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Analysis of gibberellins as free acids by ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A robust, reliable and high-throughput method for extraction and purification of gibberellins (GAs), a group of tetracyclic diterpenoid carboxylic acids that include endogenous growth hormones, from plant material was developed. The procedure consists of two solid-phase extraction steps (Oasis® MCX-HLB and Oasis® MAX) and gives selective enrichment and efficient clean-up of these compounds from complex plant extracts. The method was tested with plant extracts of *Brassica napus* and *Arabidopsis thaliana*, from which total recovery of internal standards of about 72% was achieved. A rapid baseline chromatographic separation of 20 non-derivatised GAs by ultra performance liquid chromatography is also presented where a reversed-phase chromatographic column Acquity CSH® and a mobile phase consisting of methanol and aqueous 10 mM-ammonium formate is used. This method enables sensitive and precise quantitation of GAs by MS/MS in multiple-reaction monitoring mode (MRM) by a standard isotope dilution method. Optimal conditions, including final flow rate, desolvation temperature, desolvation gas flow, capillary and cone voltage for effective ionisation in the electrospray ion source were found. All studied GAs were determined as free acids giving dominant quasi-molecular ions of $[M-H]^-$ with limits of detection ranging between 0.08 and 10 fmol and linear ranges over four orders of magnitude. Taking advantage of highly effective chromatographic separation of 20 GAs and very sensitive mass spectrometric detection, the presented bioanalytical method serves as a useful tool for plant biologists studying the physiological roles of these hormones in plant development.

1. Introduction

Gibberellins (GAs) are a class of diterpenoid carboxylic acids present in plants and some bacterial and fungal species, and which include compounds that act as endogenous growth regulators in higher plants. The main physiological effects of biologically active GAs include the induction of germination and flowering, stimulation of stem elongation through enhanced cell division and elongation, parthenocarpic (seedless) development of some fruit in the absence of fertilisation and delay of senescence in leaves and citrus fruits [1–3].

Structurally, GAs possess either the *ent*-gibberellane (containing 20 carbon atoms), or a 20-nor-*ent*-gibberellane (containing only 19 carbon atoms) carbon skeletons. The simplest examples of C_{20}- and C_{19}-GAs are GA_{12} and GA_9, respectively, the latter being formed biosynthetically from the former by loss of C-20 (Fig. 1). The C_{19}-GAs include the biologically active forms, which must contain an hydroxyl group on C-3β, a γ-lactone between C-4 and C-10 and a free carboxyl group on C-6 for optimal binding to the GID1 receptor, while hydroxylation on C-2β causes loss of binding and biological activity [4,5]. Mechanisms for GA inactivation, which as well as 2β-hydroxylation include epoxidation on C-16–C-17, methylation of the carboxylic group and glucosylation, are necessary for regulating the size of the pool of active hormone [6].

Gibberellins are present in plant tissues at very low concentrations, normally in the range 10^{-9}–10^{-15} mol g^{-1} fresh weight, depending on the tissue. Therefore very high enrichment of the GAs is essential prior to their detection by standard analytical techniques. The extraction and preconcentration steps are often tedious, labour intensive and time-consuming, comprising usually liquid–liquid extraction, solid-phase extraction (SPE) using general

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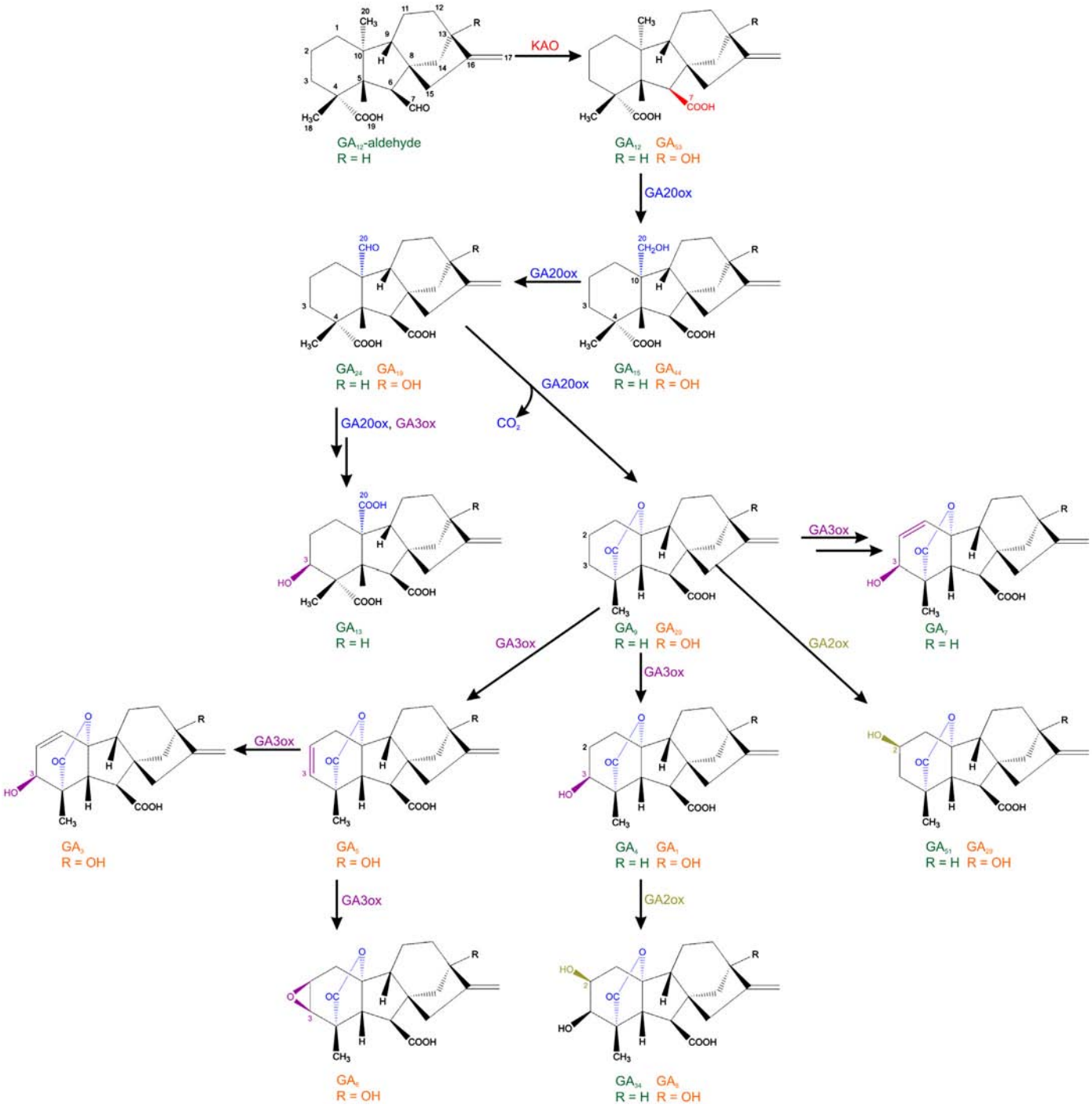

Fig. 1. Structures and biosynthetic relationship of the 20 GAs including in this study, showing the enzymes responsible for each reaction (above the arrow). KAO: *ent*-kaurenoic acid oxidase; GA20ox: GA 20-oxidase; GA3ox: GA 3-oxidase and GA2ox: GA 2-oxidase.

purpose sorbents, ion-exchange and high performance liquid chromatography (HPLC). Ideally, the extraction solvent should extract the GAs efficiently, whilst the quantity of interfering substances extracted should be as low as possible. The extraction efficiency of GAs depends on the extent to which they are associated with such as phenolics, lipids, pigments or proteins. The purification of plant extracts is then usually achieved by one of two main approaches: either a combination of solvent partitioning between water and ethyl acetate, strong anion-exchange chromatography and C_{18} solid phase extraction (SPE) followed by HPLC fractionation [7,8], or liquid–liquid extraction can be replaced by SPE step [9]. The low concentrations of GAs necessitate very sensitive analytical tools, the most common approaches involving gas or liquid chromatography combined with mass spectrometry (GC–MS, LC–MS). Pioneering work on GA analysis by GC–MS originates already from the 1960s [10–12] and this method is still widely used for both the identification and quantitation of GAs as volatile methyl ester trimethylsilyl ether derivatives. The quantitative analysis of GAs is usually achieved by isotope dilution giving accurate determination [13]. Highly selective multiple reaction monitoring (MRM) can be used to provide sufficient selectivity in quantitative analysis, which needs to overcome problems arising from the occurrence of many interfering compounds [14,15]. Since the early 1990s, LC–MS is slowly replacing GC–MS as the routine

Table 1
Tabulated negative-ESI precursor ion mass spectra of authentic gibberellin and deuterium labelled gibberellin standards with spectra normalised to the most abundant product ion (bold entries).

Compound	Precursor ion $[M-H]^-$	Diagnostic fragment ions: *m/z* (relative intensity, %)
GA_8	363	321(40), **275(100)**, 257(45), 119(55)
$[^2H_2]$-GA_8	365	**277(100)**, 321(70), 229(50), 158(50)
GA_{29}	347	**303(100)**, 259(50), 163(70)
$[^2H_2]$-GA_{29}	349	305(50), **261(100)**, 219(20), 173(10)
GA_3	345	301(30), **239(100)**, 143(95)
$[^2H_2]$-GA_3	347	**241(100)**, 143(15)
GA_1	347	303(50), **259(100)**
$[^2H_2]$-GA_1	349	**261(100)**, 231(50), 143(5)
GA_6	345	301(35), 257(80), 239(40), 161(40), **119(100)**
$[^2H_2]$-GA_6	347	259(90), 241(70), 161(80), **119(100)**
GA_5	329	285(40), 241(20), 223(25), **145(100)**
$[^2H_2]$-GA_5	331	**287(100)**, 269(90), 243(80), 225(45), 145(70)
GA_{20}	331	**287(100)**, 250(10), 225(10), 219(15), 173(50), 147(30)
$[^2H_2]$-GA_{20}	333	**289(100)**, 271(75)
GA_{13}	377	**359(100)**, 303(45), 259(5), 215(15)
GA_{44}	345	**301(100)**, 273(60), 187(50), 111(40)
$[^2H_2]$-GA_{44}	347	**303(100)**, 257(50)
GA_{19}	361	317(70), **273(100)**, 255(45), 229(45), 203(60), 133(40)
$[^2H_2]$-GA_{19}	363	**275(100)**, 232(70), 185(60), 147(80)
GA_{34}	347	303(25), **259(100)**, 241(35), 199(20)
$[^2H_2]$-GA_{34}	349	305(10), **261(100)**, 243(20), 201(10)
GA_{51}	331	**287(100)**, 243(75), 219(60), 182(20)
$[^2H_2]$-GA_{51}	333	**289(100)**, 261(50)
GA_7	329	287(5), **223(100)**, 211(15), 168(10)
$[^2H_2]$-GA_7	331	287(20), **225(100)**, 195(10)
GA_4	331	313(50), **287(100)**, 257(75)
$[^2H_2]$-GA_4	333	289(50), **259(100)**, 245(50), 213(30)
GA_{53}	347	**329(100)**, 303(75), 233(35), 189(70)
$[^2H_2]$-GA_{53}	349	**331(100)**, 305(80), 233(60)
GA_{24}	345	301(90), **257(100)**, 213(60), 135(35)
$[^2H_2]$-GA_{24}	347	302(20), 284(30), **259(100)**, 219(40),16(30)
GA_9	315	**271(100)**, 253(20), 121(10)
$[^2H_2]$-GA_9	317	**273(100)**, 257(70)
GA_{15}	329	285(40), **257(100)**, 220(15), 185(50), 123(20)
$[^2H_2]$-GA_{15}	331	287(15), **259(100)**, 187(70), 123(30)
GA_{12}	331	**313(100)**, 270(50), 287(80), 201(20)
$[^2H_2]$-GA_{12}	333	**315(100)**, 289(60), 220(50)
GA_{12}-ald	315	**271(100)**, 227(7), 163(30), 121(10)
$[^2H_2]$-GA_{12}-*ald*	317	**273(100)**, 257(10), 165(25), 135(20)

analytical method for quantitative analysis of GAs [16–19] and is now the most common analytical method [20], usually utilising electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) [9,21–24]. A separation method for GAs based on capillary electrophoresis coupled to MS has also been described [25]. Importantly however, none of these methods has dealt with the analysis of more than ten GAs in one chromatographic run although from physiological perspective, there are at least 23 GAs already identified in the most studied model plant *Arabidopsis thaliana* (Arabidopsis), while 12 GAs in rice (*Oryza sativa*) and 14 in oilseed rape (*Brassica napus*) have been identified in these important crop plants [26]. Although it is essential to know the concentrations of the bioactive forms of GAs (such as GA_1, GA_3 and GA_4), knowledge of the concentrations of their precursors and metabolites provides important information on GA metabolism and its regulation by, for example, genetic or environmental factors. In the present study, we report on the development of a method for extraction and pre-concentration of 20 GAs as free acids (Table 1), and their quantitation by ultra performance liquid chromatography (UPLC) followed by (−)ESI-MS/MS with limits of detection ranging between 0.08 and 10 fmol. The structures and biosynthetic relationship of the analysed GAs are shown in Fig. 1. The method was successfully applied for the quantitation of GAs in *B. napus* flowers by isotope dilution analysis [27].

2. Material and methods

2.1. Chemicals and reagents

Authentic gibberellins (GA_1, GA_3, GA_4, GA_5, GA_6, GA_7, GA_8, GA_9, GA_{12} and GA_{12}-aldehyde, GA_{13}, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{29}, GA_{34}, GA_{44}, GA_{51} and GA_{53}) and their corresponding 17-$^2H_2$-labelled analogues were purchased from OlChemIm (Olomouc, Czech Republic). Tritium-labelled GAs ([1,2-$^3H_2$]GA_1, [1,2-$^3H_2$]GA_4 and [1,2,3-$^3H_3$]GA_{20}) were produced as custom synthesis by Amersham Radiolabelling Services (Cardiff, UK) and in the case of [^{3}H]GA_1 and [^{3}H]GA_4 were generous gifts from Professor Makoto Matsuoka (Nagoya University, Japan). Formic acid (FA) and methanol (MeOH, HPLC grade) were purchased from Merck (Darmstadt, Germany). Deionised (Milli-Q) water obtained from a Simplicity 185 water system (Millipore, Bedford, MA, USA) was used to prepare all aqueous solutions. All other chemicals (analytical grade or higher purity) were from Sigma-Aldrich Chemie (Steinheim, Germany).

2.2. Biological material

Arabidopsis thaliana Columbia-0 (Arabidopsis) was grown for 4 weeks on soil at 22 °C, with an 18-h photoperiod, photon density 130 μE m^{-1} s^{-1}. The winter cultivar of oil seed rape plants *Brassica napus* (L.) var. napus f. biennis was grown in the field from August to May when fully expanded flowers were harvested at different times during the day. Detached flowers of *B. napus* in 50 mL Falcon tubes and whole shoots of Arabidopsis weighing 100 mg in 1.5 mL Eppendorf tubes were immediately immersed in liquid nitrogen after harvesting, and then stored at −80 °C until preparation.

2.3. Extraction and purification of gibberellins

Frozen plant tissues (100 mg) were ground to a fine consistency using a MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany) after adding 1 mL of ice-cold 80% AcCN containing 5% FA as extraction solution and 3-mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany). Internal standard mixture containing 50 pmol each of $^2H_2$-labelled GAs was also added to the samples. For optimisation of the purification procedure, ^{3}H- together with $^2H_2$-labelled GAs were used. For ^{3}H-labelled standards we used 555 kBq each of [$^3H_2$]GA_1, [$^3H_2$]GA_4 and [$^3H_3$]GA_{20}. The samples were extracted for 12 h at 4 °C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK). The homogenates were then centrifuged (14 000 rpm, 10 min, 4 °C; Beckman Avanti™ 30) and the resultant pellets were re-extracted in the same way for 60 min with rotation at 4 °C. The combined extracts were evaporated to the water phase *in vacuo* (CentriVap® Acid-Resistant benchtop concentrator, Labconco Corp., MO, USA) and purified using joint Oasis® MCX and Oasis® HLB cartridges (150 mg and 60 mg, respectively, both Waters, Milford, MA, USA) activated with MeOH and pre-equilibrated with 5% aqueous MeOH (v/v). The evaporated samples were dissolved in 5% aqueous MeOH (3 mL), loaded onto the joint cartridges, which were washed with 5.75 mM FA (pH 3) and 5% aqueous MeOH (each 9 mL). The coupled columns were then run to dryness, disconnected and GAs were eluted from the HLB cartridge with MeOH/diethyl ether (20:80 v/v) (3 mL). The eluates were evaporated to dryness under a stream of nitrogen using evaporation system TurboVap® LV (Caliper Life Sciences, Hopkinton, MA, USA). The sample was dissolved in

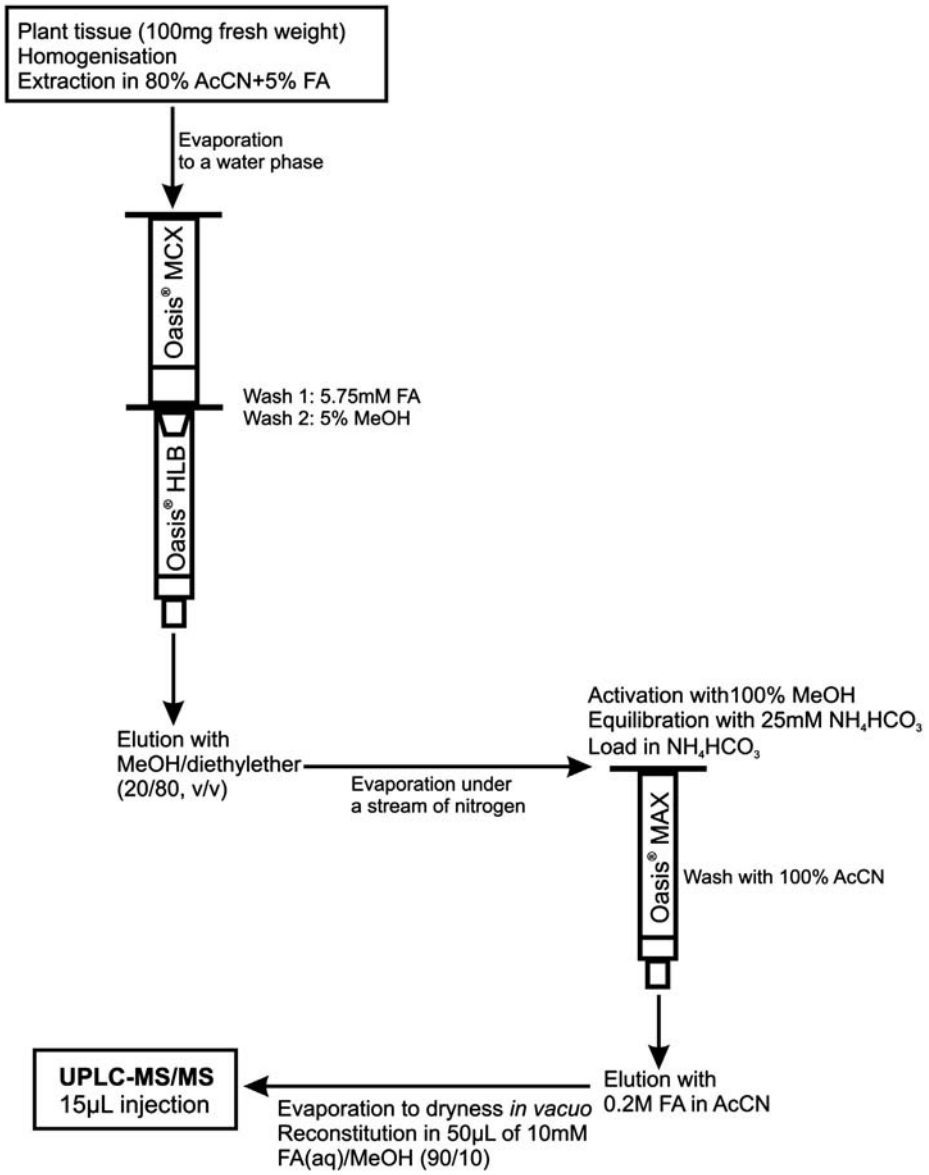

Fig. 2. Scheme of extraction and purification procedure for GAs from plant tissues.

50 μL of MeOH by vortexing and ultrasonication for 5 min and made up to 3 mL with 25 mM NH_4HCO_3 before loading onto Oasis® MAX polymer-based mixed mode columns. The sorbent in these columns was first activated with MeOH (3 mL) and then equilibrated with 25 mM NH_4HCO_3 (6 mL) before loading the sample. The MAX columns were then washed with AcCN (3 mL) and GAs eluted with 0.2 M FA in AcCN (3 mL), which was evaporated to dryness in vacuo. The Visiprep™ Solid Phase Extraction Vacuum Manifold (Supelco®, Bellefonte, PA, USA) was used for the purification stated above. If not analysed immediately, the evaporated samples were stored in a freezer (−20 °C) until UPLC–MS/MS analysis.

The radioactivity of ^{3}H-labelled GA standards during the purification procedure optimisation was measured after addition of a 50 μL aliquot from 3 mL sample to 3 mL of liquid scintillation cocktail Ultima Gold™ using a multi-purpose scintillation counter LS 6500 (both Beckman Coulter, Brea, CA, USA).

2.4. 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 electrospray (ESI) interface and the collision cell—ScanWave™ was utilised for GA quantitation. The entire LC–MS system was controlled by Masslynx™ Software (version 4.1, Waters, Manchester, UK).

2.5. UPLC–ESI-MS/MS conditions

The dried samples were reconstituted in 50 μL of mobile phase (initial conditions), and 15 μL of each sample was then injected onto the reversed-phase UPLC column (Acquity CSH®, 2.1 mm × 50 mm, 1.7 μm; Waters) coupled to the ESI-MS/MS system. Gibberellins were analysed in negative ion mode as $[M-H]^-$, the product and precursor ions for each GA and ^{2}H-labelled internal standard are listed in Table 1. The compounds of interest were separated in a linear gradient of MeOH (A) and 10 mM FA (B) at a flow rate of 0.25 mL min^{-1}, from 10:90 A:B (v/v) to 60:40 (v/v) over 15 min. Finally, the column was washed with 100% MeOH (0.75 mL) and equilibrated to initial conditions (10:90 A:B, v/v) for 2.5 min. For retention time of each GA studied see supplementary Table S1.

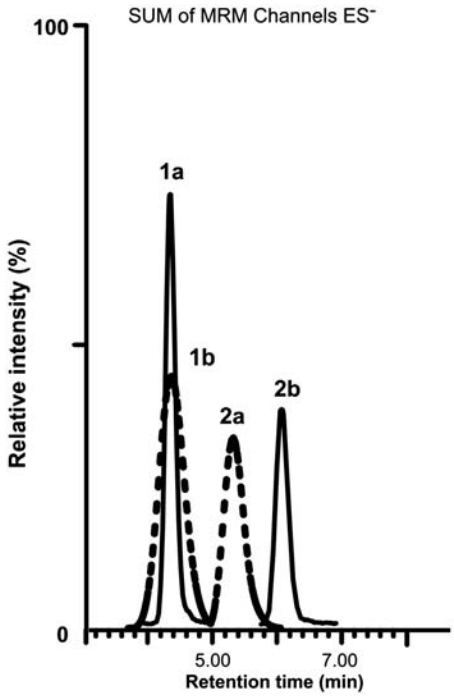

Fig. 3. Comparison of peak shape and peak-to-peak resolution of two selected GAs (GA_8-1a, 1b and GA_{29}-2a, 2b) on column Acquity UPLC® CSH (solid line) and Acquity UPLC®BEH column (dashed line).

The column was kept at 40 °C. Tandem mass spectra of all GAs included in this study were acquired by continuous infusion of 10^{-3} mol L^{-1} solution in MeOH at a flow rate of 15 μL min^{-1}. Capillary voltage, cone voltage, collision cell energy, and ion source temperatures were optimised for each individual compound using the same setup. The MS settings were as follows: capillary voltage 1.5 kV, cone voltage 30 V, source temperature 150 °C, desolvation gas temperature 650 °C, cone gas flow 2 L h^{-1}, desolvation gas flow 650 L h^{-1} and collision cell energy 25 eV. MS data were recorded in the multiple reaction monitoring (MRM) mode, the dwell time of each channel calculated to provide 16 scan points per peak, with an inter channel delay of 0.1 s. All data were processed by the Masslynx™ Software (ver. 4.1, Waters).

3. Results and discussion

3.1. Extraction and purification procedure

In order to quantify GAs, their precursors and metabolites in plant tissue, the samples have to be firstly homogenised and extracted with a suitable solvent under the conditions under which these substances are stable and enzymatic degradation is minimised. The extraction and purification procedures should be performed in solutions between pH 2.5 and 8.5 to avoid rearrangement of the C/D rings and hydration of the 16, 17-double bond (under acidic conditions) and a reversible retro-aldol rearrangement resulting in epimerisation of the 3β-hydroxyl when present, and rearrangement of 1,2-dehydro C_{19}-GAs (such as GA_3) to the 19,2β-lactones with a shift of the double bond (under alkaline conditions). Furthermore, the solutions containing GAs (especially aqueous ones) should not exceed 40 °C. The extraction of plant tissues with an aqueous solution of organic solvent with higher water content might be preferred due to the relatively low pigment content of crude plant extracts, but more hydrophobic GAs (i.e. GA_9, GA_{15}, GA_{12} and GA_{12}-aldehyde for instance) may not be efficiently extracted under these hydrophilic conditions. We compared extraction of GAs from homogenised biological material using ice-cold MeOH or acidified (5% FA) AcCN, both with different water content, with rotation to achieve high extraction efficiency. In order to determine the content of the most abundant plant pigments in the extraction solution, the levels of chlorophylls a (chl_a) and b (chl_b) were measured using a previously described standard spectrophotometric method [28]. Extracts of 100 mg shoots from 3-week old Arabidopsis were prepared in triplicate for each extraction solution. In extracts prepared in 0–80% MeOH, the level of chl_a ranged between 2.78 and 22.07 mg L^{-1}, while that of chl_b between 5.10 and 17.90 mg L^{-1}. When performing the same experiment with 0–80% AcCN as the extraction solvent, the equivalent ranges were 1.5–22.26 mg L^{-1} for chl_a 2.68–15.13 mg L^{-1} for chl_b. Thus, 80% AcCN extracted about 15% less interfering plant pigments than 80% MeOH as the more polar chlorophyll, chl_b was extracted relatively less efficiently by 80% AcCN as compared to chl_a. Furthermore, recovery of ^{3}H-labelled GA standards after purification from extracts of green plant material with acidified 80% AcCN was on average $>73\%$, compared with only 55% recoveries when the solvent was 80% MeOH (data not shown). Comparing acidified 80% AcCN and 80% AcCN without addition of acid, we obtained similar losses during purification for all GAs tested except the most hydrophobic, for which recoveries of 80% were achieved after extraction with AcCN with 5% FA compared to non-acidified AcCN (cca 60% recoveries). Therefore, this latter solvent was used for tissue extraction in all subsequent optimisation procedures.

In general, the supernatants after centrifugation of the crude extracts require further purification before analysis by standard methods for the reasons mentioned above and also for prevention of contamination and overloading the UPLC column. Gibberellin isolation can be usually achieved by SPE in one or two steps when interfering compounds are not retained on the sorbent in a prepacked cartridge or they are washed out before the GAs are eluted. Commercial C_{18} cartridges of different optional format and bed sizes are suitable for purification of aqueous methanolic extracts to effectively separate nonpolar impurities such as chlorophyll and carotenoids from the GAs depending on sample weight and volume of extract [29]. Recently, polymer based hydrophilic–lipophilic-balanced (HLB) reversed-phase SPE columns and mixed mode cartridges have become increasingly popular [10,30,31].

We tested different purification schemes for our extracts in acidified 80% AcCN. All purification procedures were examined for the matrix effect and the recoveries of the endogenous GAs, their deuterium-labelled as well as tritium-labelled analogues in extracts with and without biological background (100 mg 3-week old seedlings of Arabidopsis 3 replicates). At first, we tested recovery of GAs (free acids) on the cartridge commercially prepacked with polymer-based mixed mode anion-exchange sorbent (Oasis MAX®, Waters). The extracts where evaporated to the water phase and diluted in 25 mM NH_4HCO_3. GAs form negatively charged ions in this alkaline medium that would interact with the polymeric MAX sorbent bearing positively charged tertiary amine groups ($pK_a > 18$). Under these experimental conditions, the recovery mean for GAs/[$^2H_2$]-GAs was found to be about 70% except for the more hydrophobic GAs (GA_{53}, GA_{24}, GA_9, GA_{15} and GA_{12}) for which recoveries varied between 20% and 57% (Table S2).

In case of GA_{12}-ald, only 16% was recovered. It is probably due to the fact that this very hydrophobic compound is not fully dissolved in aqueous NH_4HCO_3 as loading medium. When using [^{3}H]GA_1, [^{3}H]GA_4 and [^{3}H]GA_{20}, the recoveries from the MAX column ranged between 97 and 100%. Finally, we evaluated GA recoveries on MAX sorbent to be satisfactory and suitable for further optimisation of the purification procedure. Since the sample after MAX purification was not sufficiently pure for the subsequent UPLC–MS/MS analysis (visible presence of some plant pigments residue), we decided to examine some additional purification step prior to MAX. In the first

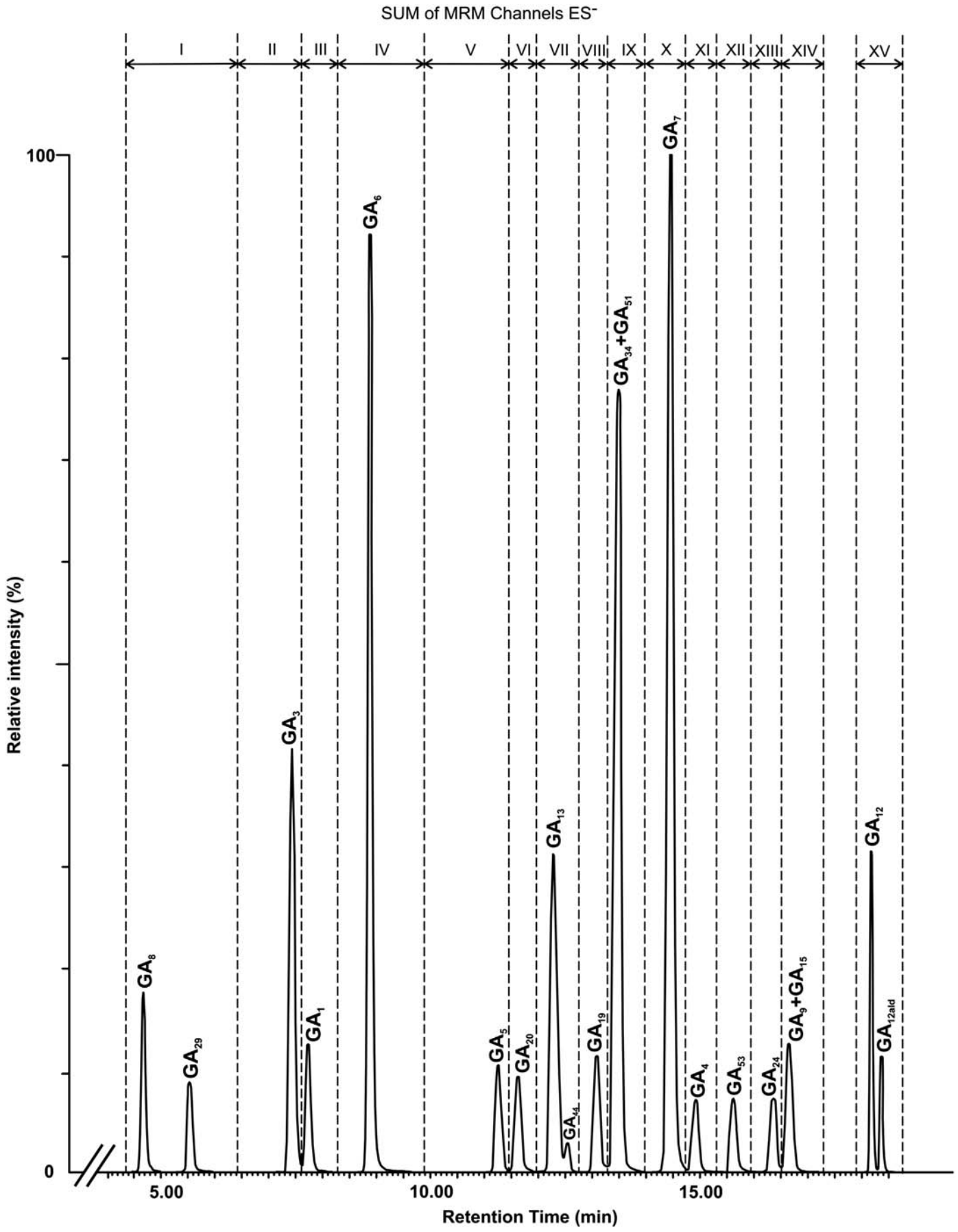

Fig. 4. Separation of 20 GAs by ultra-performance liquid chromatography (UPLC). UPLC–MS chromatogram of free acids standard mixture divided into 15 MRM channels (I–XV) containing 10 pmol of each GA per injection.

Table 2
Optimised MS conditions for each of the analysed gibberellins.

Compound	Diagnostic transition	Confirmation transition*	Cone voltage (V)	Collision energy (V)	Dwell time (s)	Retention time window (min)	Channel
GA_8	363 > 275	363 > 119	25	18	0.150	4.00–5.50	I
$[^2H_2]$-GA_8	365 > 277	–	25	18	0.150		
GA_{29}	347 > 259	347 > 303	30	15	0.150		
$[^2H_2]$-GA_{29}	349 > 261	–	30	15	0.150		
GA_3	345 > 239	345 > 143	25	14	0.290	6.80–7.40	II
$[^2H_2]$-GA_3	347 > 241	–	25	14	0.290		
GA_1	347 > 259	347 > 303	32	18	0.290	7.15–7.70	III
$[^2H_2]$-GA_1	349 > 261	–	32	18	0.290		
GA_6	345 > 119	345 > 257	30	26	0.250	8.30–8.80	IV
$[^2H_2]$-GA_6	347 > 119	–	30	28	0.250		
GA_5	329 > 145	329 > 285	28	24	0.127	10.78–11.25	V
$[^2H_2]$-GA_5	331 > 287	–	25	18	0.127		
GA_{20}	331 > 287	331 > 173	32	19	0.335	11.00–11.65	VI
$[^2H_2]$-GA_{20}	333 > 289	–	32	19	0.335		
GA_{13}	377 > 359	377 > 303	25	21	0.190	11.70–12.50	VII
GA_{44}	345 > 301	345 > 273	32	23	0.190		
$[^2H_2]$-GA_{44}	347 > 303	–	32	23	0.190		
GA_{19}	361 > 273	361 > 317	32	27	0.240	12.65–13.00	VIII
$[^2H_2]$-GA_{19}	363 > 275	–	32	27	0.240		
GA_{34}	347 > 259	347 > 241	30	17	0.120	12.95–13.50	IX
$[^2H_2]$-GA_{34}	349 > 261	–	30	17	0.120		
GA_{51}	331 > 287	331 > 243	33	18	0.120		
$[^2H_2]$-GA_{51}	333 > 289	–	33	18	0.120		
GA_7	329 > 223	329 > 211	25	18	0.300	13.95–14.40	X
$[^2H_2]$-GA_7	331 > 225	–	25	18	0.300		
GA_4	331 > 257	331 > 287	33	24	0.300	14.30–15.00	XI
$[^2H_2]$-GA_4	333 > 259	–	33	24	0.300		
GA_{53}	347 > 329	347 > 303	35	26	0.300	15.20–15.60	XII
$[^2H_2]$-GA_{53}	349 > 331	–	35	26	0.300		
GA_{24}	345 > 257	345 > 301	35	25	0.300	15.85–16.35	XIII
$[^2H_2]$-GA_{24}	347 > 259	–	35	25	0.300		
GA_9	315 > 271	315 > 253	32	23	0.150	16.10–16.70	XIV
$[^2H_2]$-GA_9	317 > 273	–	32	23	0.150		
GA_{15}	329 > 257	329 > 185	37	22	0.150		
$[^2H_2]$-GA_{15}	331 > 259	–	37	22	0.150		
GA_{12}	331 > 313	331 > 287	35	23	0.107	17.95–18.90	XV
$[^2H_2]$-GA_{12}	333 > 315	–	35	23	0.107		
GA_{12ald}	315 > 271	315 > 163	32	27	0.056		
$[^2H_2]$-GA_{12}-ald	317 > 273	–	32	24	0.056		

* Optimised MS conditions (cone voltage, collision energy and dwell time) are not shown.

instance, we decided to test GA recoveries on C_{18} SPE cartridges (Bond Elut® C18, Agilent Technologies). Silica-based C_{18} columns were activated with 100% MeOH and equilibrated by ultra pure water. After a washing step (ultra pure water), GAs were eluted with 100% MeOH. This procedure gave overall recoveries of about 46%. In order to improve recoveries of GAs, polymeric StrataX sorbent (Phenomenex, Torrance, CA, USA) was examined as an alternative reverse-phase medium to C_{18}. Functionalized polymeric sorbent of StrataX allows generally strong retention even under high organic wash conditions required when unwanted contaminants including phospholipids and diverse pigments need to be removed, while C_{18} sorbent offers strong hydrophobic retention with negligible secondary polar interactions from active silanol groups. However, about 53% of GAs were washed out from the StrataX sorbent when washing the cartridge with 5.75 mM FA and 5% MeOH, with only 33% recovered in the elution fraction. The conclusion is that this polymeric reversed-phase sorbent is not suitable for GA purification from plant extracts. C_{18} SPE cartridges were evaluated also as unsuitable.

Finally, we tested a combination of polymer-based cation-exchange SPE (Oasis MCX®, Waters) directly coupled to the last generation reversed-phase SPE (Oasis HLB®, Waters). Gibberellins are not retained on the negatively charged MCX matrix, which would remove alkaloids and other basic interfering substances from plant extracts. HLB is a polymeric reversed-phase sorbent containing both hydrophilic and lipophilic ligands and would be expected to retain GAs with a wide range of polarities. After evaporation of the extraction solution to the water phase, the sample was loaded in 5% MeOH onto the MCX column pre-equilibrated with the same solvent and coupled directly with HLB column prepared in the same way. The columns were disassembled after the washing step and the sample containing GA standards was obtained after washing the Oasis® HLB cartridge with 5.75 mM FA followed by 5% MeOH and eluting with an appropriate organic solvent. MeOH and MeOH/diethyl ether (20/80, v/v) were investigated for their efficacy in eluting GAs from the HLB cartridge. MeOH/diethyl ether gave about 17% higher recoveries than MeOH alone. Moreover, when used with plant extracts, elution with MeOH/diethyl ether rather than MeOH improved overall recoveries of the tested GAs in the system MCX-HLB followed by MAX, from 37% to 72% (data not shown). The MCX-HLB procedure itself gave a recovery of about 75% with only a small reduction (about 10%) when tested in the presence of plant extracts. Thus, the combined Oasis®MCX-HLB columns were judged to provide an effective and convenient enrichment of GAs from extracts and were chosen as a first purification step before anion exchange on Oasis® MAX columns (Fig. 2).

3.2. Liquid chromatography

A solution containing a mixture of all unlabelled GA standards (20 substances) and their deuterium-labelled analogues (19 compounds) was prepared to find the optimal LC separation conditions for all 20 analytes in a single chromatographic run. For this purpose,

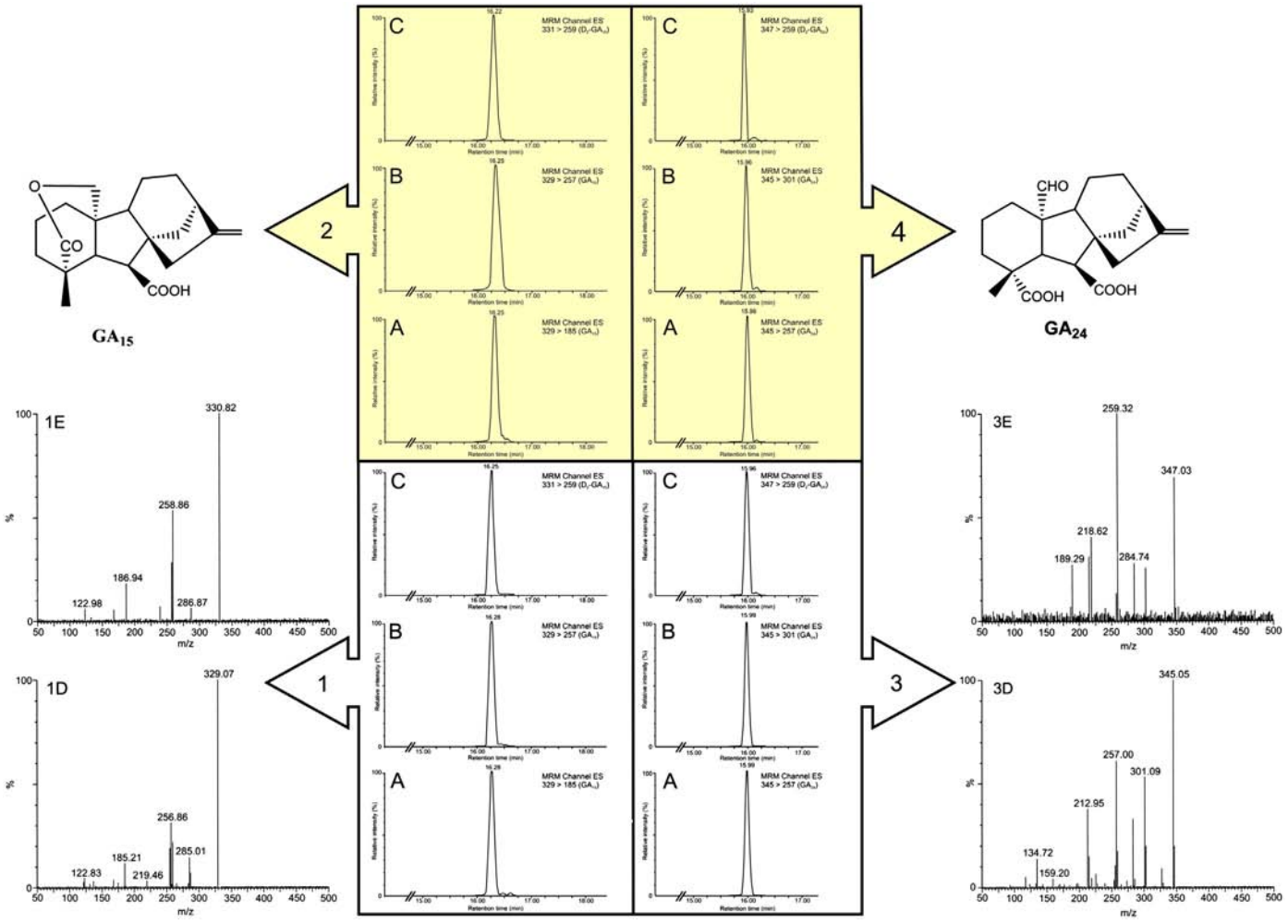

Fig. 5. MRM chromatograms of GA_{15} and GA_{24} standards (1A, B, C and 3A, B, C) and of the endogenous compounds in an extract of 100 mg *Brassica napus* flowers (2A, B, C and 4A, B, C) in the presence of $^2H_2$-labelled internal standards. MS spectra were recorded under optimised conditions for standard of GA_{15} (1D), $[^2H_2]GA_{15}$ (1E), GA_{24} (3D) and $[^2H_2]GA_{24}$ (3E).

Table 3
Method validation—selected parameters of the UPLC-MS/MS method for 50 pmol of selected GAs tested.

Compound	Diagnostic transition	LOD[a] (pmol)	LOQ[b] (pmol)	Recovery (%)	Content (pmol mg^{-1})	Content SD[c]	*n*	Analytical precision (%)	Analytical accuracy (%)
GA_{15}	329 > 257	0.06	0.19	80	0.05	0.01	6	2.33	116.11
GA_{24}	345 > 257	0.06	0.20	95	0.01	0.00	6	1.12	111.07
GA_4	331 > 287	0.04	0.14	78	0.04	0.20	6	3.68	119.32
GA_3	345 > 239	0.01	0.02	68	0.5×10^{-3}	0.02×10^{-3}	6	3.82	107.34

[a] Limit of detection LOD; $LOD = (3 \times S_b)/k$, where S_b is standard deviation of calibration equation and k is its slope.
[b] Limit of quantification (LOQ; $LOQ = (10 \times S_b)/k$), where S_b is standard deviation of calibration equation and k is its slope.
[c] Standard deviation.

two reversed-phase UPLC (RP-UPLC) columns (2.1 × 50 mm, 1.7 μm), Acquity UPLC® BEH (Ethylene Bridged Hybrid) C_{18} and Acquity UPLC® CSH (Charged Surface Hybrid) (both from Waters) were tested. The peak shape and the ionisation efficiency were found to be acceptable when using MeOH and 10 mM HCOOH as solvents A and B, respectively. Under these conditions, the Acquity UPLC® CSH column gave better peak shape and peak-to-peak resolution compared to the BEH column (Fig. 3).

The retention times ranged between 4.3 min (GA_8) and 18.3 min (GA_{12}-aldehyde) - Table S1. As expected, each unlabelled analyte/deuterated internal standard couple co-eluted with very close retention times, usually the deuterated analogue eluted earlier than the unlabelled standard (data not shown) due to the chromatographic isotope effect [32]. Sixteen of the 20 GAs studied were fully resolved under the RP-UPLC conditions (Fig. 4).

Only members of the pairs GA_{34} (*m*/*z* 347)/GA_{51} (*m*/*z* 331) and GA_9 (*m*/*z* 315)/GA_{15} (*m*/*z* 329) were not resolved and co-eluted completely, while it was not possible to achieve baseline separation of GA_{13} and GA_{44}. However, these GAs can be distinguished by the MS detector. Under our optimised conditions the stability of the retention times had a coefficient of variation between 0.60% and 0.82% (n=20) which is satisfactory. The mean of chromatographic peaks width of the studied compounds was 0.2505 min, which corresponds to a dwell time of about 0.93 s to reach the minimum required 16 data points per peak as a data sampling rate suitable for reproducible integration. Dwell time values for

appropriate analytes are listed in Table 2. Further, due to low loading capacity, it was possible to achieve excellent peak shape for all the analytes in selected low ionic strength mobile phase and rapid mobile-phase re-equilibration.

3.3. MS/MS detection

Solutions containing mixtures of standards, comprising the unlabelled GAs and their respective deuterium-labelled internal standards were used to identify the appropriate precursor-to-product ion transition for each compound using (−)ESI–MS/MS. All investigated GAs provided the background-subtracted ESI^- spectra exhibiting $[M–H]^-$ as base peaks, but varied considerably in their fragmentation pattern. Nevertheless, some fragmentation rules could be found. Twelve of the 20 GAs studied have 19 carbons and 10 of these C_{19}-GAs with no internal double bonds give the diagnostic or confirmation losses of 88 (COO^- plus CO–O from ring A) or 44 (COO^-) mass units from their precursor ions $[M–H]^-$ (see Table 2). On the other hand, two C_{19}-GAs containing double bond (GA_3 and GA_7) give the diagnostic transition corresponding to the loss of 106 mass units, reflecting the loss of water (18 m/z), in addition to the 88 *m/z* fragment cleavage. In the case of the C_{20}-GAs, only three GAs (GA_{13}, GA_{53} and GA_{12}) from eight show the same fragmentation pattern, losing water from the precursor ion $[M–H]^-$. In half of the C_{20}-GAs ($GA_{13,}$ GA_{44}, GA_{24} and GA_{15}), the loss of a CH_3 group from C-4 occurs as the second most significant fragmentation route. The C_{20}-GAs GA_{19} and GA_{24} behave in the mass spectrometer as C_{19}-GA, i.e. give the ions 273 and 257 as the most abundant fragments after loss of *m/z* 88 from the precursor ions $[M–H]^-$ 361 and 345, respectively. It is possibly due to the presence of the aldehyde functional group at C-10 that might stabilise the gibberellane skeleton and allow the cleavage of both carboxyl groups from the molecule. The double deuterium-labelled counterparts at C-17 position show no difference in fragmentation pattern compared to their unlabelled analogues. Based on the mass spectra obtained, the quasi-molecular ions $[M–H]^-$ and the most intensive fragment ions were selected for mass spectrometric detection in MRM mode (Table 1). The entire chromatographic run was then divided into 15 retention windows (channels I–XV), each characterised by defined MRM functions for appropriate analyte (Fig. 4). Examples of mass spectra of the diagnostic product ions are shown in Fig. 5. Interestingly, the intensities of GA product ions are lower than those of their deuterated GA counterparts (see Fig. 5–1D vs 1E and 3D vs 3E). To our knowledge, there is no such effect described in the literature. We might speculate that higher intensity of product ion is related to lower stability of precursor ion containing deuterium.

3.4. Method validation and application

The newly developed UPLC–MS/MS method was tested by analysing the levels of endogenous GAs in samples of different biological origin: shoots of *A. thaliana* and flowers of field-grown *B. napus*. To create calibration curves, eight solutions containing varying amounts of each unlabelled GA and a known, fixed amount of the corresponding deuterium-labelled internal standard (IS) were prepared as follows: 0.098/15; 0.5/15; 1/15; 1.5/15; 3/15; 5/15; 7.5/15 and 10/15 (endogenous/internal standard; values in pmol/15 μL). The resulting calibration curves from four separate injections were established to be linear in the selected concentration range for all 20 endogenous compounds investigated (correlation coefficient R^2 values appeared in the interval 0.9965 to 0.9995, see Table S1). For GA_{13}, in the absence of a deuterium-labelled analogue, $[^2H_2]_2GA_{44}$ was found to be an appropriate internal standard on account of their very close chromatographic behaviour and linearity of the $[^2H_2]GA_{44}/GA_{13}$ calibration curve. The linear range for all calibration curves was shown to be four orders of magnitude. The limit of detection (LOD) was evaluated using the approach based on the standard deviation s_b of the calibration curve and the slope k of a regression curve ($LOD = 3 \times s_b/k$) [49]. The LODs for selected GAs are summarised in Table 3. The limit of quantitation (LOQ) was evaluated using the standard-deviation/slope ratio approach ($LOQ = 10 \times s_b/k$) [33] and it is indicated for 4 selected GAs in Table 3.

We tested different concentrations of IS (10–100 pmol) added into the extraction media and found 50 pmol to be the most appropriate for all GAs investigated in tissues containing chlorophyll and other plant pigments. Addition of 10 pmol of GAs IS gave also satisfactory recoveries (cca 80%), however only in case of biological material without plant pigment present (seeds, data not shown). Therefore, 50 pmol of each IS was added to the samples before purification. The plant extracts were purified by SPE (MCX-HLB followed by MAX), concentrated *in vacuo* and the GAs were quantified by LC–MS as described above.

The analytical accuracy of the UPLC–MS/MS method was evaluated by spiking sample aliquots (100 mg of plant tissue in 1 mL of extraction solution, 6 replicates) with known amounts of individual compounds (50 and 75 pmol $GA_{15}/[^2H_2]$-GA_{15}, $GA_{24}/[^2H_2]$-GA_{24}, $GA_4/[^2H_2]$-GA_4 and $GA_3/[^2H_2]$-GA_3) prior to sample purification. The assessment of analytical accuracy ranged within 107 and 120% of the true amounts value (Table 3). The analytical precision was determined in the range of 1.1 and 3.8% for the 4 above-mentioned GAs selected (Table 3).

4. Conclusion

In this report, we describe a method for the simultaneous analysis of 20 GAs, which have been identified in Arabidopsis, the most frequently used model plant. The developed UPLC–ESI-MS/MS method is based on effective chromatographic separation combined with a suitable extraction and purification procedure for plant samples. The solvent extraction process followed by two solid-phase based procedures, allows these 20 GAs to be isolated effectively and rapidly from plant material. This method was then successfully applied to the analysis of biologically active GAs, their metabolites and precursors in *B. napus* flowers and Arabidopsis shoots. The developed LC–MS/MS method offers a fast separation, high chromatographic resolution, with sufficient selectivity and a satisfactory sensitivity for studying the distribution of GAs and their physiological roles in plants.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.068.

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