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RESEARCH ARTICLE

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A new UHPLC-MS/MS method for the direct determination of strigolactones in root exudates and extracts

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Abstract

Introduction: Strigolactones (SLs) are the most representative germination stimulants for seeds of root parasitic plants, and they show activity even at concentrations below 10^{-10} M. The low amounts of stimulants produced by the host and their rapid degradability make it crucial to develop analytical methods with very low limits of quantification.

Objective: To develop a sensitive and validated analytical method for the simultaneous quantification of seven SLs [7-oxoorobanchyl acetate (**1**), solanacol (**2**), orobanchol (**4**), strigol (**5**), fabacyl acetate (**6**), orobanchyl acetate (**7**), and 5-deoxystrigol (**8**)].

Methods: SLs were analysed using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), with (±)-GR24 (**3**) employed as internal standard (IS). Validation was based on selectivity, linearity, precision of the peak areas (repeatability and intermediate precision), detection and quantification limits, and stability.

Results: A simple, rapid and reliable UHPLC-MS/MS method has been validated for the routine analysis of seven SLs and has been successfully applied to quantify them in exudates and extracts from tomato roots (*Solanum lycopersicum*). The limits of quantifications range from 0.05 μg/L for 5-deoxystrigol to 0.96 μg/L for solanacol.

Conclusion: The method provides a useful tool for research in all the fields related to SLs, both for studies related to their function as hormones, and signalling molecules in the rhizosphere, without sample preparation required for extracts and root exudates in less than 11 minutes.

KEYWORDS

parasitic plants, quantification, *Solanum lycopersicum*, stimulators, strigolactones, tomato, UHPLC-MS/MS, validation

1 | INTRODUCTION

Striga, *Orobanche* and *Phelipanche* are three root parasitic plant genera that are some of the most damaging agricultural pests. These plants acquire nutrients and water from their host through an organ called the haustorium and this causes significant losses in crops. For instance, *Striga* spp. parasitise crops such as maize and rice, while *Orobanche* spp. and *Phelipanche* spp. parasitise legumes, crucifers, sunflower, hemp, tobacco and tomato.[1-3]

Seeds of these parasites only germinate when they perceive chemical signals, ie, germination stimulants produced and released from the roots of host and non-host plants.[4] Several germination

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stimulants have been identified in root exudates.[5-8] The most representative germination stimulants are the strigolactones (SLs). In plants, SLs play important roles as plant hormones that regulate shoot and root architecture as modulators of plant development and these compounds are extensively studied. These compounds were originally described as inducers of the germination of root parasitic plants, a role that was later linked to encouraging the beneficial symbiosis with arbuscular mycorrhizal (AM) fungi.[9] Recently, the focus has shifted to examining the role of SLs in other plant–microbe interactions and such work has revealed roles for SLs in the association of legumes with nitrogen-fixing rhizobacteria and in plant interactions with disease-causing pathogens.[10]

The use of stimulants to induce suicidal germination,[11] recently called the honey-pot strategy,[12] of parasitic plants prior to crop sowing has been proposed as a strategy to reduce the seed bank of these pests on the soil. With this aim in mind, SL derivatives and mimics have been synthesised. GR24, a synthetic SL analogue,[13,14] is currently used in most scientific studies as a control for the germination viability of seeds of root parasitic plants.

The low amount of stimulants produced by the host – these compounds have shown activity even at concentrations below 10^{-10} M – and their rapid degradability make it crucial to develop fast analytical methods with very low limits of quantification. One of the methods proposed uses parasitic plant germination bioassay as an indirect method to quantify SLs.[15] Nowadays, almost all studies in this area are based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as a fast and sensitive method to identify and quantify SLs. Almost all studies only identify SLs and others only can quantify them in relative mode comparing their areas between samples, with an external calibration curve or fortifying with known amounts of each SLs.[1,4,16-18] These methods do not take into account the matrix effect, which is very important in LC-MS/MS quantification. This effect had been corrected by Jamil et al using an internal standard (IS) to quantify orobanchol and 2′-epi-5-deoxystrigol.[19] Recently, an analytical method to quantify three different SLs from pea has been published.[20] This method involves the use of two novel deuterium labelled SLs to quantify fabacyl acetate and orobanchyl acetate, and (±)-GR24 was used as IS to quantify orobanchol in root extracts after a solid phase extraction (SPE) step. That method allows for a reliable determination; however, it is limited because of the limited availability of deuterium labelled SLs. Additionally, the sample preparation step by SPE produces a large analysis time (more than 24 h).

The study described here was mainly aimed at the development, optimisation and validation of a simple, fast and sensitive analytical method for the simultaneous quantification of seven natural SLs (7-oxoorobanchyl acetate (**1**),[21] solanacol (**2**),[22] orobanchol (**4**),[6] strigol (**5**),[23,24] fabacyl acetate (**6**),[25] orobanchyl acetate (**7**)[8] and 5-deoxystrigol (**8**)[9]) by ultra-high performance (UHP) LC-MS/MS using (±)-GR24 (**3**) as single IS (Figure 1)[26] directly in root exudates and extracts, without additional sample preparation. These compounds were selected because they were found as the most common SLs in root exudates from different plants.[1,27,28]

2 | EXPERIMENTAL

2.1 | Reagents and plant material

The SLs orobanchol, solanacol, orobanchyl acetate, fabacyl acetate and 7-oxoorobanchyl acetate were generously provided by Professor Xiaonan Xie and Professor Koichi Yoneyama (Weed Science Centre, Utsunomiya University, Japan). Strigol and 5-deoxystrigol were purchased from StrigoLab (Turin, Italy). (±)-GR24 was supplied by Professor Binne Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands). The solvents for UHPLC-MS/MS analysis were UHPLC-grade. Methanol (MeOH) and formic

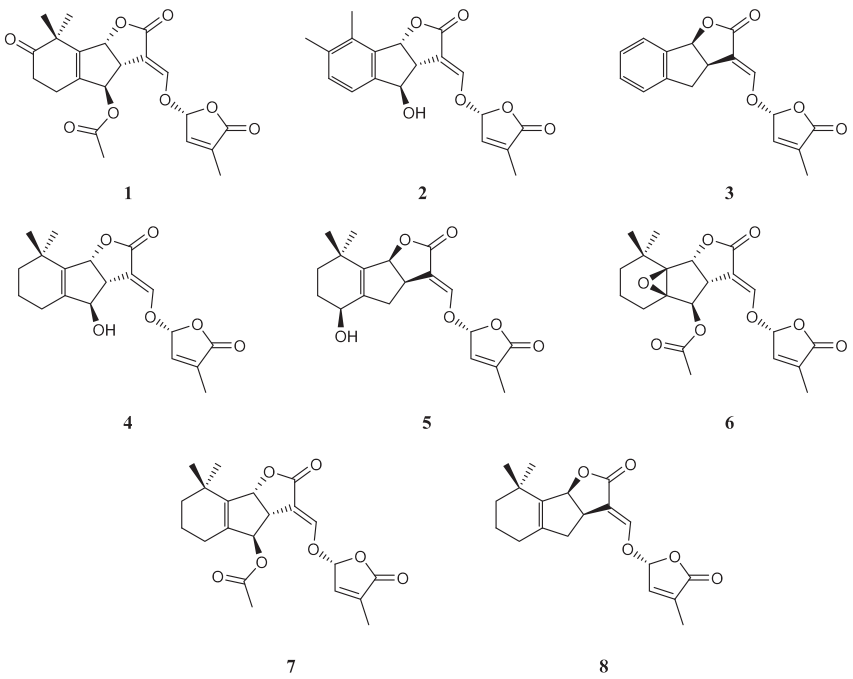

FIGURE 1 Structures of the strigolactones analysed by UHPLC-MS/MS: (**1**) 7-oxoorobanchyl acetate, (**2**) solanacol, (**3**) (±)-GR24, (**4**) orobanchol, (**5**) strigol, (**6**) fabacyl acetate, (**7**) orobanchyl acetate and (**8**) 5-deoxysrtrigol

acid were obtained from Fischer Chemicals (Geel, Belgium). Water for UHPLC-MS/MS analysis was type I obtained from an Ultramatic system from Wasserlab (Barbatáin, Spain).

Tomato root exudates were obtained, purified and concentrated from fresh plants using the procedure previously described by López-Ráez et al.[1] Samples from plants grown with and without phosphate starvation were analysed.

2.2 | Tomato roots extract

In addition to root exudate, pools of roots from fresh plants grown with and without phosphate starvation were used to extract the germination stimulants from the tomato roots. For this, 100 mg of roots were ground in a mortar with liquid nitrogen and then extracted with 1 mL of ethyl acetate in an ultrasonic bath (Selectra Ultrasonics, Barcelona, Spain) for 10 min. Samples were centrifuged for 10 min at 5000 rpm in a Selecta centrifuge (Selecta, Barcelona, Spain). The organic phase was carefully transferred to glass vials. This procedure was repeated three times. Finally, samples were concentrated in a rotavapor and stored at −80 °C.

2.3 | Calibration curves

A stock standard solution of 10 mg/L was prepared in MeOH. The external standard calibration curve was prepared by the serial dilution of the working standard solution as follows: 7-oxoorobanchyl acetate from 75 to 10 μg/L (five levels), solanacol from 100 to 7 μg/L (six levels), orobanchol from 100 to 0.5 μg/L (nine levels), strigol from 100 to 1 μg/L (eight levels), fabacyl acetate from 100 to 0.5 μg/L (nine levels), 5-deoxystrigol from 100 to 0.1 μg/L (10 levels) and orobanchyl acetate from 100 to 5 μg/L (six levels). Also, a stock solution of the IS (±)-GR24 of 10 mg/L was prepared in MeOH, and this was added to all samples to give a final concentration of 10 μg/L. The concentration of IS was optimised to obtain similar signal intensities for the IS and the analysed compound in the sample. A 5-μL aliquot of each standard solution was injected three times onto the UHPLC column. The calibration curve was constructed by plotting the peak area ratio (y) of standard to IS versus the ratio of their concentrations (x). The curve was fitted to a linear function with a weight of $1/nx$ ($R^2 > 0.99$), being "n" the calibration level. The compounds in the sample were determined by their peak area ratio with respect to the IS and by reference to the standard curve. All standards and stock solutions were filtered through a PTFE syringe filter (0.22 μm) prior to analysis and samples were stored at −80 °C.

2.4 | Sample preparation

Exudates were used without any prior treatment. Extracts were dissolved with MeOH to achieve a ratio of 1/1 g/L. IS was added to each sample to give a final concentration of 10 μg/L. The concentration of IS was optimised to obtain a similar signal intensity for the IS and the analyte in the sample. A 5-μL aliquot of each sample was injected three times onto the UHPLC column. All samples were filtered through a PTFE syringe filter (0.22 μm) prior to analysis and samples were stored at −80 °C.

2.5 | Data analysis

Data acquisition, calibration curves and the statistical analysis of the data from the quantification was performed with the software MS Data Review (Bruker Chemical Analysis, Karlsruhe, Germany).

2.6 | UHPLC-MS/MS method

Samples were analysed on a Bruker EVOQ Triple Quadrupole Mass Spectrometer with an electrospray ionisation (ESI) source in positive mode. Samples were injected and separated using an ACE Excel 1.7 C18 (100 mm × 2.1 mm, 1.7 μm particle size) (Advanced Chromatography Technologies Ltd, Aberdeen, UK) maintained at 40 °C. The mobile phase consisted of solvent A (water, 0.1% formic acid) and solvent B (MeOH, 0.1% formic acid) and the flow rate was set to 0.3 mL/min, SLs have shown to be stable under the chromatographic conditions.[29] The optimised linear gradient system was as follows: 0–0.5 min, 50% B; 0.5–5 min, to 100% B; 5–7 min, 100% B; 7–7.5 min, to 50% B; 7.5–10.5 min, 50% B. The autosampler was set at 5 °C to preserve the samples. The injection volume was 5 μL. The instrument parameters were as follows: spray voltage +4500 V, cone temperature 300 °C, cone gas flow 15 psi, heated probe temperature 400 °C, heated probe gas flow 15 psi, nebuliser gas flow 55 psi and collision pressure 2.0 mTorr.

3 | RESULTS AND DISCUSSION

3.1 | Analysis of SLs with the multiple reaction monitoring method

The compound-dependent parameters for each of the seven standards and the IS were optimised by direct infusion to the mass spectrometer to achieve maximum multiple reaction monitoring (MRM) signal intensities for the protonated molecule $[M + H]^+$ or the sodium adduct $[M + Na]^+$. The parent or precursor ions for each standard, the fragments obtained by MRM analysis and the collision energy to achieve each fragmentation are provided in Table 1. The most stable fragments were: the loss of a water molecule (−18), the loss of an acetate group (−59), the loss of the D-ring moiety (−97) or different combinations of these. In the literature, the loss of the D-ring is reported as the most intense fragmentation[1,4] but, under our conditions, the loss of water, an acetate molecule or the loss of both water (or acetate) and the D-ring, was more favourable if they are present in the molecule. The different fragmentation pathways detected are shown in Figure 2.

In the cases where more than one fragmentation was possible, the most intense signal was used as a quantifier ion, while the other was used as a qualifier ion to improve the identification of the standards. When sodium adducts showed a more intense signal, a fragmentation of the proton adduct was selected in order to confirm the identity of the standard. In the cases of strigol and orobanchol, the fragmentations were optimised to identify each isomer clearly. The sodium adduct of strigol gave a more intense signal, while the orobanchol signal was more intense for the proton adduct. In addition, the fragmentation 347 > 215 showed selectivity for strigol, whereas the

TABLE 1 Mass pair (*m/z*) and compound-dependent parameters of the standards

Compound name	t_R (min)	Quantifier ion (*m/z*)			Qualifier ion (*m/z*)		
		Q1 Precursor ion	Q3 Product ion	CE[a] (eV)	Q1 Precursor ion	Q3 Product ion	CE[a] (eV)
7-Oxoorobanchyl acetate	2.47	425	268	15	425	328	12
Solanacol	2.95	343	228	14	365	268	14
(±)-GR24 (IS)	3.47	321	224	11	–	–	–
Orobanchol	3.67	347	233	12	347	329	6
Strigol	3.78	369	272	17	347	215	13
Fabacyl acetate	4.13	405	231	10	405	345	7
Orobanchyl acetate	4.40	411	255	14	389	233	17
5-Deoxystrigol	4.91	353	256	11	353	241	20

[a]CE, collision energy.

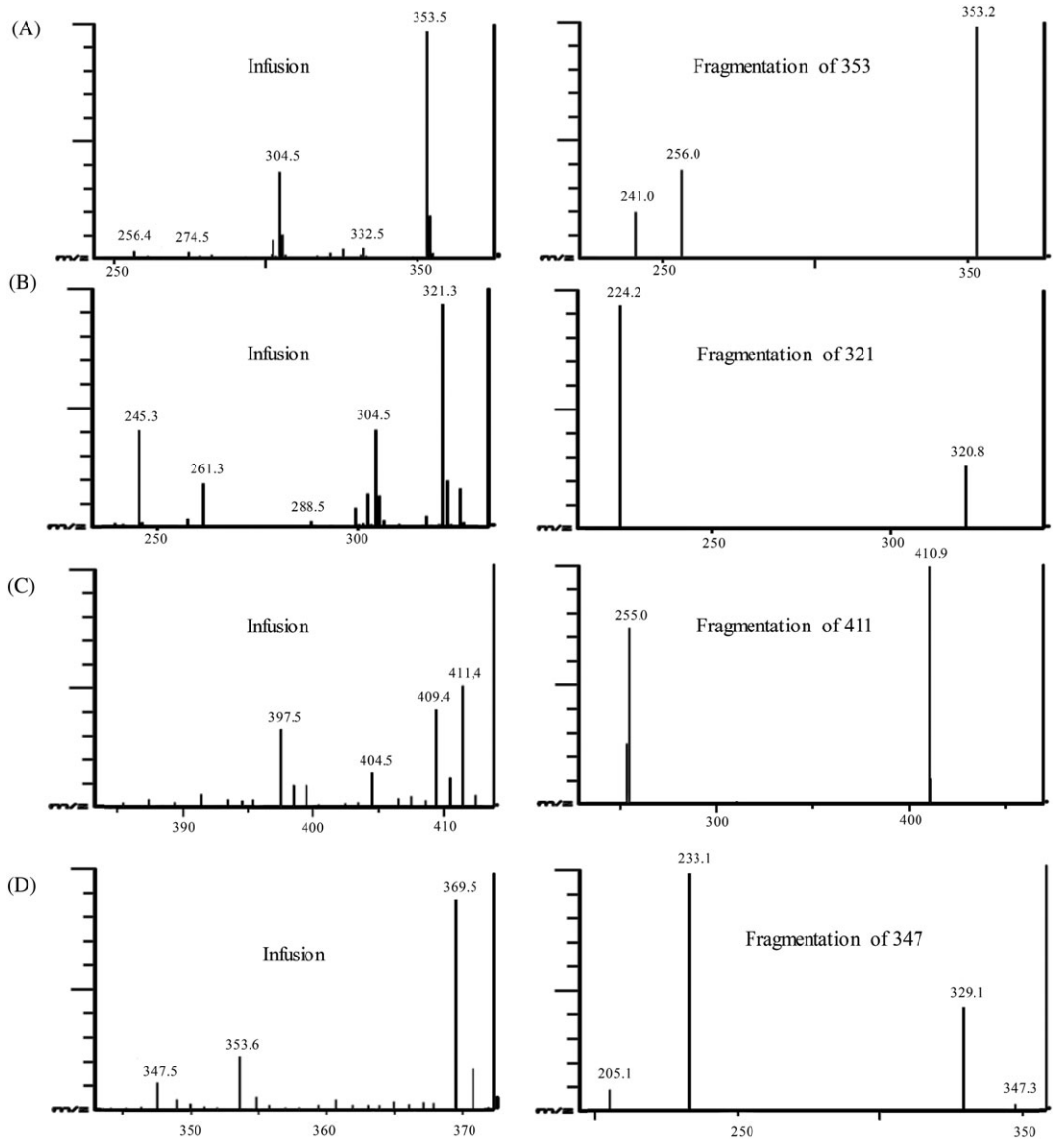

FIGURE 2 Mass spectra and fragmentation obtained by direct infusion of: (A) 5-deoxystrigol, (B) (±)-GR24, (C) orobanchyl acetate and (D) orobanchol

fragmentation 347 > 233 was selective to orobanchol. The two channels 369 > 272 and 347 > 215 for strigol and 347 > 233 for orobanchol are shown in Figure 3 for a mixture of orobanchol and strigol. In cases where a hydroxyl group was not present in the structure, the proton adduct gave either a very low intensity or it was not detected at all.

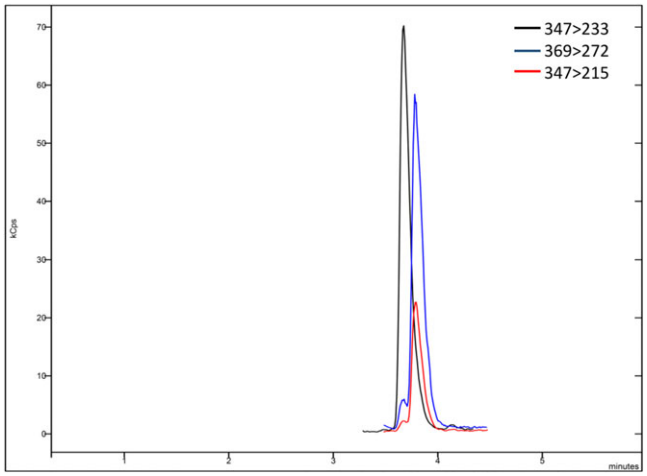

FIGURE 3 Peaks of the transitions 369 > 272 and 347 > 215 for strigol and the transition 347 > 233 for orobanchol in a mixture of strigol and orobanchol [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Development of the UHPLC method

Research on different chromatographic conditions was focused on achieving the best peak separation and resolution for all of the standards. The chromatographic separation obtained with the final conditions is shown in Figure 4. Strigol and orobanchol were not completely separated by UHPLC, but they could be quantified clearly by the different MRM. The UHPLC method has been optimised to quantify the seven SLs in just 10.5 minutes.

3.3 | Method validation

Validation of the quantitative analytical method for the simultaneous determination of all standards followed ICH recommendations.[30] Validation was based on the following parameters: selectivity, linearity, precision of the instrumental system (repeatability and intermediate precision), detection and quantification limits, and stability. The analytical properties of the calibration curves prepared are provided in Table 2, which also contains the limit of quantification (LOQ) and limit of detection (LOD) determined by nine replicate analyses and considered to be 10 and 3 times the standard deviation of baseline noise. The precision of the method was studied in an intra- and inter-day assay (n = 9) for each compound. Repeatability was assessed by analysing replicates of the same sample with all standards. The method was found to be precise with relative standard deviation (RSD) values below 5% in all cases. Intermediate precisions were below 6% for all analytes injected on three different days.

3.4 | Validation with real samples

As previously described by López-Ráez and co-workers,[1,18] phosphate starvation stimulates the biosynthesis of SLs. For this reason, exudates and extracts from tomato roots with and without phosphate starvation were analysed. The samples were named as follows: –P for phosphate starvation samples and +P for samples grown under standard conditions. A total of seven samples were analysed for exudates and seven samples for extracts, five replicates for phosphate starvation samples and two replicates for standard samples. No SLs were

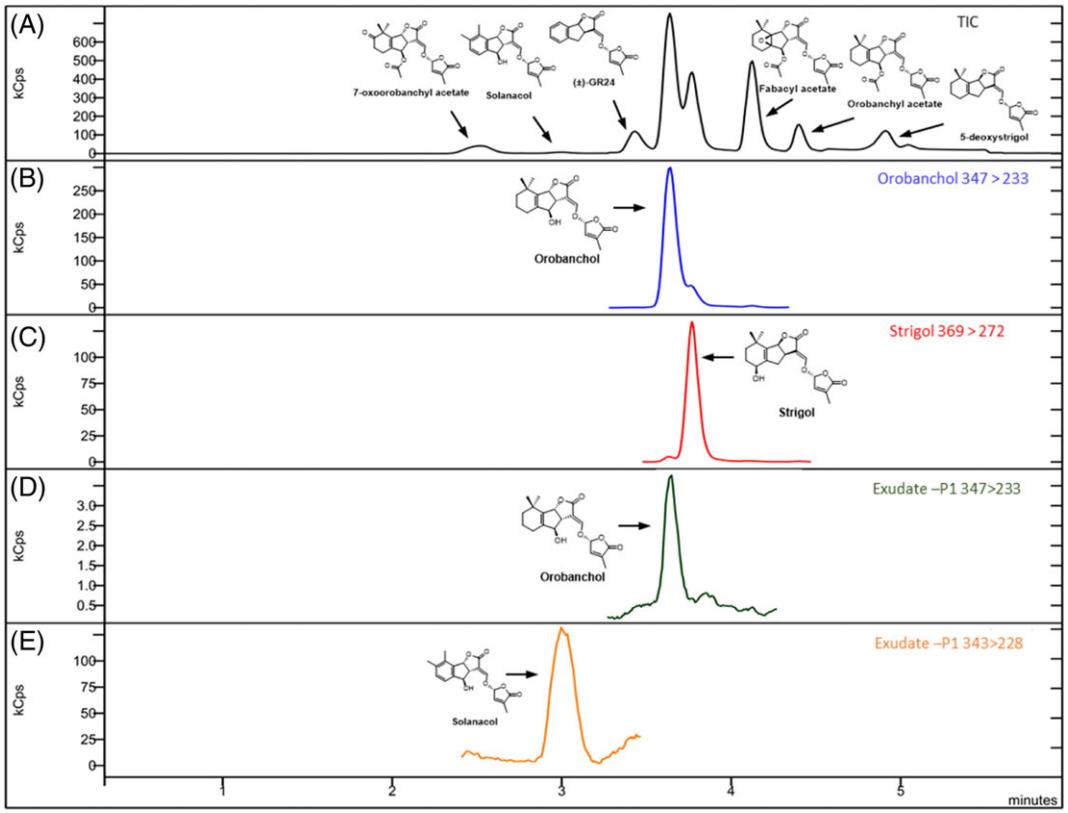

FIGURE 4 Chromatographic separation of standards (A,B,C) and orobanchol (D) and solanacol (E) traces of –P1 tomato exudate [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Analytical characteristics of the chromatographic method

Compound	Intercept	Slope	Regression coefficient	LOD[a] (μg/L)	LOQ[b] (μg/L)	Repeatability RSD %[c]		Intermediate precision RSD %[c]	
						Area	t_R	Area	t_R
7-Oxoorobanchyl acetate	−0.2021	0.3175	0.9928	0.14	0.48	3.5	0.5	4.7	0.5
Solanacol	−0.0116	0.0335	0.9964	0.96	3.19	4.7	0.4	4.9	0.4
Orobanchol	−0.0137	0.6155	0.9974	0.06	0.18	4.4	0.3	4.6	0.3
Strigol	−0.0371	1.1838	0.9957	0.26	0.87	4.5	0.4	5.6	0.4
Fabacyl acetate	−0.0105	0.5802	0.9954	0.04	0.13	3.0	0.3	5.5	0.3
Orobanchyl acetate	−0.0070	0.0394	0.9986	0.85	2.84	4.8	0.3	5.4	0.3
5-Deoxystrigol	−0.0178	3.1273	0.9940	0.02	0.05	3.7	0.3	4.2	0.3

[a]LOD, limit of detection.

[b]LOQ, limit of quantification.

[c]RSD %, relative standard deviation.

detected in samples grown under normal phosphorous conditions. The concentrations of the SLs identified in the samples from plants subjected to phosphate starvation – orobanchol and solanacol – are provided in Table 3. The concentration of solanacol was approximately 35 times higher than that of orobanchol. Exudate sample –P5 was diluted 1:1 with MeOH because the concentration was outside the range of the calibration curve. Our method confirms the results previously reported, phosphate starvation stimulates the biosynthesis of SLs, and solanacol and orobanchol are the major characterised SLs present in tomato plants.

To evaluate the robustness of the analytical method against the matrix effect, known amounts of all the SLs not identified in tomato roots were added to a real sample to achieve a concentration of 50 μg/L and the recovery was measured. Hence, –P1 extract sample was selected for this study. Recoveries were calculated as the differences between the true (spiked) and calculated values obtained using the external calibration method, relative to the true (spiked) value. Results are shown in Table 4. These values (< 10%) show the reliability of this method against the matrix effect. In this condition, the method was also validated regarding the repeatability for the area and t_r values. As it can be seen in Table 4, RSD values (2.3–9.6% for areas and 0.2–0.7% for t_r) are quite similar to the values obtained for standard solutions (Table 2: 3.0–4.8% for areas and 0.3–0.5% for t_r). The results confirm that the new method is robust against the matrix effect.

TABLE 3 Concentration of strigolactones (SLs) identified in tomato samples

Sample		Orobanchol (μg/L (RSD %)[a])	Solanacol (μg/L (RSD %)[a])
Exudates	–P1	1.26 (1.3)	60.77 (2.6)
	–P2	1.85 (2.5)	69.39 (5.8)
	–P3	2.56 (0.7)	62.50 (2.1)
	–P4	1.49 (5.8)	63.04 (3.5)
	–P5	4.72 (1.6)	180.60 (4.5)
	+P1	n.d.	n.d.
	+P2	n.d.	n.d.
Extracts	–P1	1.89 (4.6)	59.73 (6.1)
	–P2	1.84 (3.3)	65.77 (6.6)
	–P3	n.d.	88.08 (1.0)
	–P4	2.87 (4.7)	41.11 (5.7)
	–P5	n.d.	44.07 (6.4)
	+P1	n.d.	n.d.
	+P2	n.d.	n.d.

[a]RSD %, relative standard deviation.

n.d., not detected.

TABLE 4 Recoveries calculated for the matrix effect of strigolactones (SLs)

Compound	Recovery %[a]	Repeatability RSD % [b]	
		Area	t_R
7-Oxoorobanchyl acetate	−7.7	2.3	0.2
Strigol	8.1	6.7	0.3
Fabacyl acetate	−9.8	5.2	0.4
Orobanchyl acetate	−7.6	9.6	0.7
5-Deoxystrigol	4.9	2.5	0.2

[a]The value of recovery is positive when the presence of matrix promotes the signal and negative when it lowers the signal (n = 9).

[b]RSD %, relative standard deviation.

In conclusion, a simple, rapid and reliable UHPLC-MS/MS method has been validated for the routine analysis of seven SLs [7-oxoorobanchyl acetate (**1**), solanacol (**2**), orobanchol (**4**), strigol (**5**), fabacyl acetate (**6**), orobanchyl acetate (**7**), and 5-deoxystrigol (**8**)], using (±)-GR24 as the IS. The analytical method was proven to be selective, linear, precise, and accurate in the ranges studied for all standards. The detection and quantification limits achieved allow the direct identification and quantification of the compounds in root exudates and extracts.

The validated analytical method described above provides a useful tool for research in all the fields related to SLs, both for studies related to their function as hormones, and signaling molecules in the rhizosphere, without sample preparation required in less than 11 minutes.

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