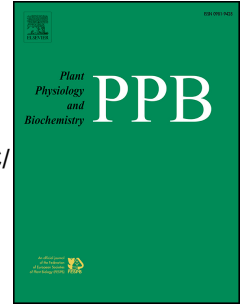


Accepted Manuscript

Rapid and reproducible determination of active gibberellins in Citrus tissues by UPLC/ESI-MS/MS

Matías Manzi, Aurelio Gómez-Cadenas, Vicent Arbona



PII: S0981-9428(15)30016-4

DOI: [10.1016/j.plaphy.2015.04.015](https://doi.org/10.1016/j.plaphy.2015.04.015)

Reference: PLAPHY 4187

To appear in: *Plant Physiology and Biochemistry*

Received Date: 10 February 2015

Revised Date: 22 April 2015

Accepted Date: 28 April 2015

Please cite this article as: M. Manzi, A. Gómez-Cadenas, V. Arbona, Rapid and reproducible determination of active gibberellins in Citrus tissues by UPLC/ESI-MS/MS, *Plant Physiology et Biochemistry* (2015), doi: 10.1016/j.plaphy.2015.04.015.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title

Rapid and reproducible determination of active gibberellins in *Citrus* tissues by UPLC/ESI-MS/MS

Authors

Matías Manzi, Aurelio Gómez-Cadenas, Vicent Arbona*
Ecofisiologia i Biotecnologia. Dept. Ciències Agràries i del Medi Natural.
Universitat Jaume I. E-12071 Castelló de la Plana. Spain

Research highlights

- A rapid and reproducible method to determine gibberellins in citrus fruitlets was developed.
- The efficiency of the method relies in the use of ultrapure water that reduces interferences.
- Coeluting GA₁/[²H₂]-GA₃ and GA₄/[²H₂]-GA₇ could interfere in their determination.
- Deuterium-labeled GA₁ and GA₄ can be used for quantitation of four bioactive GAs.

1 **Title**

2 Rapid and reproducible determination of active gibberellins in *Citrus* tissues by
3 UPLC/ESI-MS/MS

4 **Authors**

5 Matías Manzi, Aurelio Gómez-Cadenas, Vicent Arbona*
6 Ecofisiología y Biotecnología. Dept. Ciències Agraries i del Medi Natural,
7 Universitat Jaume I.E-12071 Castellón de la Plana, Spain
8
9 manzi@uji.es, aurelio.gomez@uji.es, arbona@uji.es

10 **Corresponding author:**

11 Vicent Arbona
12 Dept. Ciències Agraries i del Medi Natural
13 Universitat Jaume I
14 E-12071 Castellón de la Plana. Spain
15 Ph. +34 964 72 8101
16 Fax. +34 964 72 8216

17

18 **Abbreviations**

19 amu Arbitrary Mass Units

20 ESI Electrospray Ionization

21 GA Gibberellin

22 GC Gas Chromatography

23 HPLC High Performance Liquid Chromatography

24 IS Internal Standard

25 LC Liquid Chromatography

26 LOB Limit Of Blank

27 LOD Limit Of Detection

28 LOQ Limit Of Quantification

29 *m/z* mass-to-charge ratio

30 MRM Multiple Reaction Monitoring

31 MS Mass spectrometry

32 MS/MS Tandem Mass Spectrometry

33 RSD Relative Standard Deviation, coefficient of variation of the mean

34 SPE Solid Phase Extraction

35 UPLC Ultra Performance Liquid Chromatography

36

37 **Abstract**

38 Phytohormone determination is crucial to explain the physiological mechanisms
39 during growth and development. Therefore, rapid and precise methods are
40 needed to achieve reproducible determination of phytohormones. Among many
41 others, gibberellins (GAs) constitute a family of complex analytes as most of
42 them share similar structure and chemical properties although only a few hold
43 biological activity (namely GA₁; GA₃; GA₄ and GA₇). A method has been
44 developed to extract GAs from plant tissues by mechanical disruption using
45 ultrapure water as solvent and, in this way, ion suppression was reduced
46 whereas sensitivity increased. Using this methodology, the four active GAs
47 were separated and quantified by UPLC coupled to MS/MS using the isotope-
48 labelled internal standards [²H₂]-GA₁ and [²H₂]-GA₄. To sum up, the new method
49 provides a fast and reproducible protocol to determine bioactive GAs at low
50 concentrations, using minimal amounts of sample and reducing the use of
51 organic solvents.

52 **Key words:** ion suppression; matrix effect; phytohormones; tandem mass
53 spectrometry; ultrapure water

54

55

56

57

58

59

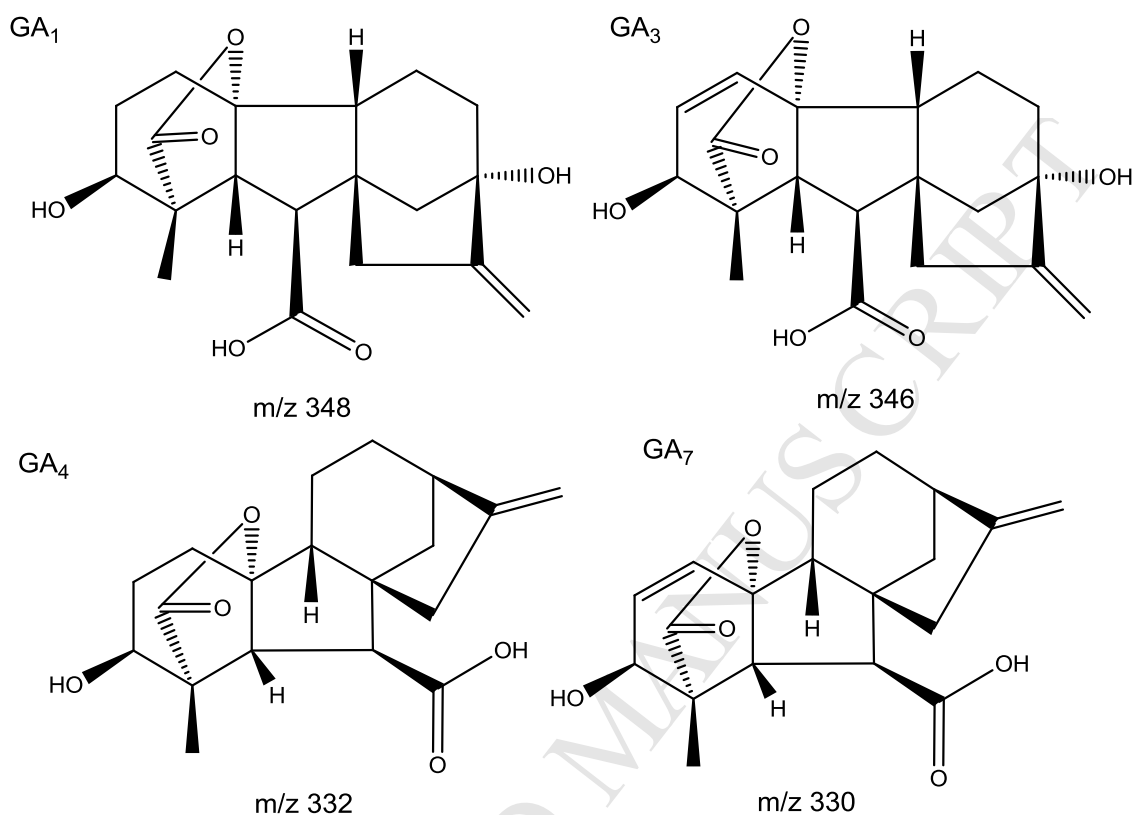
60 1. Introduction

61

62 The determination of phytohormones in plants has been of great interest for
63 researchers in plant biology and physiology. For the last half-century,
64 phytohormone fluctuations have been used to explain the physiological changes
65 in response to stresses and throughout different stages of plant growth and
66 development. Moreover, this knowledge has become essential for the
67 development of many agricultural practices and has allowed growers to improve
68 crop production. In citrus, application of different plant growth regulators alters
69 flower induction, fruit set, ripening, and fruit quality parameters [1]. In this sense,
70 gibberellins (GAs) are the main group of plant growth regulators used for these
71 purposes, particularly the commercially available gibberellic acid (GA₃; [2]). This
72 molecule was isolated in 1935 from the fungus *Gibberella fujikuroi* and named
73 GA₃ after it. So far, 136 GAs have been identified in plants, fungi and bacteria
74 being a group of compounds which shares a common ent-gibberellane ring [3]
75 but, despite their structural similarity, only a small set of GAs displays biological
76 activity in plants. Hence, GA₁; GA₃, GA₄ and GA₇ are considered the “active
77 GAs” in vegetative tissues of plants (Figure 1), whereas the rest of molecules
78 are synthesized in seeds or reproductive organs and few of them constitute
79 biosynthetic intermediates or inactive forms. Presence and concentration of
80 endogenous active GAs varies between plant species, tissues and
81 developmental stages, playing a key role in different physiological processes.
82 Hence, GA₁, GA₄ and GA₇ have been identified as the main active GAs in the
83 genus *Citrus* [4,5], whereas GA₃ has only been detected occasionally in this
84 plant species but no clear role could be assigned [5]. In general, GA₁ levels are

85 higher in developing fruitlets of citrus and especially in those fruitlets with a
86 higher setting probability whereas low amounts are found in shedding fruitlets
87 [4,6]. On the other hand, GA₄ has been associated with rind color break of
88 'Washington Navel' sweet orange fruits [7]. However, for GA₇ no clear role has
89 been assigned in citrus fruiting or other physiological processes although it is
90 often quantitated together with GA₄ and presented as GA₇+GA₄ [8].
91 Nevertheless, it is clear that different GAs could have specific roles in plant
92 physiology and therefore the ability to quantitate individual molecules is of
93 special interest [7]. The high similarities in chemical structure and properties of
94 different GAs (i.e. similar mass and hydrophobicity) constitutes an important
95 drawback in the development of accurate analytical methodologies. Indeed,
96 previous works found in the literature presented their results as GA-like activity
97 [9] and as mentioned above, in some cases it was not possible to differentiate
98 GA₁ from GA₃ and GA₄ from GA₇, therefore reporting them as two groups: GA_{1/3}
99 and GA_{4/7} [8,9]. An additional problem relies on the low hormone levels usually
100 found in plants, which is the reason why most of them were first identified in
101 organisms different from plants [10]. Therefore, hormone extraction and
102 purification has to be carried out accurately and highly sensitive detection and
103 quantitation procedure is required [11,12]. Moreover, in the particular case of
104 GAs, the analytical technique needs to be selective enough to ensure the
105 differentiation of structural isomers. Until recently, analytical procedures used
106 high amounts of plant material (more than 2 g) along with tedious purification
107 processes to get rid of interfering compounds [13,14]. In most cases, higher
108 specificity and sensitivity were reached with radioactive tracers and
109 immunological detection methods [15] which have an extremely narrow linear

110 range for quantitation and do not allow the simultaneous determination of
111 multiple compounds [16].



112

113

Figure 1

114 In recent years, mass analyzers (MS) coupled to either GC or LC have been
115 developed and implemented for phytochemical analysis allowing an increase in
116 sensitivity and selectivity and, in addition, enabling simultaneous determination
117 of multiple compounds in a single run [17,18]. For these applications, the most
118 widespread mass analyzers are triple quadrupoles that perform detection of
119 target compounds by monitoring specific precursor-to-product ion transitions
120 after collision-induced fragmentation of selected molecular ions [19]. These
121 specific transitions (precursor ion>fragment ion) can be monitored throughout
122 the chromatographic run and implemented in multiresidue methods allowing a
123 highly selective and sensitive detection of several compound classes [11,16,20].

124 Regarding analysis of phytohormones, LC has the advantage (over GC) that
125 samples do not need to be derivatized prior to analysis, avoiding considerable
126 time-consuming steps and the use of toxic chemicals. The accurate quantitation
127 of compounds is achieved by using stable isotope-labeled analogues of
128 phytohormones as IS showing the same elution time and fragmentation pattern
129 that the endogenous compound but differing in mass-to-charge (m/z) values
130 [13,21]. Spiking of samples with IS compensates for possible analyte losses
131 during the extraction procedure and matrix effects during LC/MS acquisition
132 [16]. In addition, the development of very small particles for column packing
133 (around 1.8 μm) has reduced analysis time and enhanced resolution and
134 sensitivity compared to conventional HPLC [22].

135 The main goal of this work was to develop a simple, fast and robust method to
136 extract and quantitate active GAs (GA_1 ; GA_3 ; GA_4 and GA_7) from a particularly
137 complex matrix such as citrus tissues. For this purpose, several published
138 extraction methods were compared in terms of sensitivity, accuracy and
139 specificity to that implemented in the present work.

140

141 **2. Materials and methods**

142 *2.1. Optimization of MS parameters*

143 Individual standard solutions of phytohormones GA_1 ; GA_3 ; GA_4 ; and GA_7 as well
144 as IS [$^2\text{H}_2$]- GA_1 ; [$^2\text{H}_2$]- GA_3 ; [$^2\text{H}_2$]- GA_4 and [$^2\text{H}_2$]- GA_7 (Olchemim Ltd, Olomouc,
145 Czech Republic) at 5.0 $\mu\text{g mL}^{-1}$ in 50% (v/v) acetonitrile:water supplemented
146 with formic acid (0.1% v/v) were infused at a flow rate of 10 $\mu\text{L min}^{-1}$ into a triple
147 quadrupole MS analyzer (TQDTM, Waters Micromass Ltd., Manchester, UK). All

148 the analyses were performed using the following settings: capillary voltage: 3.5
149 kV, nebulizer gas (N₂) 30 L hr⁻¹, desolvation gas (N₂) 400 L hr⁻¹, desolvation
150 temperature 250°C and setting an acquisition time of 30 s. Full scan from *m/z*
151 100 to 360 was performed in negative and positive ionization modes and cone
152 voltage optimized to maximize precursor ion signal. Selected precursor ions
153 were then fragmented adjusting collision energy within the hexapole chamber
154 (filled with Ar gas) and the population of product ions recorded in order to select
155 the highest intensity fragment. After setting up all transitions for analytes, a
156 MRM method was implemented and used to selectively monitor selected GAs in
157 plant extracts.

158 2.2. Extraction procedure

159 To optimize phytohormone extraction from plant tissues, four reported
160 procedures for hormone analysis were tested using developing fruitlets of
161 mandarin (*Citrus clementina* Tan.). Samples were harvested and frozen
162 immediately in liquid N, and then converted into a fine powder. Six replicates
163 were used to test the efficiency of each of the extraction procedures chosen,
164 described below. All procedures were performed using 200 mg of sample
165 (unless otherwise indicated) that was spiked with 25.0 µL of IS solution
166 containing 1.5 mg L⁻¹ of [²H₂]-GA₁ and [²H₂]-GA₄ before extraction. The final
167 concentration of each IS compound was 37.5 ng/sample.

168 The specific extraction procedures were: 1) following [20]: samples were
169 combined with 4.0 mL of a isopropanol:acetic acid 99:1 (v/v) mixture and
170 extracted (Ultra-Turrax, Ika-Werke, Staufen, Germany) for 1 min. Afterwards,
171 samples were centrifuged for 30 min at 4700 ×g and 4°C. Supernatants were

172 collected and 9.0 mL of ultrapure water was added to each sample. The
173 resulting solutions were passed through a C₁₈ SPE cartridge (Scharlab,
174 Barcelona, Spain). Retained analytes were then eluted with 0.5 mL of a
175 methanol:water:acetic acid (80:19:1, v/v/v) solution that was evaporated until
176 complete dryness in a vacuum concentrator (Jouan, Saint-Herblain, France).
177 Finally, dry residues were redissolved by ultrasonication in 0.5 mL of a
178 methanol:water:acetic acid (80:19:1, v/v/v) solution for 10 min. 2) following the
179 procedure described in [11]; samples were extracted in 5.0 mL of ultrapure
180 water and centrifuged as described above. After recovery of supernatants, pH
181 was adjusted to 3.0 with an acetic acid solution (30%). The aqueous solution
182 was partitioned twice against 3.0 mL of di-ethyl ether (Panreac, Barcelona,
183 Spain) and the upper organic layers collected, combined and evaporated to
184 dryness. Each sample was resuspended in 0.5 mL of methanol:water, (10:90,
185 v/v) by sonication for 10 min. 3) Following the procedure described in [7],
186 samples were extracted in 3.0 mL of methanol:water:acetic acid (80:19:1, v/v/v)
187 as indicated in previous procedures. The homogenate was partitioned twice
188 against 3.0 mL of ethyl acetate supplemented with acetic acid to a 1% (v/v)
189 concentration. The organic layer was recovered and evaporated to dryness and
190 then, reconstituted in 50% methanol:water solution prior to SPE purification with
191 a preconditioned C₁₈ cartridge. The purified extract was dried again and
192 reconstituted in 0.5 mL of the same extraction solution by sonication. 4) Finally,
193 as in [12], 50 mg of samples were extracted in 0.2 mL of
194 methanol:isopropanol:acetic acid solution (20:79:1, v/v/v) by ultrasonication for
195 30 min. After centrifugation at 10000 ×g during 15 min at 4°C, supernatant was
196 collected and meanwhile sample pellet was re-extracted three more times with

197 0.1 mL of extraction solution each time, achieving a final volume of 0.5 mL of
198 extracted solution.

199 Before injection onto the UPLC system, each sample was filtered through a 0.2
200 μm PTFE membrane filter (Whatman International Inc., Kent, United Kingdom)
201 in all cases.

202 Selection of the most suitable method to determine GAs was performed by
203 comparing the peak areas obtained for the four GAs as well as for IS ($[\text{}^2\text{H}_2\text{]}\text{-GA}_1$;
204 $[\text{}^2\text{H}_2\text{]}\text{-GA}_4$) among procedures. Also, matrix effect produced for each method
205 was calculated. Since the impossibility to find any citrus tissue without GAs,
206 matrix effect was assessed for the IS compounds ($[\text{}^2\text{H}_2\text{]}\text{-GA}_1$; $[\text{}^2\text{H}_2\text{]}\text{-GA}_4$) by the
207 comparison of the recoveries between the spiked extracts in samples tissues
208 and reference standards in solvent.

209 *2.3. Internal standard selection*

210 Since deuterium-labeled IS have the same physicochemical properties as non-
211 deuterated compounds, these were used to correct for any analyte losses
212 during extraction or matrix effects. For that purpose, three replicates of fresh
213 mandarin fruitlets were fortified at two levels (50 and 100 ng g^{-1}) with GA_1 ; GA_3 ;
214 GA_4 and GA_7 and an extra set of samples was extracted without any
215 fortification. Addition of IS ($[\text{}^2\text{H}_2\text{]}\text{-GA}_1$; $[\text{}^2\text{H}_2\text{]}\text{-GA}_3$; $[\text{}^2\text{H}_2\text{]}\text{-GA}_4$ and $[\text{}^2\text{H}_2\text{]}\text{-GA}_7$) was
216 performed at the beginning of extraction. To select the appropriate IS for each
217 GA, the recovery value of each GA assessed by the IS must fall between 70
218 and 120% and the relative standard deviation [$\text{RSD}=(\text{standard deviation}/\text{mean})$
219 $\times 100$] must be lower than 20%, as has been established in [20].

220 2.4. Method validation and analysis of plant samples

221 Linearity, limit of blank (LOB), limit of detection (LOD) and limit of quantitation
222 (LOQ) were assessed for each analyte. Standard curves with increasing
223 amounts of each plant hormone, ranging from 0.4 to 200.0 $\mu\text{g L}^{-1}$ were
224 analyzed. Linearity was considered satisfactory when R^2 values were higher
225 than 95%. LOB was estimated according to [23] by analyzing ($n=10$) a blank
226 sample (without analyte) using the following formula:
227 $\text{LOB} = \text{Mean}_{\text{blank}} + 1.654 \times (\text{SD}_{\text{blank}})$.

228 LOD is defined as the lowest analyte concentration that can be consistently
229 distinguished from LOB [23] and was determined by measuring ($n=6$) a low and
230 known analyte concentration using the following calculation:
231 $\text{LOD} = \text{LOB} + 1.654 \times (\text{SD}_{\text{low concentration sample}})$.

232 LOQ defined as the lowest concentration that the analytical procedure can
233 reliably differentiate from background noise allowing unequivocal quantitation,
234 was estimated according to [23]: $\text{LOQ} = \text{LOD} + 0.2 \times (\text{RSD}_{\text{samples at LOD concentration}})$.

235 The accuracy of the procedure was determined by means of the standard
236 addition method by spiking citrus fruitlets at two hormone concentrations (50
237 and 100 ng g^{-1} FW). Then, the endogenous concentration of each hormone
238 ($n=6$) was determined by interpolation of the response value
239 ($\text{area}_{\text{endogenous}} / \text{area}_{\text{ISTD}}$) in the calibration curve. Mean concentration value in
240 non-fortified (Mean_{nsp}) samples was subtracted from that in fortified samples
241 (Mean_{sp}) and the result was expressed as percentage of the expected
242 theoretical concentration:

243 $\% \text{Recovery} = [(\text{Mean}_{\text{sp}} - \text{Mean}_{\text{nsp}})] \times 100 / \text{expected theoretical concentration}$

244 Precision was estimated in terms of repeatability of the method (%RSD) for
245 each fortification level and analyte.

246 Sample stability was also tested by analyzing three replicate extracts from citrus
247 leaf samples that were injected twice within a daytime, the second spanning
248 24h from the first and a third injection one week after. Between injections,
249 samples were kept at 4°C. RSD was calculated among the initial injection and
250 after the storage, considering satisfactory when RSD values were below 30%
251 as in [24].

252 Additionally, the resulting method was validated in developing fruitlets of
253 mandarin that were taken at three different developmental stages: pre-anthesis
254 (non-pollinated ovaries), anthesis and S2 (10 days after anthesis). Three
255 lyophilized replicates (200 mg each) per sample group were spiked with IS (as
256 mentioned above), extracted and analyzed to confirm the presence of each
257 phytohormone. Transitions as well retention time for each compound were
258 compared to that of standards. A positive confirmation of each analyte was
259 established if retention time deviation from standards was $\pm 2.5\%$.

260 *2.5. Optimized chromatographic conditions*

261 Sample aliquots (20 μL) were injected onto an Acquity UPLC system (Waters,
262 Mildford, MA, USA) coupled to a triple quadrupole MS instrument through an
263 orthogonal Z-spray electrospray interface (TQD, Micromass Ltd., Manchester,
264 UK). Analytes were separated by reversed-phase LC on a C_{18} column
265 (Nucleodur Gravity, EC50/2.0 50 mm \times 2.0 mm id, 1.8 μm particle size,

266 Macherey–Nagel, Germany) maintained at 40°C during chromatographic runs.
267 Ultrapure water and methanol were used as mobile phase at a flow rate of 0.3
268 mL min⁻¹, both supplemented with CH₃COOH to a concentration of 0.1% (v/v). A
269 gradient elution program was established as follows: isocratic conditions of
270 90:10 (water/methanol) for 2 min and from that point methanol concentration
271 was linearly increased to 80% in 4 min. Afterwards, isocratic conditions were
272 maintained for 1 min and then the solvent composition was restored to initial
273 conditions to allow column re-equilibration for 2 min. Samples were analyzed in
274 MRM mode setting an interscan time of 1.0 s and a dwell time for each function
275 of 0.025 s. Source block and desolvation gas (N₂) temperatures were set at
276 120°C and 350°C, respectively. Nebulization and desolvation gas flows were set
277 at 60 and 800 L h⁻¹, respectively. A two min solvent delay period was included
278 at the beginning of the run to avoid build-up of poorly-retained contaminants in
279 the mass spectrometer. Analysis of data was carried out with MassLynx v. 4.1
280 software (Micromass Ltd) and differences in mean GA concentration values
281 among samples assessed by means of ANOVA followed by Tukey's post-hoc
282 test considering a $p \leq 0.05$ *p*-value cutoff.

283 3. Results and discussion

284 Plant hormone determination is of key interest in plant physiology and LC
285 coupled to tandem mass spectrometry (MS/MS) is the preferred technique due
286 to short sample processing time and compatibility with semipolar compounds
287 [11]. However, current analytical platforms must ensure high sensitivity,
288 throughput, accuracy and precision and allow a satisfactory quantification using
289 small sample amounts [25]. In addition, analytical platforms must allow

290 differentiation of target compounds with similar structures and chemical
291 properties such as GAs.

292 3.1. Selection of precursor-to-product ion transitions.

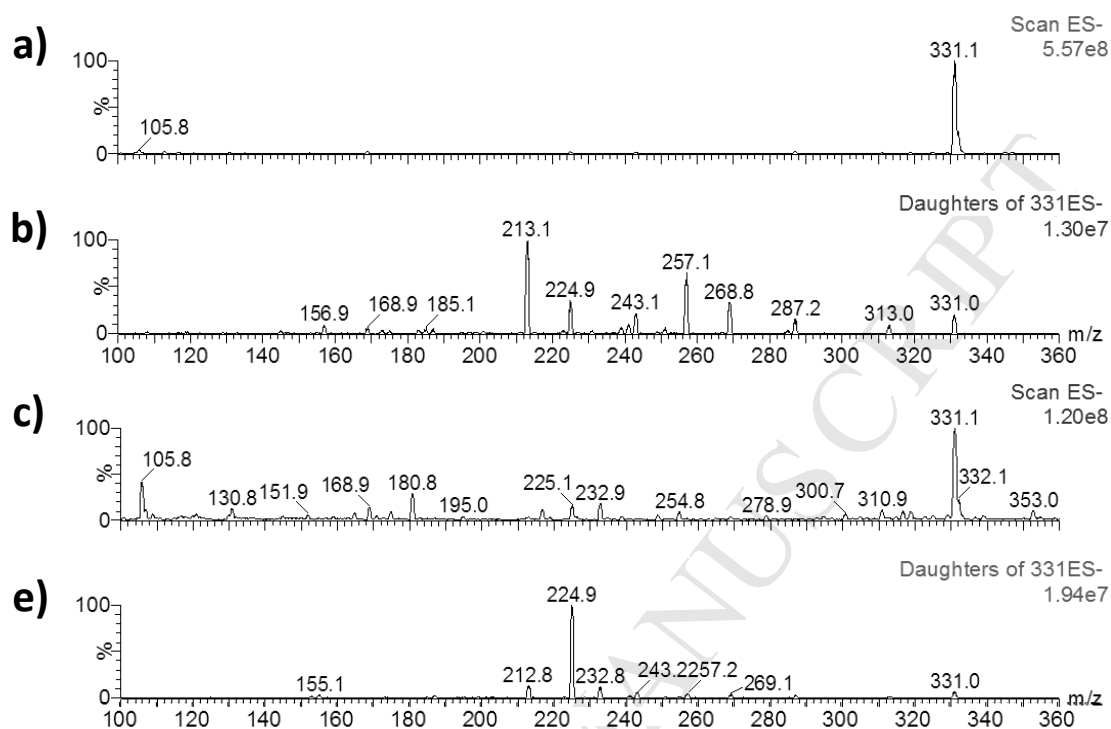
293 The optimized precursor-to-product ion transitions as well as ionization modes
294 for each analyte are summarized in Table 1. After direct injection of standard
295 solutions into the MS system, identification of parent ions was performed in full-
296 scan mode by recording mass spectra from m/z 100 to 360 in both negative and
297 positive ionization modes, adjusting cone voltages to maximize precursor ion
298 intensity. Afterwards, collision energy was adjusted to maximize intensity of
299 product ions. Negative ionization mode (ESI-) was chosen due to the higher
300 intensity found for labeled and non-labeled compounds. In this work, transitions
301 chosen were identical to those previously reported using similar equipment [7,
302 12,26,27], but although differing in cone voltage and collision energy, suggests
303 the existence of a specific fragmentation pattern for this molecules despite of
304 the MS/MS instrument. On the other side, as known, chromatographic
305 separation for these compounds is difficult since their retention times are similar
306 due to the chemical structure (Table 1 and Figure 3). Even extending run time
307 did not completely resolve these compounds. Thus, the maximum resolution
308 achieved between GA₁ and GA₃ was only of 0.2 min [28], or even lower
309 between GA₄ and GA₇ using nanoflow-LC-MS/MS [21]. Indeed, at the initial
310 stages of use of LC-MS for metabolite determination, some researchers could
311 only report joint data for GA₁ and GA₃ (GA₁₊₃) and GA₄ and GA₇ (GA₄₊₇; [8]) or
312 in cases, some reports did not determine GA₃ and GA₇ when GA₁ or GA₄ were
313 quantitated [12,28,29]. Overall, our results indicate that a proper selection of the
314 precursor-to-product ion transition is essential to achieve a precise and

315 selective hormone determination by MS/MS, whereas chromatographic
 316 separation has to be considered less important. Molecular weights of
 317 endogenous GAs usually differ from their respective commercially-available
 318 deuterium-labelled analogues in 2 amu (see Figures 1 and 2). This results in an
 319 inconvenient overlapping of metabolite transitions in some cases. Thus, the [M-
 320 H]- ions for [²H₂]-GA₃ and ²H₂]-GA₇ have the same nominal masses as those for
 321 GA₁ and GA₄, respectively, and these cannot be resolved by the mass analyser.
 322 Moreover, these similar molecules also share a common fragmentation pattern
 323 and thus daughter ions. As signaled above, the similarity in fragmentation
 324 pattern among GAs lead to interference among molecules as shown in figure 2
 325 as exposed in previous works [30]. Furthermore, this could be the reason
 326 behind the exclusion of [²H₂]-GA₃ and [²H₂]-GA₇ as it is when GA₁ and GA₄ are
 327 quantitated along with GA₃ and GA₇ [21,31,32]. Therefore, due to this double
 328 chromatographic (similar retention times) and spectrometric (similar
 329 monoisotopic mass and fragmentation pattern) interference among analytes
 330 and some deuterium-labelled compounds, we excluded [²H₂]-GA₃ and [²H₂]-GA₇
 331 as internal standards for further analyses.

332 **Table 1.** Optimized MS conditions for gibberellins and deuterated gibberellins under
 333 negative electrospray ionization (ESI) mode.

Compound	Quantification transition Q	Cone voltage (V)	Collision energy (eV)	Retention time (min)
GA ₁	347 > 229	25	30	4.63
[² H ₂]-GA ₁	349 > 231	35	25	4.62
GA ₃	345 > 143	35	20	4.56
GA ₄	331 > 213	35	27	5.97
[² H ₂]-GA ₄	333 > 215	35	25	5.97

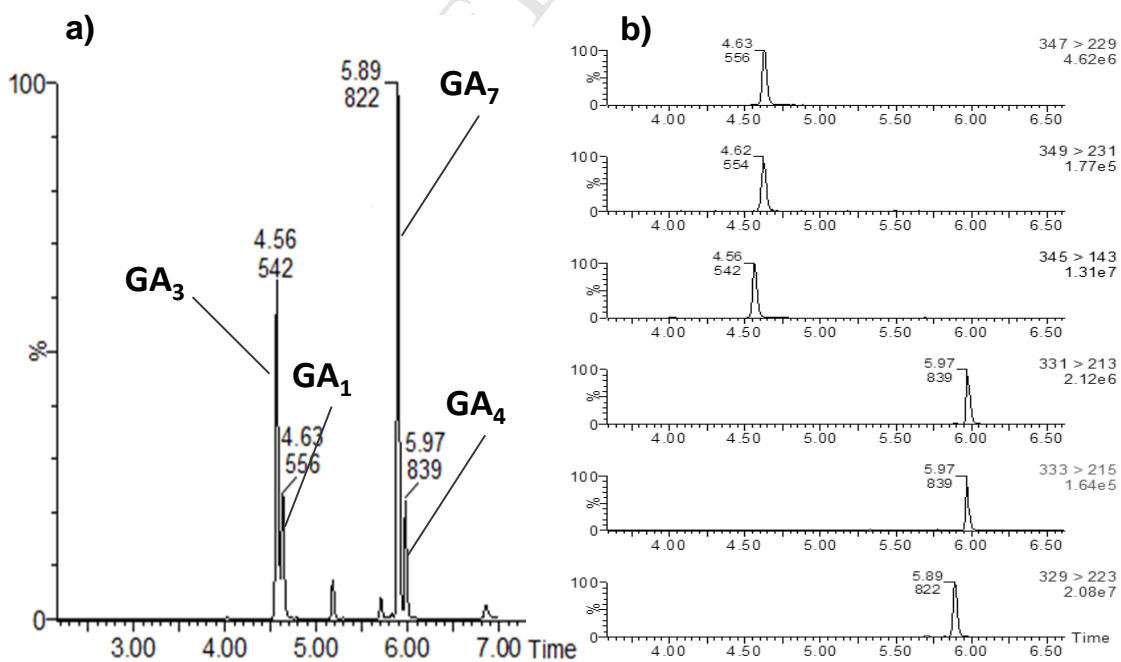
GA ₇	329 > 223	27	17	5.89
[² H ₂]-GA ₇	331 > 225	30	20	5.89



334

335

Figure 2



336

337

Figure 3

338 3.2. Selection of the extraction procedure

339 An efficient extraction procedure is crucial for a successful determination of
340 phytohormones avoiding the effect of matrix-related compounds that may be
341 also co-extracted with target analytes. In this sense, many different extraction
342 procedures have been published and most of them included a pre-concentration
343 step followed by liquid-liquid partition or SPE extraction. In this work, some
344 procedures that differ in the composition of the extraction solution were tested:
345 a) a mix of organic solvents such as methanol:isopropanol:acetic acid (20:79:1,
346 v/v/v; [12]); b) methanol:water:acetic acid (80:19:1, v/v/v) followed by partition
347 with ethyl acetate [7]; c) water as extraction solvent followed by partition with di-
348 ethyl ether [11] and; d) isopropanol:acetic acid (99:1, v/v) as a extracting solvent
349 followed by a C₁₈-based SPE pre-concentration step [22].

350 The comparison of extraction methods is presented in Figure 4. Retention time
351 and specific transitions confirmed the presence of the target analytes in
352 samples extracted following each of the methods. Results showed that the
353 highest peak area values for most target analytes were found in samples
354 extracted with water (Figures 4 and 5). Indeed, peak areas for all analytes,
355 except for GA₇, were higher using water in the extraction than using any organic
356 solvent mixture. For GA₇, on the contrary, higher peak areas were achieved
357 using Isop:AA or MeOH:H₂O:AA (Figure 4). Moreover, peak area of [²H₂]-GA₄ in
358 samples extracted in Isop:AA showed similar values to those found in samples
359 extracted with water. As expected, GA₃ was not detected in mandarin fruitlet
360 samples [5]. From these results, a preliminary estimation of GA concentrations
361 could be performed: GA₁ content was highest in samples extracted with water
362 whereas in methods using organic solvents it was significant lower or even non-

363 detectable (Table 2). For GA₄, levels were similar among samples extracted
364 using water, ISOP:AA or MeOH:H₂O:AA whereas MeOH:ISOP:AA mixture
365 rendered significantly lower amounts. Moreover, GA₇ concentration was lower
366 in samples extracted with water; ISOP:AA and MeOH:ISOP:AA, but when
367 samples were extracted using the MeOH:H₂O:AA mixture, estimated contents
368 were significantly higher. Besides this, relative standard deviation (RSD) of the
369 average GA levels has to be considered to evaluate the precision of the
370 method. Therefore, when water was used as solvent RSD values were always
371 low (<13%) compared to other extraction methods (ISOP:AA from 20.7 to
372 71.4%; MeOH:ISOP:AA from 70.3 to 84.1% and MeOH:H₂O:AA 43.8 to 76.8%)
373 indicating a higher precision of water extraction. Besides the efficiency of each
374 solvent mixture to extract GAs from plant tissues, data suggests that the solvent
375 mixture used in the extraction strongly influences the sensitivity of the
376 procedure through changes in ion suppression. This aspect becomes the main
377 drawback especially in highly complex matrices such as apple fruit in which the
378 target analytes could be efficiently recovered from the matrix [34]. In this
379 respect, absolute quantitation of endogenous GAs relies on the interpolation of
380 sample response factors for each GA/IS pair within the respective calibration
381 curves. Hence, a balanced ion suppression affecting endogenous GAs as well
382 as their respective IS leads to correct estimation of the hormone amount in
383 samples. Thus, the procedure using water extraction rendered the highest peak
384 areas and recoveries for GAs (Figures 4 and 5) of all procedures tested. This is
385 likely to be a result of lower ion suppression rather than higher extraction
386 efficiency. Most of the extraction protocols for phytohormones found in the
387 literature employ different mixtures of organic solvents (such as methanol or

388 isopropanol or combinations); however, the results presented in this and
 389 previous works [11,16,35] show clear evidence that the extraction performed
 390 with water could be more effective. This higher recovery could be explained in
 391 part by the low extraction capability of water that reduces the incidence of
 392 interfering compounds (such as chlorophylls, carotenoids, lipids, etc.) that could
 393 act as suppressors of ionization [26]. Moreover, most of the GAs described here
 394 have pKa values around 4.0 which indicate a clear tendency to dissociation in
 395 aqueous solution, which makes water a more selective solvent for this kind of
 396 compounds. Therefore, water performance could be also partially associated
 397 with a better recovery of GA and a reduction in the incidence of interfering
 398 compounds. In this respect, water provided a better signal-to-noise ratio,
 399 allowing a more reproducible GA quantification. Indeed, the extremely low area
 400 values of IS in samples extracted with MeOH:H₂O:AA and MeOH:Isop:AA
 401 (Figure 5) led to an overestimation of recovery. Therefore, for subsequent tests
 402 the water-based procedure was chosen.

403 **Table 2.** Analysis of gibberellins in developing citrus fruitlets using different extraction
 404 methodologies.

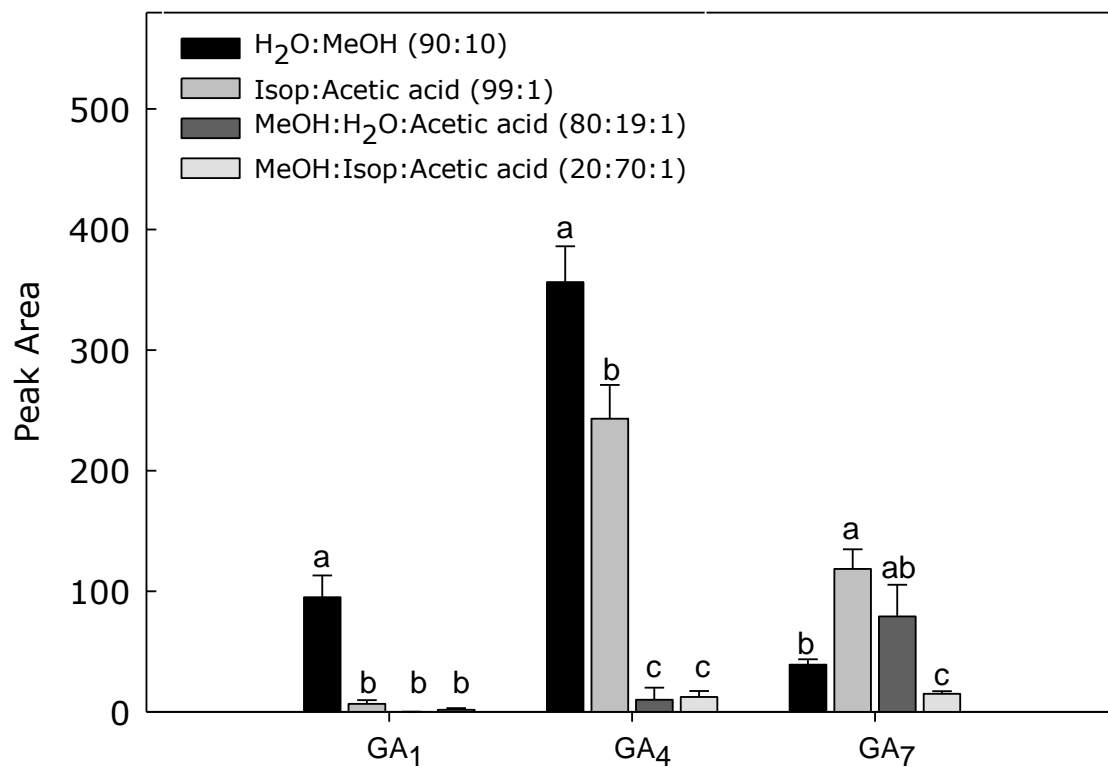
Hormone	ng.g ⁻¹ FW		
	GA ₁	GA ₄	GA ₇
H ₂ O (100%)	10.9 ± 1.5 a	56.5 ± 2.9 a	0.95 ± 0.1 b
Isop:AA (99:1)	0.7 ± 0.5 b	45.5 ± 9.4 a	0.78 ± 0.4 b
MeOH:H ₂ O:AA (80:19:1)	nd -	42.7 ± 33 ab	22.6 ± 9.9 a
MeOH:ISOP:AA (20:70:1)	5.6 ± 4.2 ab	18.2 ± 15 b	3.7 ± 2.6 b

405

406 H₂O: milliQ water; MeOH: methanol; Isop: isopropanol; AA: acetic acid; nd; non-
 407 detectable. Data are means ± standard errors and letters indicate statistical differences
 408 among methods for each analyte (n=3; p≤0.05).

409

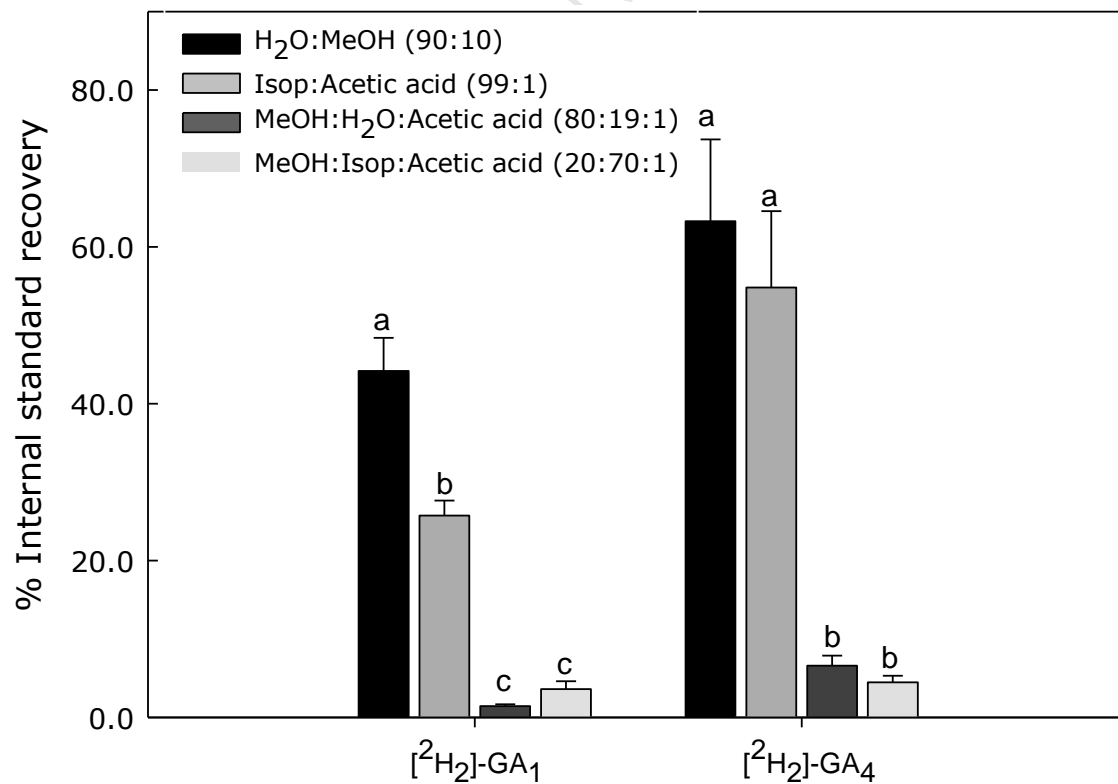
410 The results presented here support the selection of water extraction for plant
411 hormone extraction and quantitation. Use of water as extraction solvent, also
412 reduces the use of flammable and toxic solvents, being a readily available,
413 cheap and environment-friendly solvent [36,37], however, safety precaution
414 needs to be considered when using di-ethyl ether. Despite these advantages,
415 water has not been widespread used as a solvent for phytohormone extraction.
416 Nevertheless, water has been used as the extraction solvent successfully for
417 the quantification of ABA [11,16], jasmonic acid [38] or cytokinins [39]. In the
418 method presented here, a clean-up and pre-concentration step involving di-ethyl
419 ether partition was included. In previous reports, partition against di-ethyl ether
420 showed excellent results in yielding high recovery of GAs and other
421 phytohormones from aqueous solutions at pH between 2.5 and 3.5 [40]. Acetic
422 acid was used to reduce the pH of the extract to 3.0, below the pKa of the GAs
423 ensuring the recovery in the organic phase. Solvent partition is a convenient
424 step since it also removes many polar interfering compounds present in plant
425 tissues which are discarded in the aqueous phase, such as sugars and amino
426 acids [11] representing the biggest portion of metabolites found in developing
427 citrus fruitlets [41]. Moreover, organic solvents, and especially di-ethyl ether, are
428 easily evaporated *in vacuo* which allows reconstitution in smaller volumes,
429 increasing concentration of analytes.



430

431

Figure 4



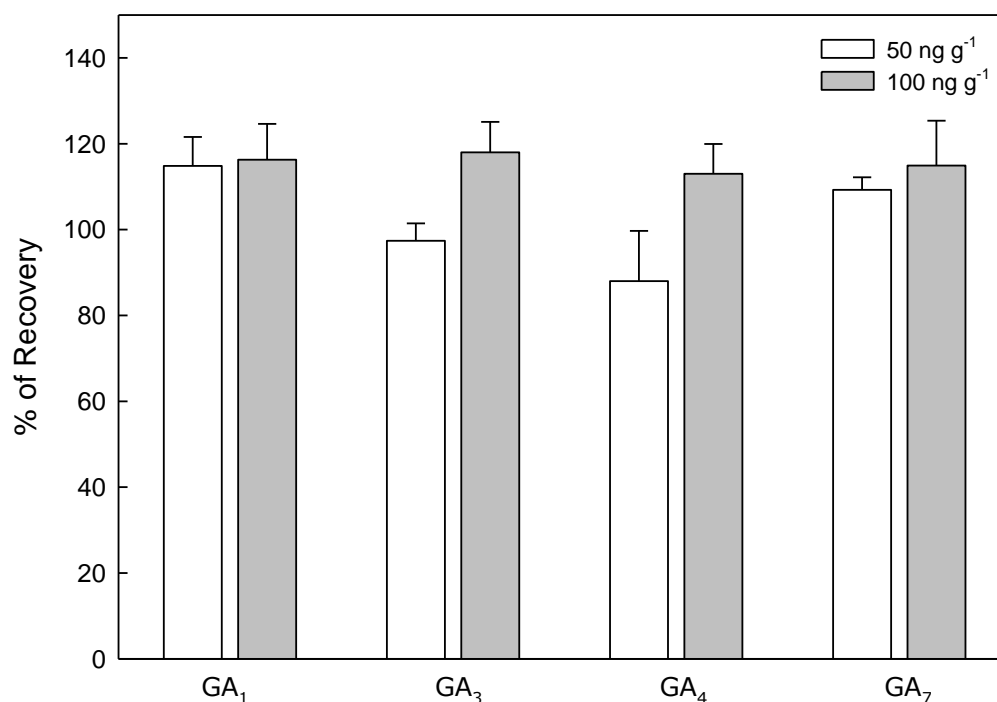
432

433

Figure 5

434 3.3 Internal standard selection

435 The addition of IS with identical chemical properties as the target analyte(s)
436 compensates for analyte losses during extraction and purification, as well as
437 metabolite stability, ion suppression and run-to-run variations [11,16].
438 Commercially available labeled compounds contain deuterium, ^{13}C or another
439 stable isotope and identical amounts are added to every sample before
440 extraction. Isotope-labelled compounds show identical chromatographic
441 behavior as non-labeled analytes but differ in their mass values. In this sense,
442 most of the usually analyzed GA is available as deuterium-labelled standard
443 including the bioactive GA_1 , GA_3 , GA_4 and GA_7 . As mentioned above, $[\text{}^2\text{H}_2]\text{-GA}_3$
444 and $[\text{}^2\text{H}_2]\text{-GA}_7$ could not be added as IS because these IS interfere
445 chromatographically and spectrometrically (similar retention time and mass
446 transition compared to GA_1 and GA_4 , respectively). Instead, $[\text{}^2\text{H}_2]\text{-GA}_1$ and $[\text{}^2\text{H}_2]\text{-}$
447 GA_4 were used as IS [21,32], improving recovery, which is essential for an
448 accurate quantitation of the four bioactive GAs (Figure 6). Moreover, RSD
449 values for these compounds corrected by the IS fell within the limits established
450 (<20%), evidencing the high precision of the method and the recoveries
451 obtained using $[\text{}^2\text{H}_2]\text{-GA}_1$ and $[\text{}^2\text{H}_2]\text{-GA}_4$ as IS. Furthermore, the use of $[\text{}^2\text{H}_2]\text{-GA}_4$
452 as IS of GA_7 seems to amend the low initial recovery found for this
453 phytohormone (Figure 4).



454

455

Figure 6

456

457 *3.4. Method validation and analysis of samples*

458 Method validation was accomplished by calculation of LOB, LOD and LOQ.

459 First, calibration curves (from 0.4 to 200.0 ng mL⁻¹) were analyzed in triplicate,460 including increasing GA concentrations and keeping IS amount constant ([²H₂]-461 GA₁ and [²H₂]-GA₄). The correlation coefficients (R²) for the calibration curves462 were always higher than 0.98 for each GA, 0.9809 for GA₁; 0.9859 for GA₃;463 0.9901 for GA₄; and 0.9959 for GA₇ and therefore considered acceptable.

464 Sensitivity parameters (LOB, LOQ and LOD) were calculated from the standard

465 curves as in [23]. LOB values ranged from baseline to 0.4 ng g⁻¹, LOD levels466 varied from 0.4 ng g⁻¹ to 1.9 ng g⁻¹ and LOQ from 0.8 ng g⁻¹ to 2.0 ng g⁻¹ (Table

467 3). It can be concluded that the limits of the procedure ensure the detection of

468 GAs at very low concentrations in plant samples. Indeed, physiological levels of
 469 this compounds are usually higher than 10.0 ng g⁻¹ [12,28].

470 **Table 3.** Sensitivity parameters and sample stability for bioactive gibberellins.

Hormone	LOB	LOD	LOQ	Stability (%RSD)		
				Within 24 h	Within 1 week	
		ng g ⁻¹				
GA ₁	0.3	1.1	2.0	3.2	28.3	
GA ₃	0.0	1.2	1.2	16.5	21.0	
GA ₄	0.4	1.9	2.0	15.0	22.3	
GA ₇	0.3	0.4	0.8	9.7	20.5	

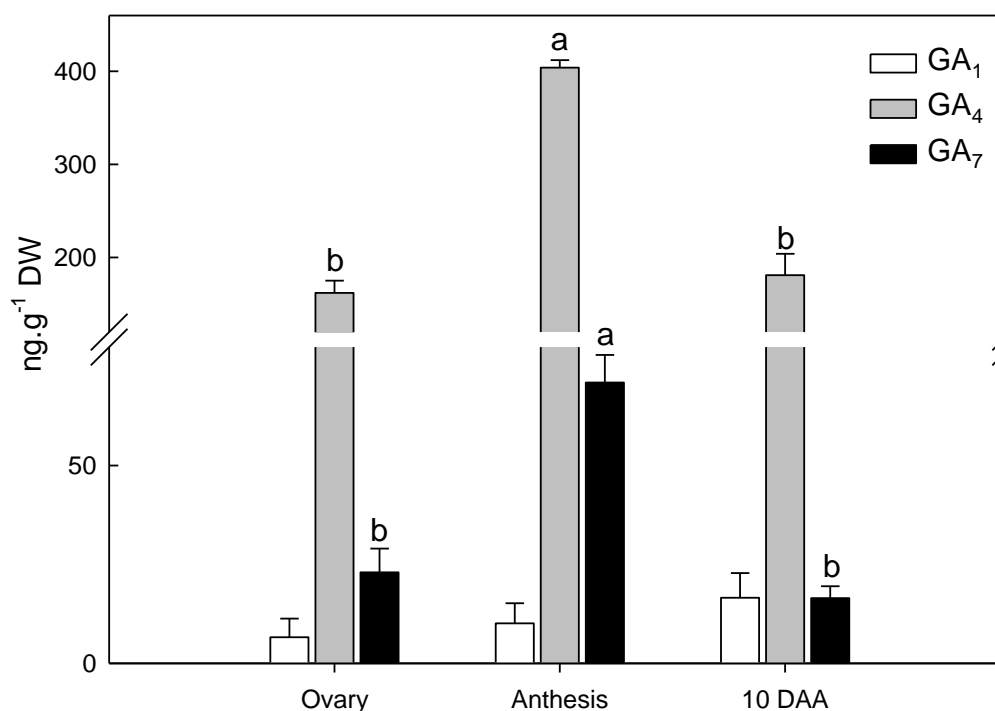
471

472 As indicated above, accuracy for each hormone was satisfactory at both spiking
 473 levels showing values within the range considered as acceptable (70 to 120%)
 474 (Figure 6). In addition, precision was acceptable for each GA as evidenced by
 475 RSD values that were always below 20% (commonly accepted threshold for
 476 these procedures, [20,34]). Taking results globally, it can be concluded that the
 477 method could be used for the quantification of the four bioactive GAs in citrus
 478 samples after spiking samples with IS prior to extraction.

479 When several samples have to be analyzed, they usually stand in queue for
 480 hours until they are injected onto the LC. For this reason, stability of citrus
 481 samples was performed at two time points after storage at 4°C (Table 3).
 482 Results indicate that no significant variation was found after 24 h of cold
 483 storage. However, after one week variation coefficient became slightly higher
 484 than 20% (Table 3). For samples stored for one week, RSD values higher than
 485 30% are acceptable [24].

486 The method was tested in fresh mandarin fruitlets that constitute a particularly
 487 difficult matrix for phytohormone analysis. GA content in these samples was

488 assessed in three stages of fruitlet development: pre-anthesis (non-pollinated
489 ovaries), anthesis and S2 (10 days after anthesis, 10 DAA; Figure 7). Levels of
490 GA₄ (the main bioactive GA in citrus) and GA₇ were higher on anthesis,
491 reaching 404 and 72 ng g⁻¹ DW, respectively. On the other hand, levels of GA₁
492 were low compared to the other GAs analyzed and similar among the three
493 stages chosen, ranging from 6.4 to 16.6 ng g⁻¹ DW. As expected, GA₃ was not
494 detected in these samples as reported before in citrus [5]. The GA levels found
495 with the optimized methodology are consistent with previous reports [5,6,42],
496 although measured levels of GA₄ were substantially higher than those
497 previously reported [5,6]. However, we could not ascertain whether these
498 differences were attributable to environmental factors such as water deficit [8],
499 or previous fruit load that influences the number of flowers and subsequently
500 fruit set ability, processes in which GAs play a key role [1]. During fruitlet
501 development, GA content increases about two-fold during anthesis and then
502 decreases again to pre-anthesis values [6]. In this respect, final fruit set in citrus
503 seems to be linked to the decrease of GA content (GA₄ and GA₇) after the
504 anthesis stage, mainly in seedless and self-incompatible varieties such as
505 clementines [6,42].



506

507

Figure 7

508

509 3. Conclusions

510 In summary, procedures to attain gibberellin determination face several issues
 511 such as the high structural similarity among the different components of this
 512 family and the extremely low concentrations found in plant tissues. In this work,
 513 ultrapure water as extraction solvent for plant phytohormones proved to be
 514 more efficient than organic solvents, which allows skipping time-consuming
 515 clean-up steps. This was related to the low extraction potential of water that
 516 reduces the occurrence of interfering compounds that might induce ion
 517 suppression leading to increased sensitivity. Quantitation of endogenous GAs
 518 was achieved by spiking samples with deuterium-labeled analogues. In
 519 addition, it has been shown that [²H₂]-GA₃ and [²H₂]-GA₇ could interfere with
 520 GA₁ and GA₄ determination due to its similar *m/z* values and fragmentation

521 patterns and retention times, thus excluding their use as IS. However, [²H₂]-GA₁
522 and [²H₂]-GA₄ were efficient to assess the recovery of GA₁ and GA₄ but also for
523 GA₃ and GA₇, respectively. Finally, the method was validated and confirmed in
524 different fruit growth developing stages, proving the robustness of the method.

525 **Acknowledgements**

526 Financial support provided through concession of grants No. AGL2013-42038-R
527 and P1IB2013-23 from Ministerio de Economía (MINECO) and Universitat
528 Jaume I, respectively, is acknowledged. MM was supported by a "Santiago
529 Grisolia" grant from Generalitat Valenciana and VA was recipient of a "Ramón y
530 Cajal" contract from MINECO. Mass Spectrometry analyses were performed at
531 the central facilities (Servei Central d'Instrumentació Científica, SCIC) of
532 Universitat Jaume I.

533 **References**

- 534 [1] D.J. Iglesias, M. Cercós, J.M. Colmenero-Flores, M.A. Naranjo, G. Ríos, E.
535 Carrera, O. Ruiz-Rivero, I. Lliso, R. Morillon, F.R. Tadeo, M. Talón,
536 Physiology of citrus fruiting, *Braz. J. Plant Physiol.* 19 (2007) 333-362.
- 537 [2] V.M. Sponsel, P. Hedden, Gibberellin biosynthesis and inactivation, in: P.J.
538 Davies (Ed.), *Plant Horm. Biosynthesis, Signal Transduct. Action!*, Kluwer
539 Academic Publishers, Dordrecht, NL, 2004: pp. 63–94.
- 540 [3] S. Yamaguchi, Gibberellin metabolism and its regulation, *Annu. Rev. Plant*
541 *Biol.* 59 (2008) 225–51.
- 542 [4] M. Talon, M. Koornneef, J.A.D. Zeevaart, Accumulation of C 19-gibberellins
543 in the gibberellin-insensitive dwarf mutant *gai* of *Arabidopsis thaliana* (L.)
544 Heynh, *Planta* 182 (1990) 501–505.
- 545 [5] M. Talon, L. Zacarías, E. Primo-Millo, Gibberellins and parthenocarpic
546 ability in developing ovaries of seedless mandarins, *Plant Physiol.* 99
547 (1992) 1575–1581.

- 548 [6] W. Ben-Cheikh, J. Perez-Botella, F.R. Tadeo, M. Talón, E. Primo-Millo,
549 Pollination increases gibberellin levels in developing ovaries of seeded
550 varieties of citrus, *Plant Physiol.* 114 (1997) 557–564.
- 551 [7] G. Gambetta, A. Martínez-Fuentes, O. Bentancur, C. Mesejo, C. Reig, A.
552 Gravina, et al., Hormonal and nutritional changes in the flavedo regulating
553 rind color development in sweet orange [*Citrus sinensis* (L.) Osb.], *J. Plant*
554 *Growth Regul.* 31 (2011) 273–282.
- 555 [8] Y. Koshita, T. Takahara, T. Ogata, A. Goto, Involvement of endogenous
556 plant hormones (IAA, ABA, GAs) in leaves and flower bud formation of
557 satsuma mandarin (*Citrus unshiu* Marc.), *Sci. Hortic.* 79 (1999) 185–194.
- 558 [9] A.A. Egorshina, R.M. Khairullin, A.R. Sakhabutdinova, M.A. Luk'yantsev,
559 Involvement of phytohormones in the development of interaction between
560 wheat seedlings and endophytic *Bacillus subtilis* strain 11BM, *Russ. J. Plant*
561 *Physiol.* 59 (2011) 134–140.
- 562 [10] P. Hedden, S.G. Thomas, Gibberellin biosynthesis and its regulation,
563 *Biochem. J.* 444 (2012) 11–25.
- 564 [11] A. Durgbanshi, V. Arbona, O. Pozo, O. Miersch, J. V Sancho, A. Gómez-
565 Cadenas, Simultaneous determination of multiple phytohormones in plant
566 extracts by liquid chromatography-electrospray tandem mass spectrometry,
567 *J. Agric. Food Chem.* 53 (2005) 8437–42.
- 568 [12] M. Müller, S. Munné-Bosch, Rapid and sensitive hormonal profiling of
569 complex plant samples by liquid chromatography coupled to electrospray
570 ionization tandem mass spectrometry, *Plant Methods* 7 (2011) 37.
- 571 [13] A. Müller, P. Düchting, E.W. Weiler, A multiplex GC-MS/MS technique for
572 the sensitive and quantitative single-run analysis of acidic phytohormones
573 and related compounds, and its application to *Arabidopsis thaliana*, *Planta*
574 216 (2002) 44–56.
- 575 [14] W. Xie, C. Han, Z. Zheng, X. Chen, Y. Qian, H. Ding, et al., Determination
576 of Gibberellin A3 residue in fruit samples by liquid chromatography-tandem
577 mass spectrometry, *Food Chem.* 127 (2011) 890–892.
- 578 [15] M.H. Beale, Immunological methods in plant hormone research, in: P.J.
579 Hooykaas, M.A. Hall, K. Libbenga (Eds.), *Biochem. Mol. Biol. Plant Horm.*,
580 Elsevier Science, New York, NY, 1999: pp. 61–88.
- 581 [16] A. Gómez-Cadenas, O.J. Pozo, P. García-Augustín, J. V Sancho, Direct
582 analysis of abscisic acid in crude plant extracts by liquid chromatography--
583 electrospray/tandem mass spectrometry, *Phytochem. Anal.* 13 (2002) 228–
584 234.

- 585 [17] S. Hou, J. Zhu, M. Ding, G. Lv, Simultaneous determination of gibberellic
586 acid, indole-3-acetic acid and abscisic acid in wheat extracts by solid-phase
587 extraction and liquid chromatography-electrospray tandem mass
588 spectrometry, *Talanta* 76 (2008) 798–802.
- 589 [18] K. Ljung, G. Sandberg, T. Moritz, Hormone analysis, in: P. Davies (Ed.),
590 *Plant Horm. Biosynthesis, Signal Transduct. Action!* Kluwer Academic
591 Publishers, Dordrecht, NL, 2004: pp. 671–694.
- 592 [19] C. Birkemeyer, A. Kolasa, J. Kopka, Comprehensive chemical derivatization
593 for gas chromatography–mass spectrometry-based multi-targeted profiling
594 of the major phytohormones, *J. Chromatogr. A* 993 (2003) 89–102.
- 595 [20] S.D.S. Chiwocha, S.R. Abrams, S.J. Ambrose, A.J. Cutler, M. Loewen,
596 A.R.S. Ross, A.R. Kermode, A method for profiling classes of plant
597 hormones and their metabolites using liquid chromatography-electrospray
598 ionization tandem mass spectrometry: an analysis of hormone regulation of
599 thermodormancy of lettuce (*Lactuca sativa* L.) seeds, *Plant J.* 35 (2003)
600 405–417.
- 601 [21] Y. Izumi, A. Okazawa, T. Bamba, A. Kobayashi, E. Fukusaki, Development
602 of a method for comprehensive and quantitative analysis of plant hormones
603 by highly sensitive nanoflow liquid chromatography-electrospray ionization-
604 ion trap mass spectrometry, *Anal. Chim. Acta* 648 (2009) 215–225.
- 605 [22] T. Obata, A.R. Fernie, The use of metabolomics to dissect plant responses
606 to abiotic stresses, *Cell Mol. Life Sci.* 69 (2012) 3225–3243.
- 607 [23] D. Armbruster, T. Pry, Limit of blank, limit of detection and limit of
608 quantitation, *Clin. Biochem. Rev.* 29 (2008) S49–52.
- 609 [24] N. Lindegårdh, W. Hanpithakpong, Y. Wattanagoon, P. Singhasivanon, N.J.
610 White, N.P.J. Day, Development and validation of a liquid chromatographic-
611 tandem mass spectrometric method for determination of oseltamivir and its
612 metabolite oseltamivir carboxylate in plasma, saliva and urine, *J.*
613 *Chromatogr. B* 859 (2007) 74–83.
- 614 [25] R. Welti, W. Li, M. Li, Y. Sang, H. Biesiada, H.-E. Zhou, C.B. Rajashekar,
615 T.D. Williams, X. Wang, Profiling membrane lipids in plant stress
616 responses. Role of phospholipase D alpha in freezing-induced lipid changes
617 in *Arabidopsis*, *J. Biol. Chem.* 277 (2002) 31994–2002.
- 618 [26] T. Urbanová, D. Tarkowská, O. Novák, P. Hedden, M. Strnad, Analysis of
619 gibberellins as free acids by ultra performance liquid chromatography-
620 tandem mass spectrometry, *Talanta* 112 (2013) 85–94.

- 621 [27]X. Pan, R. Welti, X. Wang, Simultaneous quantification of major
622 phytohormones and related compounds in crude plant extracts by liquid
623 chromatography-electrospray tandem mass spectrometry, *Phytochemistry*
624 69 (2008) 1773–81.
- 625 [28]C. Susawaengsup, M. Rayanakorn, S. Wongpornchai, S. Wangkarn,
626 Investigation of plant hormone level changes in shoot tips of longan
627 (*Dimocarpus longan* Lour.) treated with potassium chlorate by liquid
628 chromatography-electrospray ionization mass spectrometry, *Talanta*. 85
629 (2011) 897–905.
- 630 [29]M. Varbanova, S. Yamaguchi, Y. Yang, K. McKelvey, A. Hanada, R.
631 Borochoy, et al., Methylation of gibberellins by *Arabidopsis* GAMT1 and
632 GAMT2, *Plant Cell* 19 (2007) 32–45.
- 633 [30] R. G. Sharp, M. Else, W. J. Davies, R. W. Cameron, Gibberellin-mediated
634 suppression of floral initiation in the long-day plant *Rhododendron* cv.
635 Hatsugiri, *Sci Hort.* 124 (2010) 231–238.
- 636 [31] M. P. M. Derkx, E. Vermeer, C.M. Karssen, Gibberellins in seeds of
637 *Arabidopsis thaliana*: biological activities, identification and effects of light
638 and chilling on endogenous levels, *Plant Growth Regul.* 15 (1994) 223-234.
639
- 640 [32]W. Zhang, S.D.S. Chiwocha, R. Trischuk, L. V. Gusta, Profile of plant
641 hormones and their metabolites in germinated and ungerminated canola
642 (*Brassica napus*) seeds imbibed at 8°C in either GA₄₊₇, ABA, or a saline
643 solution, *J. Plant Growth Regul.* 29 (2009) 91–105.
- 644 [33] E. Stokvis, H. Rosing, J.H. Beijnen. Stable isotopically labeled internal
645 standards in quantitative bioanalysis using liquid chromatography/mass
646 spectrometry: necessity or not? *Rapid Commun. Mass Spectrom.* 19 (2005)
647 401–407.
- 648 [34] E. Beltrán, M. Ibáñez, J.V. Sancho, F. Hernández, F. Determination of
649 patulin in apple and derived products by UHPLC-MS/MS. Study of matrix
650 effects with atmospheric pressure ionisation sources, *Food Chem.* 142
651 (2014) 400–407.
- 652 [35] V. Hradecká, O. Novák, L. Havlíček, M. Strnad. Immunoaffinity
653 chromatography of abscisic acid combined with electrospray liquid
654 chromatography-mass spectrometry, *J. Chromatogr B*, 847 (2007) 162–
655 173.
- 656 [36] C. Deng, N. Li, X. Zhang, Rapid determination of essential oil in *Acorus*
657 *tatarinowii* Schott. by pressurized hot water extraction followed by solid-
658 phase microextraction and gas chromatography–mass spectrometry, *J.*
659 *Chromatogr. A* 1059 (2004) 149–155.

- 660 [37] E.S. Ong, J.S.H. Cheong, D. Goh, Pressurized hot water extraction of
661 bioactive or marker compounds in botanicals and medicinal plant materials,
662 J. Chromatogr. A 1112 (2006) 92–102.
- 663 [38] C. de Ollas, B. Hernando, V. Arbona, A. Gómez-Cadenas, Jasmonic acid
664 transient accumulation is needed for abscisic acid increase in citrus roots
665 under drought stress conditions, *Physiol. Plant.* 147 (2013) 296–306.
- 666 [39] A. Crozier, T. Moritz, Physico-chemical methods of plant hormone analysis.
667 In: P.J.J. Hooykaas, M.A. Hall, K.R. Libbenga (Eds.). *Biochemistry and*
668 *Molecular Biology of Plant Hormones*. Elsevier Science B.V. Amsterdam,
669 NL, 1999: pp. 23-59.
- 670 [40] R.C. Durley, R.P. Pharis, Partition coefficients of 27 gibberellins,
671 *Phytochemistry* 11 (1972) 317–326.
- 672 [41] A. Garcia-Luis, M. Oliveira, Y. Bordón, D.L. Siqueira, S. Tominaga, J.L.
673 Guardiola, Dry matter accumulation in citrus fruit is not limited by transport
674 capacity of the pedicel, *Annals Bot.* 90 (2002) 755–764.
- 675 [42] J. Mahouachi, D.J. Iglesias, M. Agustí, M. Talón, Delay of early fruitlet
676 abscission by branch girdling in citrus coincides with previous increases in
677 carbohydrate and gibberellin concentrations, *Plant Growth Regul.* 58 (2009)
678 15–23.
- 679

680 **Figure captions**

681 **Figure 1.** Structure and m/z values $[M-H]^-$ of the bioactive gibberellins GA₁; GA₃; GA₄
682 and GA₇.

683 **Figure 2.** Mass spectrum for GA₄ and [²H₂]-GA₇ obtained from direct flow injection
684 experiments. (a) Precursor ion of GA₄ found in Full Scan mode. (b) Product ions of GA₄
685 ($[M-H]^-331$) after fragmentation found in Daughter Scan mode. (c) Precursor ion of
686 [²H₂]-GA₇ found in Full Scan mode. (d) Product ions of the [²H₂]-GA₇ ($[M-H]^-331$) after
687 fragmentation found in Daughter Scan mode.

688 **Figure 3.** Detection and identification of gibberellins using UPLC-MS/MS. (a) Total Ion
689 Current (TIC) chromatogram showing GA₁; GA₃; GA₄ and GA₇ peaks. (b) Extracted ion
690 chromatograms of (from top to bottom) GA₁; [²H₂]-GA₁; GA₃; GA₄; [²H₂]-GA₄ and GA₇.

691 **Figure 4.** Gibberellin peak area values obtained after extracting 200 mg of mandarin
692 fruitlet samples with each method. H₂O: miliQ water; MeOH: methanol; Isop:
693 isopropanol; AA: acetic acid. Data are means, error bars represent standard errors and
694 different letters indicate statistical significance among methods for each analyte ($n=3$;
695 $p\leq 0.05$).

696 **Figure 5.** Recovery of deuterium-labelled gibberellin standards ($[^2\text{H}_2]$ -GA₁ and $[^2\text{H}_2]$ -
697 GA₄) spiked to 200 mg of mandarin fruitlet samples and extracted with each method.
698 H₂O: miliQ water; MeOH: methanol; Isop: isopropanol; AA: acetic acid. Data represent
699 mean percentage of peak area for each compound calculated on the basis of blank
700 area values and error bars are standard errors. Different letters indicate statistical
701 differences among extraction methods ($n=9$; $p\leq 0.05$).

702 **Figure 6.** Percentage of recovery for GA₁/GA₃ and GA₄/GA₇ in citrus fruitlet samples
703 spiked at known GA concentrations (50 and 100 ng g⁻¹ FW). Concentrations were
704 corrected using [²H₂]-GA₁ and [²H₂]-GA₄ as internal standards for GA₁/GA₃ and

705 GA₄/GA₇, respectively. Data are mean values of three replicates, error bars represent
706 RSD values.

707 **Figure 7.** Gibberellin content in lyophilized mandarin fruitlets at three developmental
708 stages: pre-anthesis (non-pollinated ovaries), anthesis and 10 days after anthesis (10
709 DAA). Data are means, error bars represent standard errors and different letters
710 indicate statistical significance among developmental stages ($n=3$; $p\leq 0.05$).

Title

Rapid and reproducible determination of active gibberellins in *Citrus* tissues by UPLC/ESI-MS/MS

Authors

Matías Manzi, Aurelio Gómez-Cadenas, Vicent Arbona*

Contributions

MM, AGC and VA designed and planned the experiments. MM performed experiments, measurements and analyzed data. MM, AGC and VA wrote the manuscript and performed editing and corrections. All authors have read and approved the final version of the manuscript.