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RESEARCH PAPER

The determination of 22 natural brassinosteroids in a minute sample of plant tissue by UHPLC–ESI–MS/MS

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Abstract The triterpenoid plant hormones brassinosteroids (BRs) are believed to influence almost every aspect of plant growth and development. We have developed a sensitive mass spectrometry-based method for the simultaneous profiling of twenty-two naturally occurring brassinosteroids including biosynthetic precursors and the majority of biologically active metabolites. Using ultra-high performance liquid chromatographic (UHPLC) analysis, the run time was reduced up to three times (to 9 min) in comparison to standard HPLC BRs analyses, the retention time stability was improved to 0.1–0.2 % RSD and the injection accuracy was increased to 1.1–4.9 % RSD. The procedures for extraction and for two-step purification based on solid-phase extraction (SPE) were optimised in combination with subsequent UHPLC analysis coupled to electrospray ionisation tandem mass spectrometry (ESI–MS/MS) using *Brassica* flowers and *Arabidopsis* plant tissue extracts. In multiple reaction monitoring (MRM) mode, the average detection limit for BRs analysed was close to 7 pg, and the linear range covered up to 3 orders of magnitude. The low detection limits for this broad range of BR metabolites enabled as little as 50 mg of plant tissue to be used for quantitative analyses. The results of determinations exploiting internal standards showed that this approach provides a high level of practicality, reproducibility and recovery. The method we have established will enable researchers to gain a better understanding of the dynamics of the biosynthesis and metabolism of brassinosteroids and their modes of action in plant growth and development.

Keywords Brassinosteroids · Solid-phase extraction · Ultra-high performance liquid chromatography · Tandem mass spectrometry · *Arabidopsis thaliana* · *Brassica napus*

Introduction

Brassinosteroids (BRs) are a group of naturally occurring signalling molecules with a steroidal structure and are classified as plant hormones [1]. BRs are essential growth regulators that are widespread in the plant kingdom and have structures closely related to those of animal steroid hormones. To date, over 70 BRs have been isolated and characterised since the discovery of the first BR, brassinolide (BL), in 1979 [2]. Like their animal counterparts, BRs influence many physiological processes throughout the life cycle of the plant, including germination, organ elongation, timing of senescence and flowering, male fertility and increased tolerance of stresses caused by temperature, water or salinity [3].

BRs have a common structural feature, a 5α-cholestane skeleton (Fig. 1), and they form two main groups: free BRs and BRs conjugated with glucose and fatty acids such as lauric acid and myristic acid [4]. Furthermore, like other plant sterols, BRs can be divided into three categories, C_{27}, C_{28} and C_{29} BRs, depending on the substitution of the side chain (Fig. 1). The C_{28} BRs are the most ubiquitous in nature and include, inter alia, the most biologically active compound, BL. BRs with different substituents at C-23, C-24 and C-25 have

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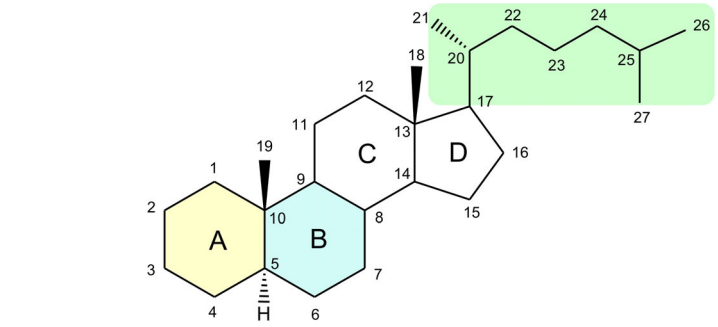
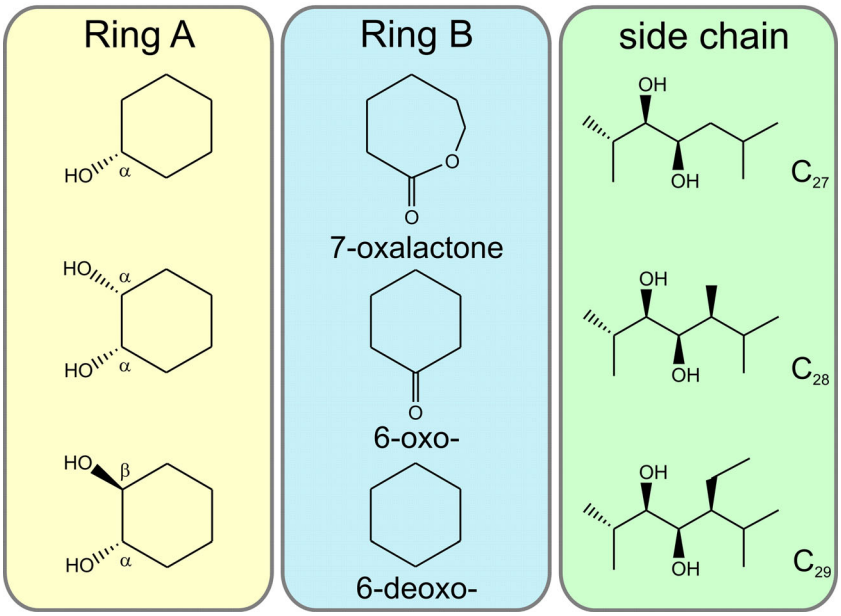


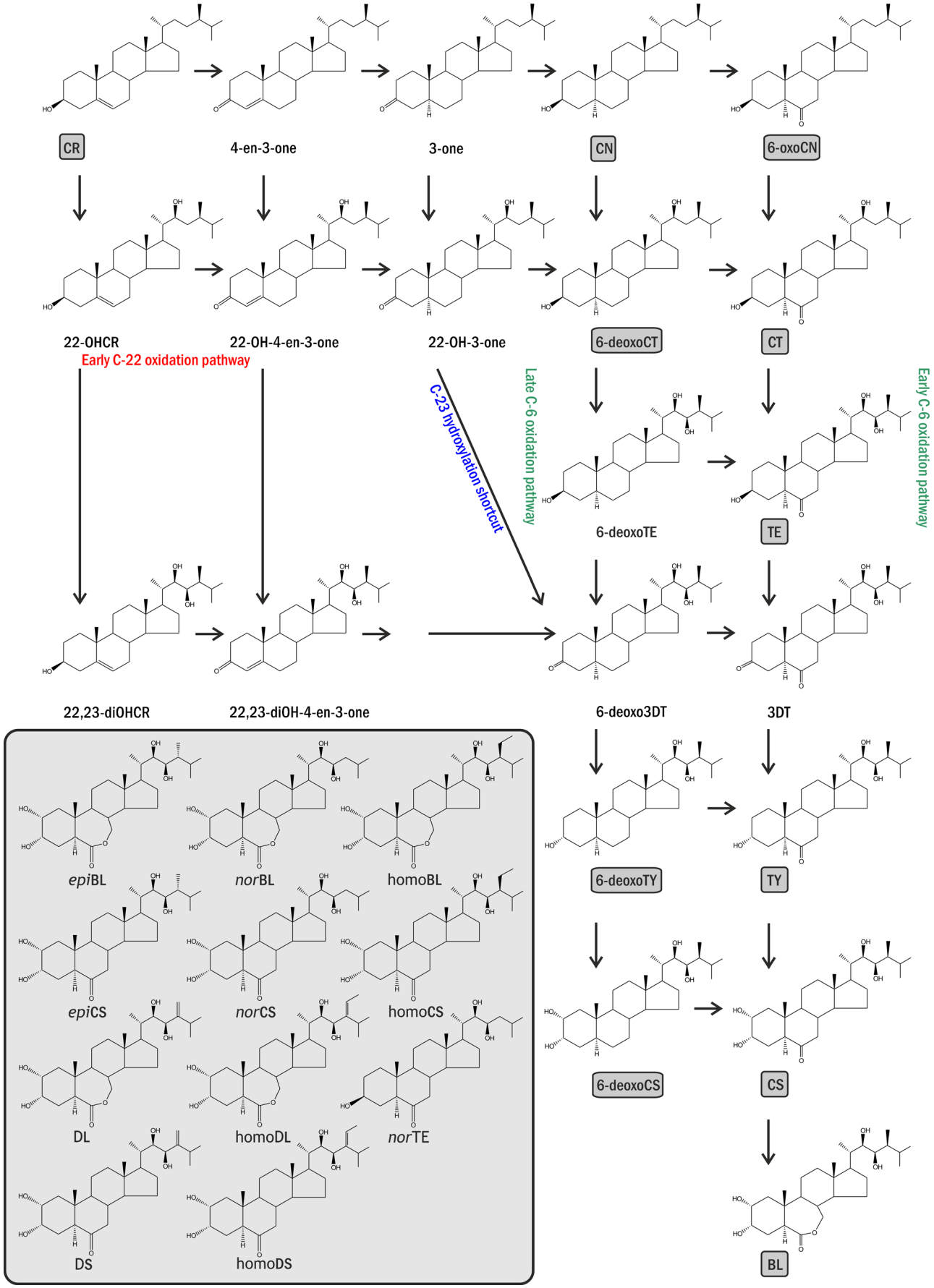
Fig. 1 Structure of 5α-cholestane and various substituents on the ring A, B and the side chain of naturally occurring brassinosteroids



also been found [5]. With respect to modifications to ring A of the skeleton (Fig. 1), BRs with α-hydroxyl and β-hydroxyl or ketone groups at position C-3 are biosynthetic precursors of BRs that have a $2\alpha,3\alpha$ vicinal diol conformation. The absence of a single OH group, or any change in configuration, results in a significant reduction in biological activity [6]. Ring B can be oxidised during biosynthesis, leading to the formation of a 6-oxo-7-oxa-7a-homocholestane skeleton (as in the case of BL, Fig. 2). Limited modifications of the B-ring significantly reduce biological activity. Thus, lactone BRs (in which oxygen is the 7th atom in the B ring, i.e. steroids with the configuration 6-oxo-7-oxa) show greater biological activity than 6-oxo types (e.g. castasterone, CS), whereas non-oxidised BRs reveal no bioactivity. The final requirement for BR activity relates to the side chain hydroxyl groups at C-22 and C-23, in which the *22R,23R*-orientation confers a higher activity than the corresponding *RS*- or *SS*-orientation.

The endogenous concentrations of BRs in samples of plant origin are extremely low, lying in the ppt to ppq range. The levels of BRs differ significantly depending on the type of plant tissue [7]. In reproductive organs (pollen, flowers and immature seeds), BR concentrations reach approximately picogram per gram fresh weight (FW) (ppt), whereas in vegetative plant organs (shoots and leaves), levels of approximately femtogram per gram FW (ppq) are typically detected. Because of the complexity of the plant tissue matrix, in which phytohormones are associated with a large number of interfering substances (e.g. plant pigments, proteins and lipids), it is very important to use an effective enrichment procedure prior to BR analysis. The purification of plant extracts (i.e. the removal of interfering compounds) is typically achieved by means of very tedious and time-consuming processes, including solvent partitioning (liquid-liquid extraction with chloroform, hexane and ethyl acetate [8]), column chromatography (Sephadex LH-20) [8], solid-phase extraction (SPE, diethylaminopropyl silica, octadecyl silica) and reversed-phase high performance liquid chromatography (RP-HPLC) [7, 8].

Fig. 2 Chemical structures and biosynthetic relationships of natural brassinosteroids (BRs). The names of twenty-two BRs included in this study are highlighted in grey ▶


CR
4-en-3-one
3-one
CN
6-oxoCN
22-OHCR
22-OH-4-en-3-one
22-OH-3-one
6-deoxoCT
CT
Early C-22 oxidation pathway
C-23 hydroxylation shortcut
Late C-6 oxidation pathway
Early C-6 oxidation pathway
6-deoxoTE
TE
22,23-diOHCR
22,23-diOH-4-en-3-one
6-deoxo3DT
3DT
epiBL
norBL
homoBL
epiCS
norCS
homoCS
DL
homoDL
norTE
DS
homoDS
6-deoxoTY
TY
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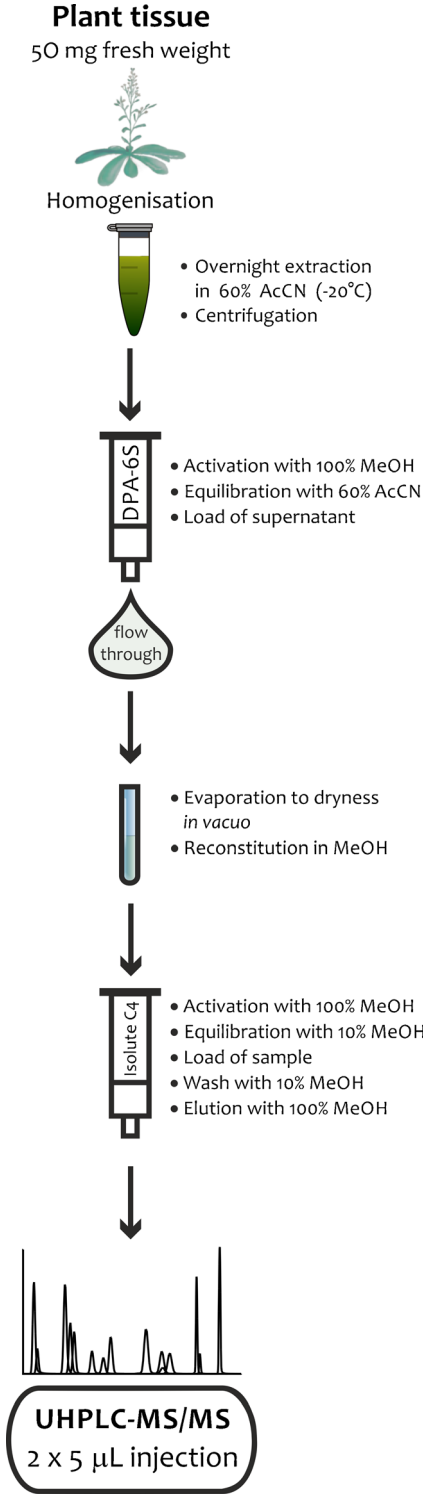

Fig. 3 Scheme of extraction and purification procedure for free naturally occurring BRs from plant tissues

For analysis of BRs, as for other non-volatile highly hydrophobic substances, liquid chromatography coupled to mass spectrometry (LC–MS) is generally the method of first choice. However, gas chromatography coupled to MS (GC-MS) after derivatisation was the first method published for analysis of BRs [9]. BRs possessing vicinal diol at ring A form *bis*-methaneboronate derivatives (BMB), which can be analysed by GC-MS at sub-nanogram levels [10, 11]. The disadvantage of this approach is that BRs lacking this conformation (e.g. campesterol, campestanol, cathasterone, 6-oxocampestanol, etc.) cannot be modified in this way and therefore cannot be quantified at all. Although several LC methods have been reported, only two have so far been used for the direct determination of free BRs [12, 13].

The other LC methods still require derivatisation (naphthalene boronates, dansyl-3-aminophenylboronates) prior to BR analysis, mainly to improve their limits of detection [14–19]. The amount of plant sample used is usually 100 mg to 2 g FW. However, it has become clear that derivatisation is not the key to successful detection of BRs when the plant matrix contains a high level of interfering substances causing a huge chemical background [7]. This problem appears when plant samples weighing 100 mg FW and more are used and it is even more pronounced if the tissue extracted is reach in pigments, lipids, starch, saccharides, etc. Therefore, the most important step in sample preparation before BRs analysis is to decrease the initial amount of plant tissue used for extraction and then separate BRs as extremely lowly abundant substances of interest from the numerous interfering compounds in crude extract using effective purification approach.

In the present study, we report the development of a method for the fast extraction and efficient pre-concentration of twenty-two free BRs originating from biosynthetic pathways that include late and early C-6 oxidation steps, including BRs lacking axial/equatorial hydroxy groups in ring A or vicinal diols in the BR side chain. We achieved sensitive quantitation of these BRs by ultra-high performance liquid chromatography (UHPLC) coupled to (+)ESI–MS/MS, with the limits of detection ranging between 0.05 and 40 pg. This method was successfully applied to the determination of BRs in 50 mg *Brassica napus* flower samples by isotope dilution analysis [20]. This is the first report of an analytical approach dealing with the analysis of such number of natural BRs in real plant samples without the need for derivatisation.

Experimental

Reagents and material

Authentic brassinosteroids (brassinolide, 24-*epi*-brassinolide, 28-*nor*brassinolide, castasterone, 24-*epi*-castasterone, 28-*nor*castasterone, 28-homocastasterone, 28-*nor*teasterone, 6-deoxocastasterone, 6-oxocampestanol and cathasterone) and deuterium-labelled brassinosteroids ([26-$^2H_3$]brassinolide, [26-$^2H_3$]castasterone, [26-$^2H_3$]*epi*brassinolide, [26-$^2H_3$]*nor*brassinolide, [26-$^2H_3$]*epi*castasterone, [26-$^2H_3$]*nor*castasterone,

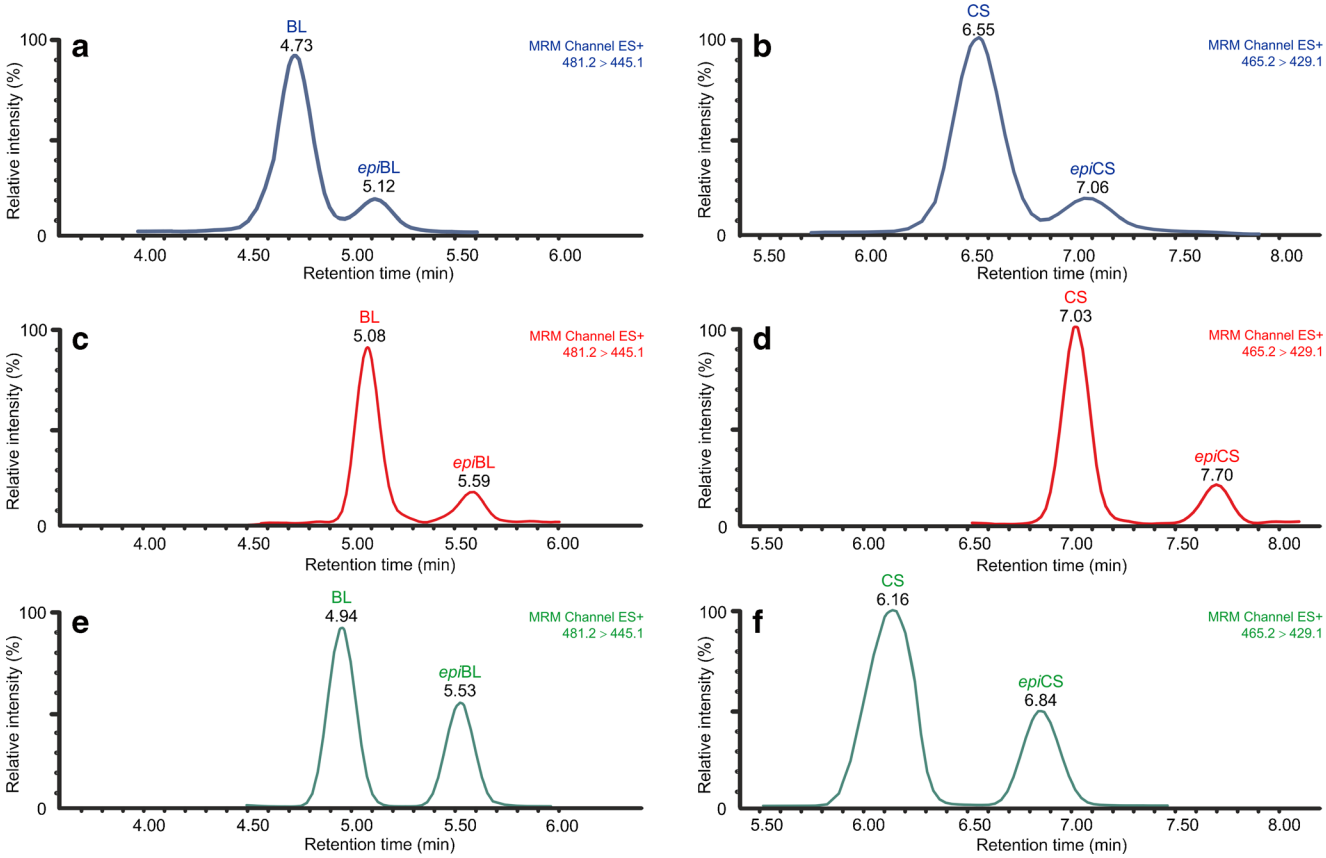

Fig. 4 The comparison of peak shape and peak-to-peak resolution of two pairs of BRs epimers BL vs. *epi*BL and CS vs. *epi*CS on column Acquity UPLC®BEH C18 column (**a**, **b**), Acquity UPLC® CSH™ C18 (**c**, **d**) and Ascentis® Express Phenyl-Hexyl (**e**, **f**)

[26-$^{2}H_3$]typhasterol, [26-$^{2}H_3$]6-deoxocastasterone, [26-$^{2}H_3$]cathasterone, [26-$^{2}H_3$]6-deoxotyphasterol, [26-$^{2}H_3$]campesterol and [26-$^{2}H_3$]campestanol) were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Other unlabelled BR standards (28-homobrassinolide, dolicholide, 28-homodolicholide, dolichosterone, 28-homodolichosterone, teasterone and typhasterol) were purchased from Chemiclones Inc. (Waterloo, Canada). The compounds 24-*epi*-brassinolide, 28-homobrassinolide, 24-*epi*-castasterone and 28-homocastasterone had side chains with the *22R,23R* conformation, as is typical of their naturally occurring forms. Tritium-labelled BRs ([5,7,7-^{3}H]*homo*castasterone, [5,7,7-^{3}H]*epi*castasterone and [5,7,7-^{3}H]*epi*brassinolide) were generous gifts from Ass. Prof. Tomáš Elbert (Laboratory of Radioisotopes, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic). HPLC grade formic acid (FA) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Analytical grade methanol (MeOH) and all other chemicals were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Deionised (Milli-Q) water obtained from a Simplicity 185 water system (Millipore, Bedford, MA, USA) was used to prepare all aqueous solutions.

Biological material

The winter cultivar of oilseed rape *B. napus* (L.) was grown in the field and fully expanded flowers were harvested at different times during the day (the first sample was taken out 1 h before sunrise and others were carried every hour until noon). Detached flowers of *B. napus* in 50-mL Falcon tubes were frozen in liquid nitrogen and stored at −80 °C until required for extraction. *Arabidopsis thaliana* Columbia-0 (Arabidopsis) was grown in 250-mL Erlenmeyer flasks containing 50 mL Murashige-Skoog basal growth medium with 3 % (*w*/*v*) sucrose and a pH of 5.6 (20–25 seeds per bottle). The flasks were agitated and maintained at 23 °C with an 8-h light/16-h dark photoperiod. After 3 weeks, the plants were harvested, immediately frozen in liquid nitrogen and stored at −80 °C prior to extraction and purification.

Extraction and purification of brassinosteroids

Frozen plant tissues were ground to a fine consistency in a mortar and pestle with liquid nitrogen. Aliquots of 50 mg FW were weighed into 2-mL Eppendorf tubes and 1 mL of ice-cold 60 % ACN, as an extraction solution, and 2-mm ceria stabilised zirconium oxide beads (Next Advance Inc., Averill

Fig. 5 Separation of 15 brassinosteroids (group A) by ultra-high performance liquid chromatography (UHPLC). UHPLC–MS chromatogram of BRs standard mixture divided into eight MRM channels (I–VIII) containing 10 pmol of each BRs per injection

Park, NY, USA) were added for further homogenisation using a MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany). An internal standard mixture containing 30 pmol of each of the ^{2}H-labelled BRs was also added to the samples at this stage. The samples were extracted overnight at 4 °C using a Stuart SB3 benchtop laboratory rotator (Bibby Scientific Ltd, Staffordshire, UK) and subsequently centrifuged (36,670×*g*, 10 min, 4 °C; Beckman Avanti™ 30). The pellets obtained were re-extracted by rotation in the same way for 60 min at 4 °C. The supernatants were combined and purified using a Discovery® DPA-6S cartridges (50 mg, Supelco®, Bellefonte, PA, USA) activated with 100 % MeOH. The flow-through fraction of each sample was evaporated to dryness in vacuo (CentriVap® Acid-Resistant benchtop concentrator, Labconco Corp., MO, USA). Each sample residue was dissolved in 100 μL 100 % MeOH by vortexing and sonicating for 5 min and made up to 1 mL with Milli-Q water before loading onto an Isolute® C4 SPE cartridge (100 mg, Isolute® C4, Biotage, UK), which was first activated with 1 mL of MeOH and equilibrated with 1 mL of 10 % MeOH. The C4 column was then washed with 10 % MeOH and BRs were eluted with 1 mL of 100 % MeOH. The elution fraction was evaporated to dryness in vacuo and stored at −20 °C until required for analysis. A Visiprep™ Solid Phase Extraction Vacuum Manifold (Supelco®, Bellefonte, PA, USA) was routinely used for the SPE sample purification step mentioned above. For optimisation of the purification procedure, the following activities of ^{3}H-labelled BRs were utilised: 3.70 kBq [5,7,7-^{3}H]homocastasterone, 3.74 kBq [5,7,7-^{3}H]*epi*castasterone and 7.3 kBq [5,7,7-^{3}H]*epi*brassinolide. The radioactivity of the tritium-labelled brassinosteroid standards was measured using a solution containing 10 μL of the sample fraction in 3 mL of a liquid scintillation cocktail, Ultima Gold™, in an LS 6500 multi-purpose scintillation counter (both Beckman Coulter, Brea, CA, USA).

LC–MS/MS apparatus

An Acquity UPLC™ System (Waters, Milford, MA, USA) consisting of a binary solvent manager and sample manager coupled to a Xevo® TQ MS triple-stage quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray (ESI) interface was utilised for BR quantitation. The entire LC–MS system was controlled by MassLynx™ Software (version 4.1, Waters, Manchester, UK).

UHPLC–ESI–MS/MS conditions

After purification, the dried plant extract samples were each reconstituted in 50 μL of 100 % MeOH (chilled to −20 °C) from which 5 μL of the sample was then injected onto a reversed-phase column (Acquity UPLC® CSH™ C18, 2.1 mm × 50 mm, 1.7 μm; Waters) coupled to an ESI–MS/MS system. The brassinosteroids were analysed in positive ion mode as $[M+H]^{+}$. The product and precursor ions for each BR and ^{2}H-labelled internal standard are listed in the Electronic Supplementary Material Table S1. Analytes belonging to group A (relatively less hydrophobic BR biosynthetic products) were separated by a linear gradient of ACN (A) and 10 mM formic acid (B) at a flow rate of 0.3 mL min^{-1}, from 35:65 A/B (*v*/*v*) to 38.5:61.5 (*v*/*v*) over 5 min and then to 70:30 A/B over 1.5 min. Under these conditions, the substances of interest were isocratically eluted in 1.5 min. Finally, the column was washed with 100 % ACN (0.45 mL) and re-equilibrated to the initial conditions (35:65 A/B, *v*/*v*) for 1.5 min. For the retention time of each BR studied, see Electronic Supplementary Material Table S2.

Substances belonging to group B (highly hydrophobic BR biosynthetic precursors) were separated using a more hydrophilic reversed-phase UHPLC column (Acquity UPLC®

Table 1 Optimised MS conditions for the quantitation of each of the brassinosteroids analysed

Compound	Diagnostic (quantitation) transition	Cone voltage (V)	Collision energy (V)	Dwell time (s)	Retention time window (min)	Channel
Group A						
Dolicholide (DL)	479.16 > 349.15	20.0	14.0	0.528	3.15–3.70	I.
28-Norbrassinolide (*nor*BL)	467.24 > 431.22	25.0	12.0	0.528		
[$^2H_3$]-*nor*BL	470.33 > 416.14	25.0	12.0	0.490		
Homodolicholide (homoDL)	493.17 > 349.18	20.0	14.0	0.400	4.35–5.30	II.
Dolichosterone (DS)	463.22 > 427.19	20.0	16.0	0.400		
28-*Nor*castasterone (*nor*CS)	451.23 > 433.29	20.0	10.0	0.400		
[$^2H_3$]-*nor*CS	454.28 > 436.31	20.0	10.0	0.460		
Brassinolide (BL)	481.20 > 445.10	22.0	12.0	0.613	5.05–5.80	III.
[$^2H_3$]-BL	484.35 > 448.20	22.0	13.0	0.613		
24-*Epi*brassinolide (*epi*BL)	481.20 > 445.10	22.0	12.0	0.425	5.60–6.70	IV.
[$^2H_3$]-*epi*BL	484.22 > 448.30	25.0	12.0	0.450		
28-Homodolichosterone (homoDS)	477.33 > 459.12	20.0	10.0	0.397		
Castasterone (CS)	465.20 > 429.10	20.0	17.0	0.778	7.1–7.90	V.
[$^2H_3$]-CS	468.32 > 432.40	20.0	18.0	0.778		
24-*Epi*castasterone (*epi*CS)	465.20 > 429.10	20.0	17.0	0.325	7.75–8.40	VI.
[$^2H_3$]-*epi*CS	468.34 > 450.27	20.0	10.0	0.325		
28-*Nor*teasterone (*nor*TE)	435.18 > 355.22	20.0	12.0	0.325		
28-Homobrassinolide (homoBL)	495.20 > 459.10	22.0	12.0	0.325		
28-Homocastasterone (homoCS)	479.20 > 443.10	21.0	17.0	0.215	8.53–8.96	VII.
Teasterone (TE)	449.47 > 283.21	20.0	16.0	0.259		
Typhasterol (TY)	449.39 > 431.24	20.0	16.0	0.205	9.30–9.56	VIII.
[$^2H_3$]-TY	451.68 > 434.32	20.0	16.0	0.205		
Group B						
6-Deoxocastasterone (6-deoxoCS)	451.61 > 433.72	20.0	14.0	0.028	4.20–5.00	I.
[$^2H_3$]-6-deoxoCS	454.87 > 331.51	20.0	16.0	0.028		
Cathasterone (CT)	433.06 > 397.08	20.0	12.0	0.028		
[$^2H_3$]-CT	435.99 > 399.94	20.0	12.0	0.028		
6-Deoxotyphasterol (6-deoxoTY)	417.10 > 398.94	20.0	14.0	0.036	5.65–5.90	II.
[$^2H_3$]-6-deoxoTY	420.16 > 285.22	20.0	20.0	0.036		
6-Oxocampestanol (6-oxoCN)	416.97 > 399.08	40.0	10.0	0.036		
6-Deoxocathasterone (6-deoxoCT)	400.98 > 382.99	20.0	16.0	0.036	5.85–6.15	III.
Campesterol (CR)	383.05 > 161.00	20.0	10.0	0.078	7.45–7.75	IV.
[$^2H_3$]-CR	386.77 > 161.00	20.0	10.0	0.078		
Campestanol (CN)	385.06 > 134.60	20.0	16.0	0.078	7.65–8.00	V.
[$^2H_3$]-CN	388.05 > 150.78	20.0	16.0	0.078		

Protein BEH C4, 2.1 mm × 150 mm, 1.7 μm; Waters). The C4 column was operated in a linear gradient of 0.1 % formic acid in ACN (A) and 0.1 % formic acid in ultra-pure water (B), at a flow rate 0.3 mL min^{-1}, from 40:60 A/B (*v*/*v*) to 100:0 (*v*/*v*) over 10 min; the elution was isocratic over 1 min of the chromatographic run. At the end of the run, the column was washed with 0.1 % formic acid in 100 % ACN (0.9 mL) and re-equilibrated to the initial conditions (40:60 A/B, *v*/*v*) for

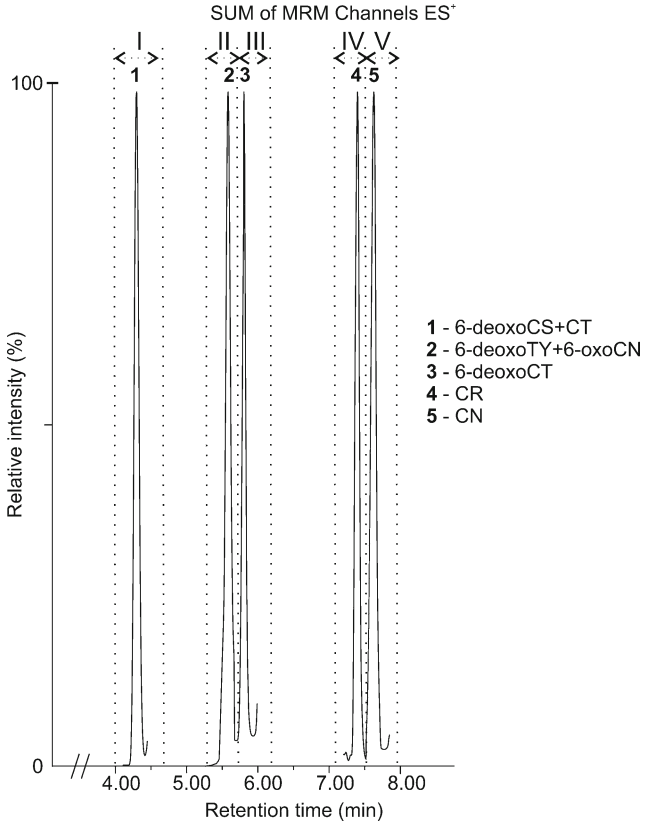

Fig. 6 Separation of seven brassinosteroids (group B) by ultra-high performance liquid chromatography (UHPLC). UHPLC–MS chromatogram of BRs standard mixture divided into five MRM channels (I–V) containing 10 pmol of each BRs per injection

3.5 min. The thermostat of the column was programmed to 40 °C and the temperature inside the autosampler was maintained at 4 °C. The capillary voltage, cone voltage, collision cell energy and ion source temperatures were optimised for each individual compound using the same setup. The mass spectrometer settings were as follows: capillary voltage, 4.0 kV; cone voltage, 20 V; source temperature, 120 °C; desolvation gas temperature, 550 °C; cone gas flow, 70 L h^{-1}; and desolvation gas flow, 600 L h^{-1}. The dwell time of each MRM channel was calculated to provide 16 scan points per peak, with an inter channel delay of 0.1. The MS data were recorded in multiple reaction monitoring mode (MRM). All of the data were processed by MassLynx™ software (ver. 4.1, Waters).

Results and discussion

Extraction and purification procedure

Generally, the optimal method for purification depends on the chemical nature of the target analyte, the type of analysis to be performed and the choice of analytical instrument. Before developing optimal extraction and purification methods for BRs (see Fig. 2 for compounds included in this study), one must be conscious of two important facts: (1) BRs are non-volatile, highly hydrophobic substances lacking ionic properties, and (2) plant tissues contain trace quantities of BRs, whereas thousands of other substances are present in far greater amounts. Taking these factors into account, two frequently used organic solvents, MeOH and ACN, containing different amounts of water (0–70 %), were chosen for optimisation of BR extraction from plant tissues, using rotation to achieve high extraction efficiency. To determine the content of the most abundant interfering plant pigments (which may result in a high level of chemical background in the final LC–MS/MS analysis) in the extraction solution, the levels of chlorophyll a (chl_a) and b (chl_b), and the total content of carotenoids, were measured using a previously described standard spectrophotometric method [21, 22]. The content of plant pigments quantitatively expresses here the amount of substances that suppress the MS signal. Similar procedure has been already earlier used for analysis of natural diterpenoid compounds of hormonal character in [23]. Given the non-ionic chemical nature of BRs, neither an acidic nor a basic modifier of the extraction solvent was tested.

Extracts from 50 mg of tissue from flowering plants of oilseed rape (*B. napus*) were prepared in quadruplicate. In extracts prepared using the mixture of water and MeOH (30–100 %, *v*/*v*), the amount of $chl_a + chl_b$ ranged between 0.70 and 1.35 μg mL^{-1}, while that of carotenoids ranged from 0.14 to 3.19 μg mL^{-1}. When the same experiment was performed with aqueous ACN (30–100 %, *v*/*v*) as the extraction solvent, the equivalent ranges were 0.09–0.62 and 0.19–3.39 μg mL^{-1} for $chl_a + chl_b$ and carotenoids, respectively. Thus, ACN extracted approximately 15–45 % less chlorophyll, represented as $chl_a + chl_b$, whereas the extraction efficiency of carotenoids was found to be comparable for both extraction solvents.

To remove representatives of both these groups of plant pigments, together with plant phenolics, from the tissue extracts, SPE cartridges containing a polyamide-based resin, Discovery® DPA-6S (Supelco), were chosen. This resin is designed to adsorb polar compounds containing hydroxyl groups (e.g. phenolics, flavonoids), as well as chlorophylls, from aqueous or organic solvent solutions by means of the reversed-phase mechanism through strong hydrogen bonding between the hydroxyl groups of the compound and the amide groups of the resin. In the next experiment, extracts from 50 mg of flowering oilseed rape tissue, prepared in quadruplicate in both of the above series of extraction solutions (30–100 % MeOH/ACN), were purified through DPA-6S SPE cartridges. The content of $chl_a + chl_b$ and the total content of carotenoids in the resulting extracts were again measured using the above spectrophotometric method. A reduction of approximately 72 % in the $chl_a + chl_b$ content and appro

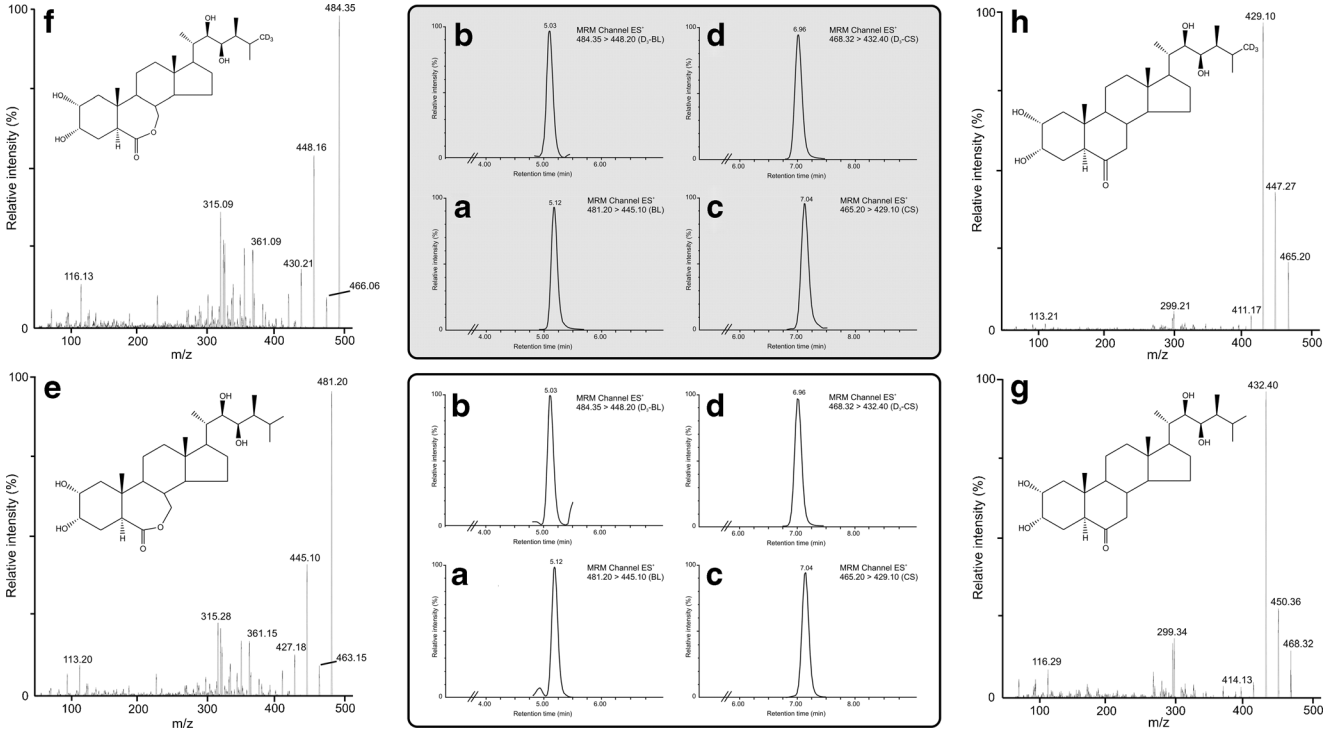

Fig. 7 MRM chromatograms of brassinolide (BL) and castasterone (CS) standards (*a*, *c* in *white frame*) and of the endogenous compounds in an extract of 50 mg *B. napus* flowers (*a*, *c* in *grey frame*) in the presence of $^{2}H_3$-labelled internal standards (*b*, *d*). MS spectra were recorded under optimised conditions for standard of BL (*e*), [$^{2}H_3$]BL (*f*), CS (*g*) and [$^{2}H_3$]CS (*h*)

ximately 48 % in the carotenoid content was observed in aqueous methanolic extracts, whereas 77 % of $chl_a + chl_b$ and 17 % of carotenoids were removed from the aqueous acetonitrile extracts. Bearing in mind that the total content of chlorophylls is generally twice as high in the methanolic extracts, the most effective method with which to extract and purify plant tissue samples in order to minimise plant pigment content is to utilise acetonitrile as the organic extraction solvent. The removal of carotenoids occurs to the same extent in both solvents.

To objectively assess the efficiency of extraction of endogenous BRs and the recovery of internal standards, we prepared a new set of 50-mg quadruplicates of *B. napus* tissue in 30–100 % MeOH/ACN with the addition of deuterium-labelled BRs and extracted them overnight at 4 °C using a laboratory rotator operating at 15 rpm. All of the extracts were further purified through a DPA-6S SPE column, evaporated to dryness and analysed by UHPLC–MS/MS after reconstitution in 50 μL of 100 % MeOH. In the case of the most hydrophobic BR from group A, typhasterol (TY), the highest extraction yield for this substance was obtained when using 50–60 % ACN (see Electronic Supplementary Material Fig. S1B). These solvents are also suitable for the extraction of one of the most hydrophilic BRs, 28-*nor*brassinolide (*nor*BL; Electronic Supplementary Material Fig. S1A). The extraction yields for brassinolide (BL) and castasterone (CS), moderately hydrophobic BRs, were found to reach the highest values when using 90 % ACN or MeOH (Electronic Supplementary Material Fig. S1C, D). Extraction in acetonitrile at a concentration of 70 % or more in the extraction solution appeared to be more suitable for deuterium-labelled BL and *epi*BL internal standards, whereas methanolic solutions of the same concentration resulted in better extraction recovery for the more hydrophobic deuterium-labelled internal standards CS and *epi*CS (Electronic Supplementary Material Fig. S1E–H). However, the differences between the yields of *nor*BL, BL and CS in 60–90 % organic solution were not significant, though in the case of TY the yield decreased dramatically when a 70 % or higher percentage of organic solvent was used for the extraction.

Taking into account the results for determining pigment content, the extraction efficiency for the endogenous BRs and the internal BRs standard recovery experiments, 60 % ACN was chosen as the most appropriate solvent for extracting BR from plant tissues, followed by subsequent purification using DPA-6S SPE cartridges (Fig. 3). At this stage, it was found that a second SPE-based purification step is needed to further decrease the chemical background in plant extracts prior to LC–MS/MS analysis. To optimise second SPE step, three tritium-labelled BR standards, [5,7,7-^{3}H]homocastasterone ([^{3}H]homoCS), [5,7,7-^{3}H]*epi*castasterone ([^{3}H]*epi*CS) and

Table 2 Method validation—selected parameters of the UHPLC–MS/MS method for determination of BRs. Extracts of 50 mg FW *B. napus* tissue were spiked with 10 pmol (a) or 30 pmol (b) of authentic BRs standards, purified by two-step SPE approach including Discovery DPA-6S and Isolute® C4 sorbent, and analysed by UHPLC–MS/MS

Nr.	Compound	Determined spiked content*[a] (pmol)	Analytical precision (a) (%)	Analytical accuracy (a) (%)	Determined spiked content[a] (b) (pmol)	Analytical precision (b) (%)	Analytical accuracy (b) (%)
1.	BL	9.9 ± 1.0	4.2	99.1	30.1 ± 0.2	1.5	100.3
2.	*nor*BL	8.9 ± 0.9	4.6	89.1	30.5 ± 0.7	3.4	101.7
3.	CS	9.7 ± 1.5	5.8	97.3	29.5 ± 0.4	5.1	98.3
4.	*nor*CS	8.8 ± 1.7	4.1	88.2	31.2 ± 0.7	2.2	104.0
5.	DL	9.8 ± 1.9	4.0	98.2	32.5 ± 1.4	2.1	108.3
6.	homoDL	9.1 ± 0.5	3.5	91.1	31.7 ± 1.0	2.7	105.7
7.	DS	10.2 ± 0.4	4.2	102.2	29.9 ± 1.2	3.1	99.7
8.	homoDS	10.5 ± 0.3	3.5	104.5	29.5 ± 0.9	2.7	98.3
9.	*epi*BL	11.2 ± 0.7	3.2	112.1	30.1 ± 0.4	1.0	100.3
10.	*epi*CS	9.7 ± 1.2	2.5	97.1	29.8 ± 0.7	1.7	99.3
11.	*nor*TE	10.3 ± 0.2	2.1	102.7	28.1 ± 1.2	2.3	93.7
12.	homoBL	10.4 ± 1.4	4.0	104.1	29.7 ± 0.7	3.5	99.0
13.	homoCS	9.2 ± 1.4	3.9	92.2	28.2 ± 1.5	4.1	94.0
14.	TE	10.0 ± 0.3	1.5	100.3	27.5 ± 0.6	1.1	91.7
15.	TY	10.8 ± 0.5	1.0	107.9	27.2 ± 0.5	1.2	90.7
16.	6-deoxoC-S	9.2 ± 1.8	4.9	92.1	29.5 ± 0.2	4.8	98..3
17.	CT	9.4 ± 1.6	4.5	94.2	27.5 ± 1.2	4.9	91.7
18.	6-deoxoT-Y	10.3 ± 1.4	3.9	103.3	27.1 ± 1.0	3.7	90.3
19.	6-oxoCN	9.5 ± 0.9	3.8	95.1	26.1 ± 0.9	2.1	87.0
20.	6-deoxoC-T	9.7 ± 0.7	4.6	96.9	24.9 ± 1.2	4.2	83.0
21.	CR	9.2 ± 1.9	4.8	91.9	27.6 ± 0.7	4.0	92.0
22.	CN	9.0 ± 0.8	4.7	90.2	25.4 ± 0.6	3.9	84.7

[a] The values represent the mean ± standard deviation obtained for six technical replicates prepared and analysed separately

[5,7,7-^{3}H]*epi*brassinolide ([^{3}H]*epi*BL) were used and their recovery from three different RP Isolute® cartridges (containing C1, C2 and C4 sorbent; bed size 100 mg/1 mL) was estimated by measuring their radioactivity by scintillation counter. All three sorbents were activated using 100 % MeOH, then equilibrated with 10 % MeOH in deionised water, and the BRs were eluted from the sorbents with 100 % MeOH after loading the extract (from 50 mg of flowering *B. napus* tissue, prepurified by DPA-6S) and carrying out a washing step with 10 % MeOH. Under these experimental conditions, the recoveries of [^{3}H]homoCS (68 %), [^{3}H]*epi*BL (67 %) and [^{3}H]*epi*CS (56 %) were found to be highest when C4 sorbent was used. To summarise, the entire sample preparation procedure for the determination of BRs in plant tissue by LC–MS/MS consisted of two SPE steps, using Discovery® DPA-6S and Isolute® C4 (Fig. 3).

Liquid chromatography

Because of the huge differences in hydrophobicity of the BRs studied here, two mixtures of unlabelled BR standards and their deuterium-labelled analogues were prepared in order to find the optimal conditions for separating them by two LC runs: group A—15 unlabelled compounds and 7 deuterium-labelled substances; group B—7 unlabelled compounds and 5 deuterium-labelled substances (see Electronic Supplementary Material Table S1 for details of both groups). For LC separation of group A compounds, three reversed-phase UHPLC columns (RP-UHPLC), Acquity UPLC® BEH C18 (Bridged Ethylene Hybrid), Acquity UPLC® CSH™ C18 (Charged Surface Hybrid) (both 2.1 × 50 mm, 1.7 μm, Waters) and Ascentis® Express Phenyl-Hexyl (2.1× 100 mm, 2.7 μm, Supelco), were tested. The ionisation efficiency

and the peak shape were found to be satisfactory when ACN (solvent A) and 10 mM HCOOH (solvent B) were used as the components of the mobile phase. Under these conditions, the Acquity UPLC® CSH™ C18 column provided significantly enhanced peak-to-peak resolution (Fig. 4c, d) compared to the BEH column (Fig. 4a, b), especially for two pairs of epimers (BL vs. *epi*BL and CS vs. *epi*CS). In resolving these pairs of epimers, the Phenyl-Hexyl column, which is generally designed for planar as well as delocalised heterocyclic ring systems, gave the best results (Fig. 4e, f); however, in this case the peak width was approximately twice the value of that obtained from the CSH and BEH columns. Similarly, the other BRs studied showed the best chromatographic characteristics when the CSH column was used as the separation medium. This column was therefore chosen as the most appropriate tool for separation of BRs belonging to group A, and it was used in the following experiments. Within group A, retention times ranged between 3.15 min (DL) and 9.56 min (TY)—Electronic Supplementary Material Table S2. Each pair, consisting of an unlabelled analyte and a deuterated internal standard, co-eluted with very close retention times (data not shown), with the deuterated analogue exhibiting a shorter retention time than its unlabelled counterpart because of the chromatographic isotope effect [24]. Ten of the 15 BRs within group A that were studied here were fully resolved under the above-mentioned RP-UHPLC conditions (Fig. 5). The DL (*m/z* 479)/*nor*BL (*m/z* 467) and DS (*m/z* 463)/*nor*CS (*m/z* 451) pairs were either unresolved or co-eluted completely. Nor was it possible to achieve a baseline separation of *epi*CS, *nor*TE and homoBL. However, these BRs can be distinguished by an MS detector because of the differences between the *m/z* values of their precursor and product ions (465 > 429; 435 > 355 and 495 > 459). The stability of the retention times fell within an acceptable range, with a coefficient of variation of 0.10–0.3 % ($n = 20$) under the optimised chromatographic conditions. The mean chromatographic peak width for the substances studied was found to be 0.15 min for substances eluted when the eluent had a high organic phase content (above 60 % ACN), whereas peaks of analytes eluted earlier showed significantly greater widths (approximately 0.50 min). To fulfil the requirement that a data sampling rate suitable for reproducible integration should yield a minimum of 16 data points per peak, a dwell time of 0.3–0.6 s was required. The dwell time values for analytes in this study are listed in Table 1.

Regarding the separation of BRs belonging to group B, seven analytes gave five peaks (Fig. 6). The pairs 6-deoxoCS (*m/z* 452)/CT (*m/z* 433) and 6-deoxoTY (*m/z* 417)/6-oxoCN (*m/z* 417) were either unresolved or co-eluted completely. In the case of the first pair, 6-deoxoCS (*m/z* 452) and CT (*m/z* 433) can be easily distinguished by an MS detector since the *m/z* values of their precursor and product ions differ (452 > 434 vs 433 > 397). To discriminate between the two members of the second critical pair by MS, it is necessary to use a very narrow span value (0.1) for each MRM transition since the difference in *m/z* between precursors and product ion is extremely small (417.10 > 398.94 for 6-deoxoTY vs. 416.97 > 399.08 for 6-oxoCN—see Table 1).

MS/MS detection

Solutions containing mixtures of standards, consisting of the unlabelled BRs and their respective deuterium-labelled internal standards, were used to select the appropriate precursor-to-product ion transition for each substance in (+)ESI–MS/MS. All of the BRs studied provided background-subtracted ESI^+ spectra exhibiting $[M+H]^+$ as the base peaks (Electronic Supplementary Material Table S1). Two main fragmentation patterns were found for both groups (A and B) of the BR derivatives investigated as hydroxylated substances. The first pattern is represented by a loss of one unit of water (*m/z* 18) from the precursor ion leading to the formation of the product ion that is the most abundant in a spectrum—this pattern was shown by *nor*CS, homoDS and TY from group A, and 6-oxoCN 6-deoxoCT, 6-deoxoTY and 6-deoxoCS from group B (see Electronic Supplementary Material Table S1). The second fragmentation pattern is defined by a loss of two units of water (*m/z* 36) from the precursor ion to produce the most abundant product ion in a spectrum—*nor*BL, DS, BL, *epi*BL, CS, *epi*CS, homoBL and homoCS from group A and CT from group B exhibited this pattern (Electronic Supplementary Material Table S1). Homodolicholide (Fig. 2) was cleaved in the collision cell giving an ion at *m/z* 349.18 as the most abundant product, which was formed as a result of the loss of *m/z* 144, corresponding to cleavage of the bond between C-20 and C-22 of the BR skeleton. The same fragmentation pattern could be also observed for dolicholide (Fig. 2), its analogue, which is not methylated at position 28. A molecular ion of *m/z* 479.16 gave its most abundant product ion at *m/z* 349.15, an ion that is formed in the ion source by the cleavage of a side chain at the C20-C22 position of the DL molecule (loss of *m/z* 130). Only teasterone (TE, Fig. 2) and $[^2H_3]$-6-deoxoTY were found to produce ions corresponding to the complete loss of their entire side chains (449.47 > 283.21 and 420.16 > 285.22—see Electronic Supplementary Material Table S1). The counterparts that were triply deuterium-labelled at the C-26 position showed no difference in fragmentation pattern compared to their unlabelled analogues [25], with the exceptions of *nor*BL, *epi*CS, 6-deoxoCS, 6-deoxoTY and CN (Table 1).

Interestingly, no molecular ion derived from CR or CN was found in their full scan MS spectra after positive ionisation by electrospray. These compounds form only ions corresponding

to the molecular ions with the loss of one unit of water, i.e. we found an ion with $m/z = 383$ for CR (molecular formula $C_{28}H_{28}O$) while its molecular weight is 400.68 g mol^{-1} (Table 1). Similarly, an ion at $m/z = 385$ was observed in the CN spectrum while the molecular mass of this substance is 402.70 g mol^{-1} (molecular formula $C_{28}H_{25}O$). Moreover, their fragmentation patterns differ from those of the other BRs studied. When measuring the daughter spectra of both dehydrated ions, we detected the highest peak intensities for ions corresponding to the skeleton containing only rings A and B of the original molecule with or without a CH_3 group at position 19 (an ion at *m/z* 134 for CN and an ion at *m/z* 161 for CR—Electronic Supplementary Material Table S1).

Based on the mass spectra obtained, the quasi-molecular ions $[M+H]^+$ and the most intensive fragment ions were selected (Electronic Supplementary Material Table S1) for mass spectrometric detection in MRM mode. The entire chromatographic run was then divided into eight retention windows (channels I–VIII) for group A (Fig. 5) and five retention windows (channels I–V) for group B (Fig. 6). Each window was characterised by defined MRM functions for the appropriate analyte (Table 1). Examples of mass spectra of the diagnostic product ions are shown in Fig. 7.

Matrix effect

As mentioned above, the targeted analysis of many mainly lowly abundant substances in crude plant extracts may be hindered by signal suppression, due to strong matrix effects (ME). To study this effect typical for the samples of biological origin, the standard mixture of BRs (30 pmol each) was added to pure extraction solvent (60 % aqueous ACN, *v*/*v*) and to the extracts of a 50 and 100 mg FW plant tissue samples after SPE process. All samples were prepared in quadruplicates. Having established a highly sensitive method for BRs analysis, we tested the extent to which the plant matrix from our samples suppressed the MS signals of interest. The peak area response for each analyte in the presence of the plant matrix ions was compared with peak area response in the absence of matrix ions to calculate matrix factor MF according to [26]. The data are summarised in Electronic Supplementary Material Table S2. A strong matrix effect was observed for 100 mg FW samples (MF mean ~31 %), while approximately two times lower ME was found for 50 mg FW samples (MF ~69 %), which corresponds with two times lower weight of plant sample tissue.

Method validation and application

The newly developed UHPLC–ESI–MS/MS method was tested by analysing the levels of endogenous BRs in samples of flowers from field-grown *B. napus*, a biological material known to be a rich source of BRs. Calibration curves were created by preparing solutions containing varying amounts of each unlabelled BR and a known, fixed amount of the corresponding deuterium-labelled internal standard (IS) across concentration ranges 0.01–0.1 to 50 pmol/5 μL of injection. Four separate injections were used to give the resulting calibration curves, which were linear in the selected concentration range for all 22 BR compounds investigated (correlation coefficient, R^2, values obtained were in the range 0.9917 to 0.9999; see Electronic Supplementary Material Table S3). For quantitation of endogenous BRs, which had no corresponding deuterium-labelled counterparts, internal standards with very similar chromatographic behaviour and falling within the linear range of the BR/[$^2H_3$]-BR calibration curve were found. [$^2H_3$]-*nor*BL was used for quantitation of DL and *nor*BL and [$^2H_3$]-*nor*CS for quantitation of homoDL, DS and *nor*CS. Similarly, the levels of *epi*BL and homoDS were determined using [$^2H_3$]-*epi*BL, and for quantitation of *epi*CS, *nor*TE and homoBL, [$^2H_3$]-*epi*CS was used. [$^2H_3$]-TY was chosen as a suitable internal standard for the quantitative analysis of TY, homoCS and TE. For analytes belonging to group B, 6-deoxoCT and 6-oxoCN were internally calibrated using [$^2H_3$]-6-deoxoTY. The linear range for all calibration curves was shown to cover 2 to 3 orders of magnitude. The limit of detection (LOD) was evaluated using an approach based on the standard deviation, s_b, of the calibration curve and the slope, k, of a regression curve ($LOD = 3 \times s_b/k$) [27]. The LODs for BRs are summarised in Electronic Supplementary Material Table S3. The limit of quantitation (LOQ) was evaluated using a standard deviation/slope ratio approach ($LOQ = 10 \times s_b/k$) [27; data not shown].

Different concentrations of IS (1–30 pmol) added to the extraction media were tested, and 10 pmol was found to be the most appropriate concentration for all BRs investigated in tissues containing chlorophyll and other plant pigments. Accordingly, 10 pmol of each IS was added to the samples before purification. The plant extracts were purified by SPE (Discovery® DPA-6S followed by Isolute® C4) and concentrated in vacuo, after which the BRs were quantified by LC–MS as described above.

Finally, the analytical accuracy of the UHPLC–(+)ESI–MS/MS method was evaluated by 'standard addition method' using two sets of samples: purified extracts of *B. napus* tissue (50 mg of plant tissue in 1 mL of extraction solution, six replicates) with the addition of 10 and 30 pmol of authentic BRs standards prior to two step sample purification. The concentration of each analyte was calculated using the standard isotope dilution method for each plant extract spiked before extraction and compared with the concentration of appropriate standard solution. The analytical accuracy values ranged between 83 and 112 % of the true amount (Table 2). The analytical precision was determined to be in the range 1.0–5.8 % (Table 2).

Our newly developed SPE/UHPLC–(+)ESI–MS/MS method has recently been successfully applied to the determination of natural BRs in many different plant materials [28–33]. The results obtained (Electronic Supplementary Material Table S4) demonstrate its usefulness for the targeted profiling of endogenous BRs present in trace amount in minute plant samples. Thus, the originality and advantage of this method consist in the ability to quantify extremely low levels of twenty-two endogenous BRs from very small amount of plant tissue without the need for derivatisation. The comparison of our newly developed method parameters with those of earlier published with respect mainly to limit of detection and amount of tissue needed for analysis is summarised in Electronic Supplementary Material Table S5. Moreover, the purification of plant tissue extracts is much less demanding (only two SPE steps) in comparison with previously published purification procedures, which are extremely time consuming and labour intensive, having up to eight steps including liquid-liquid extraction, HPLC fractionation and derivatisation.

Conclusion

In this study, we describe a method for the simultaneous analysis of twenty-two naturally occurring BRs in two independent chromatographic runs. The newly developed UHPLC–ESI–MS/MS approach is based on very fast and effective chromatographic separation of selected plant brassinosteroids obtained from extremely complex plant matrices by efficient extraction and a two-step solid-phase purification procedure. This method was successfully applied to the analysis of biologically active BRs and some of their biosynthetic precursors in *Brassicaceae* plants. The UHPLC–MS/MS method outlined here exhibits high chromatographic resolution, satisfactory sensitivity and sufficient selectivity. This technique may be classified among the methods designated 'hormone profiling', which are characterised by the quantification of not only the hormones themselves but also their biosynthetic precursors and metabolites, in plant tissues. This approach is likely to prove especially useful for the analysis of biochemical processes that involve BRs as key signalling molecules.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

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