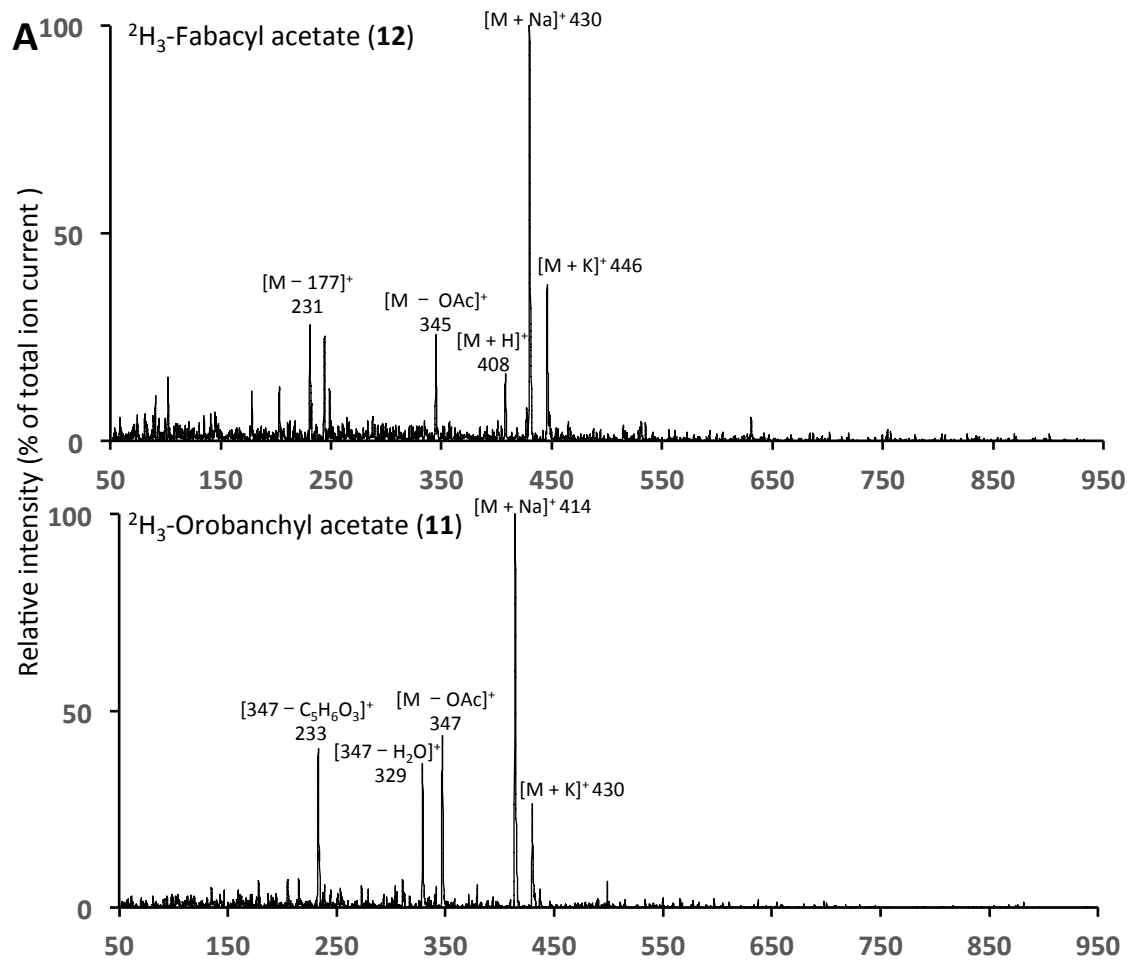


# Top of the figure





# Top of the figure



Top of the figure

