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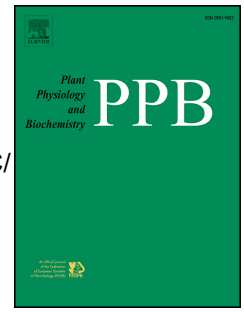

Rapid and reproducible determination of active gibberellins in Citrus tissues by UPLC/ESI-MS/MS

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PII: S0981-9428(15)30016-4

DOI: 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.

Title

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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 interferents.
- Coeluting GA_1/[$^2H_2$]-GA_3 and GA_4/[$^2H_2$]-GA_7 could interfere in their determination.
- Deuterium-labeled GA_1 and GA_4 can be used for quantitation of four bioactive GAs.

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Abbreviations

amu Arbitrary Mass Units

ESI Electrospray Ionization

GA Gibberellin

GC Gas Chromatography

HPLC High Performance Liquid Chromatography

IS Internal Standard

LC Liquid Chromatography

LOB Limit Of Blank

LOD Limit Of Detection

LOQ Limit Of Quantification

m/*z* mass-to-charge ratio

MRM Multiple Reaction Monitoring

MS Mass spectrometry

MS/MS Tandem Mass Spectrometry

RSD Relative Standard Deviation, coefficient of variation of the mean

SPE Solid Phase Extraction

UPLC Ultra Performance Liquid Chromatography

Abstract

Phytohormone determination is crucial to explain the physiological mechanisms during growth and development. Therefore, rapid and precise methods are needed to achieve reproducible determination of phytohormones. Among many others, gibberellins (GAs) constitute a family of complex analytes as most of them share similar structure and chemical properties although only a few hold biological activity (namely GA_1; GA_3; GA_4 and GA_7). A method has been developed to extract GAs from plant tissues by mechanical disruption using ultrapure water as solvent and, in this way, ion suppression was reduced whereas sensitivity increased. Using this methodology, the four active GAs were separated and quantified by UPLC coupled to MS/MS using the isotope-labelled internal standards [$^2H_2$]-GA_1 and [$^2H_2$]-GA_4. To sum up, the new method provides a fast and reproducible protocol to determine bioactive GAs at low concentrations, using minimal amounts of sample and reducing the use of organic solvents.

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

1. Introduction

The determination of phytohormones in plants has been of great interest for researchers in plant biology and physiology. For the last half-century, phytohormone fluctuations have been used to explain the physiological changes in response to stresses and throughout different stages of plant growth and development. Moreover, this knowledge has become essential for the development of many agricultural practices and has allowed growers to improve crop production. In citrus, application of different plant growth regulators alters flower induction, fruit set, ripening, and fruit quality parameters [1]. In this sense, gibberellins (GAs) are the main group of plant growth regulators used for these purposes, particularly the commercially available gibberellic acid (GA_3; [2]). This molecule was isolated in 1935 from the fungus *Gibberella fujikuroi* and named GA_3 after it. So far, 136 GAs have been identified in plants, fungi and bacteria being a group of compounds which shares a common ent-gibberellane ring [3] but, despite their structural similarity, only a small set of GAs displays biological activity in plants. Hence, GA_1; GA_3, GA_4 and GA_7 are considered the “active GAs” in vegetative tissues of plants (Figure 1), whereas the rest of molecules are synthesized in seeds or reproductive organs and few of them constitute biosynthetic intermediates or inactive forms. Presence and concentration of endogenous active GAs varies between plant species, tissues and developmental stages, playing a key role in different physiological processes. Hence, GA_1, GA_4 and GA_7 have been identified as the main active GAs in the genus *Citrus* [4,5], whereas GA_3 has only been detected occasionally in this plant species but no clear role could be assigned [5]. In general, GA_1 levels are

higher in developing fruitlets of citrus and especially in those fruitlets with a higher setting probability whereas low amounts are found in shedding fruitlets [4,6]. On the other hand, GA_4 has been associated with rind color break of 'Washington Navel' sweet orange fruits [7]. However, for GA_7 no clear role has been assigned in citrus fruiting or other physiological processes although it is often quantitated together with GA_4 and presented as GA_7+GA_4 [8]. Nevertheless, it is clear that different GAs could have specific roles in plant physiology and therefore the ability to quantitate individual molecules is of special interest [7]. The high similarities in chemical structure and properties of different GAs (i.e. similar mass and hydrophobicity) constitutes an important drawback in the development of accurate analytical methodologies. Indeed, previous works found in the literature presented their results as GA-like activity [9] and as mentioned above, in some cases it was not possible to differentiate GA_1 from GA_3 and GA_4 from GA_7, therefore reporting them as two groups: $GA_{1/3}$ and $GA_{4/7}$ [8,9]. An additional problem relies on the low hormone levels usually found in plants, which is the reason why most of them were first identified in organisms different from plants [10]. Therefore, hormone extraction and purification has to be carried out accurately and highly sensitive detection and quantitation procedure is required [11,12]. Moreover, in the particular case of GAs, the analytical technique needs to be selective enough to ensure the differentiation of structural isomers. Until recently, analytical procedures used high amounts of plant material (more than 2 g) along with tedious purification processes to get rid of interfering compounds [13,14]. In most cases, higher specificity and sensitivity were reached with radioactive tracers and immunological detection methods [15] which have an extremely narrow linear

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

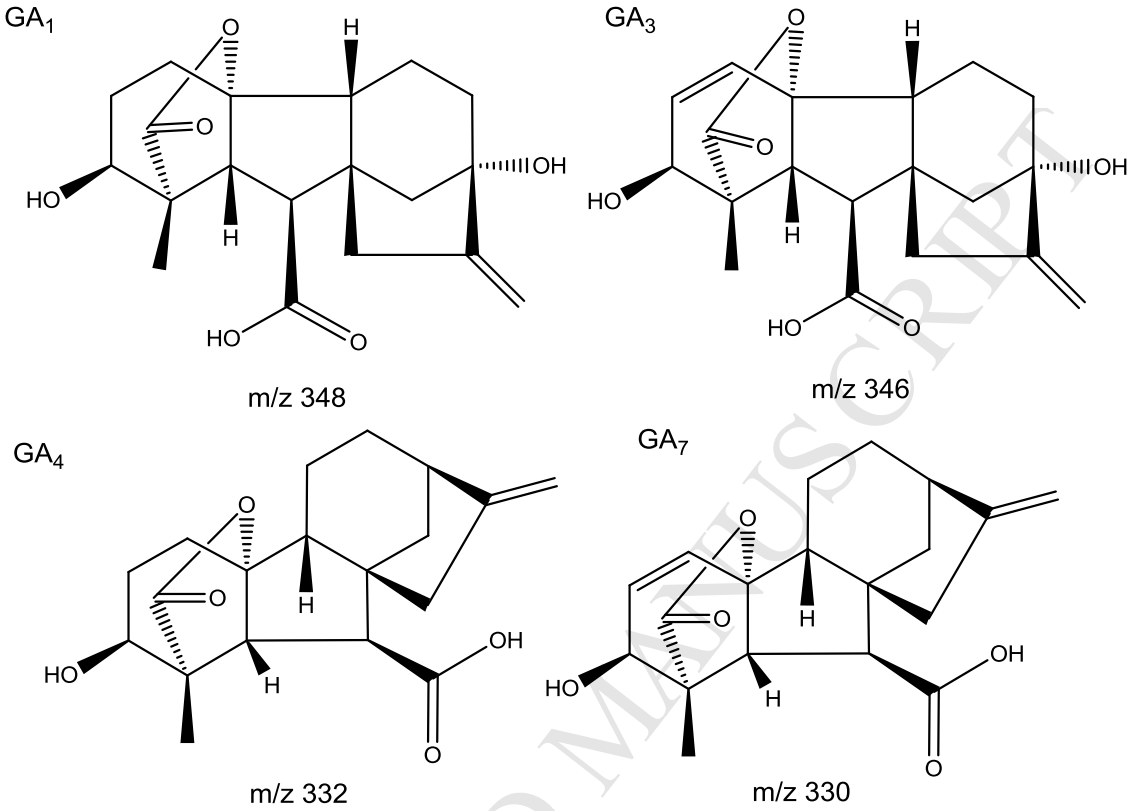


Figure 1

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

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

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

2. Materials and methods

2.1. Optimization of MS parameters

Individual standard solutions of phytohormones GA_1; GA_3; GA_4; and GA_7 as well as IS [$^2H_2$]-GA_1; [$^2H_2$]-GA_3;[$^2H_2$]-GA_4 and [$^2H_2$]-GA_7 (Olchemim Ltd, Olomouc, Czech Republic) at 5.0 µg mL^{-1} in 50% (v/v) acetonitrile:water supplemented with formic acid (0.1% v/v) were infused at a flow rate of 10 µL min^{-1} into a triple quadrupole MS analyzer (TQD^{TM}, Waters Micromass Ltd., Manchester, UK). All

the analyses were performed using the following settings: capillary voltage: 3.5 kV, nebulizer gas (N_2) 30 L hr^{-1}, desolvation gas (N_2) 400 L hr^{-1}, desolvation temperature 250ºC and setting an acquisition time of 30 s. Full scan from *m/z* 100 to 360 was performed in negative and positive ionization modes and cone voltage optimized to maximize precursor ion signal. Selected precursor ions were then fragmented adjusting collision energy within the hexapole chamber (filled with Ar gas) and the population of product ions recorded in order to select the highest intensity fragment. After setting up all transitions for analytes, a MRM method was implemented and used to selectively monitor selected GAs in plant extracts.

2.2. Extraction procedure

To optimize phytohormone extraction from plant tissues, four reported procedures for hormone analysis were tested using developing fruitlets of mandarin (*Citrus clementina* Tan.). Samples were harvested and frozen immediately in liquid N, and then converted into a fine powder. Six replicates were used to test the efficiency of each of the extraction procedures chosen, described below. All procedures were performed using 200 mg of sample (unless otherwise indicated) that was spiked with 25.0 µL of IS solution containing 1.5 mg $L^{-1}$of [$^2H_2$]-GA_1 and [$^2H_2$]-GA_4 before extraction. The final concentration of each IS compound was 37.5 ng/sample.

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

collected and 9.0 mL of ultrapure water was added to each sample. The resulting solutions were passed through a C_{18} SPE cartridge (Scharlab, Barcelona, Spain). Retained analytes were then eluted with 0.5 mL of a methanol:water:acetic acid (80:19:1, v/v/v) solution that was evaporated until complete dryness in a vacuum concentrator (Jouan, Saint-Herblain, France). Finally, dry residues were redissolved by ultrasonication in 0.5 mL of a methanol:water:acetic acid (80:19:1, v/v/v) solution for 10 min. 2) following the procedure described in [11]; samples were extracted in 5.0 mL of ultrapure water and centrifuged as described above. After recovery of supernatants, pH was adjusted to 3.0 with an acetic acid solution (30%). The aqueous solution was partitioned twice against 3.0 mL of di-ethyl ether (Panreac, Barcelona, Spain) and the upper organic layers collected, combined and evaporated to dryness. Each sample was resuspended in 0.5 mL of methanol:water, (10:90, v/v) by sonication for 10 min. 3) Following the procedure described in [7], samples were extracted in 3.0 mL of methanol:water:acetic acid (80:19:1, v/v/v) as indicated in previous procedures. The homogenate was partitioned twice against 3.0 mL of ethyl acetate supplemented with acetic acid to a 1% (v/v) concentration. The organic layer was recovered and evaporated to dryness and then, reconstituted in 50% methanol:water solution prior to SPE purification with a preconditioned C_{18} cartridge. The purified extract was dried again and reconstituted in 0.5 mL of the same extraction solution by sonication. 4) Finally, as in [12], 50 mg of samples were extracted in 0.2 mL of methanol:isopropanol:acetic acid solution (20:79:1, v/v/v) by ultrasonication for 30 min. After centrifugation at 10000 ×*g* during 15 min at 4ºC, supernatant was 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 ($[^2H_2]$-GA_1;
204 $[^2H_2]$-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 ($[^2H_2]$-GA_1; $[^2H_2]$-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 ($[^2H_2]$-GA_1; $[^2H_2]$-GA_3; $[^2H_2]$-GA_4 and $[^2H_2]$-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 [RSD=(standard deviation/mean)
219 × 100] must be lower than 20%, as has been established in [20].

2.4. Method validation and analysis of plant samples

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

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

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

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

243 $$\%Recovery=[(Mean_{sp}–Mean_{nsp})]\times100/expected\ theoretical\ concentration$$

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 4ºC. 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 ±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 ×2.0 mm id, 1.8 μm particle size,

Macherey–Nagel, Germany) maintained at 40ºC during chromatographic runs. Ultrapure water and methanol were used as mobile phase at a flow rate of 0.3 mL min^{-1}, both supplemented with CH_3COOH to a concentration of 0.1% (v/v). A gradient elution program was established as follows: isocratic conditions of 90:10 (water/methanol) for 2 min and from that point methanol concentration was linearly increased to 80% in 4 min. Afterwards, isocratic conditions were maintained for 1 min and then the solvent composition was restored to initial conditions to allow column re-equilibration for 2 min. Samples were analyzed in MRM mode setting an interscan time of 1.0 s and a dwell time for each function of 0.025 s. Source block and desolvation gas (N_2) temperatures were set at 120ºC and 350ºC, respectively. Nebulization and desolvation gas flows were set at 60 and 800 L h^{-1}, respectively. A two min solvent delay period was included at the beginning of the run to avoid build-up of poorly-retained contaminants in the mass spectrometer. Analysis of data was carried out with MassLynx v. 4.1 software (Micromass Ltd) and differences in mean GA concentration values among samples assessed by means of ANOVA followed by Tukey´s post-hoc test considering a $p \leq 0.05$ *p*-value cutoff.

3. Results and discussion

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

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

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

The optimized precursor-to-product ion transitions as well as ionization modes for each analyte are summarized in Table 1. After direct injection of standard solutions into the MS system, identification of parent ions was performed in full-scan mode by recording mass spectra from *m*/*z* 100 to 360 in both negative and positive ionization modes, adjusting cone voltages to maximize precursor ion intensity. Afterwards, collision energy was adjusted to maximize intensity of product ions. Negative ionization mode (ESI-) was chosen due to the higher intensity found for labeled and non-labeled compounds. In this work, transitions chosen were identical to those previously reported using similar equipment [7, 12,26,27], but although differing in cone voltage and collision energy, suggests the existence of a specific fragmentation pattern for this molecules despite of the MS/MS instrument. On the other side, as known, chromatographic separation for these compounds is difficult since their retention times are similar due to the chemical structure (Table 1 and Figure 3). Even extending run time did not completely resolve these compounds. Thus, the maximum resolution achieved between GA_1 and GA_3 was only of 0.2 min [28], or even lower between GA_4 and GA_7 using nanoflow-LC-MS/MS [21]. Indeed, at the initial stages of use of LC-MS for metabolite determination, some researchers could only report joint data for GA_1 and GA_3 (GA_{1+3}) and GA_4 and GA_7 (GA_{4+7}; [8]) or in cases, some reports did not determine GA_3 and GA_7 when GA_1 or GA_4 were quantitated [12,28,29]. Overall, our results indicate that a proper selection of the precursor-to-product ion transition is essential to achieve a precise and

selective hormone determination by MS/MS, whereas chromatographic separation has to be considered less important. Molecular weights of endogenous GAs usually differ from their respective commercially-available deuterium-labelled analogues in 2 amu (see Figures 1 and 2). This results in an inconvenient overlapping of metabolite transitions in some cases. Thus, the [M-H]- ions for [$^2H_2$]-GA_3 and $^2H_2$]-GA_7 have the same nominal masses as those for GA_1 and GA_4, respectively, and these cannot be resolved by the mass analyser. Moreover, these similar molecules also share a common fragmentation pattern and thus daughter ions. As signaled above, the similarity in fragmentation pattern among GAs lead to interference among molecules as shown in figure 2 as exposed in previous works [30]. Furthermore, this could be the reason behind the exclusion of [$^2H_2$]-GA_3 and [$^2H_2$]-GA_7 as it is when GA_1 and GA_4 are quantitated along with GA_3 and GA_7 [21,31,32]. Therefore, due to this double chromatographic (similar retention times) and spectrometric (similar monoisotopic mass and fragmentation pattern) interference among analytes and some deuterium-labelled compounds, we excluded [$^2H_2$]-GA_3 and [$^2H_2$]-GA_7 as internal standards for further analyses.

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

Compound	Quantification transition *Q*	Cone voltaje (V)	Collision energy (eV)	Retention time (min)
GA_1	347 > 229	25	30	4.63
[$^2H_2$]-GA_1	349 > 231	35	25	4.62
GA_3	345 > 143	35	20	4.56
GA_4	331 > 213	35	27	5.97
[$^2H_2$]-GA_4	333 > 215	35	25	5.97

GA_7	329 > 223	27	17	5.89
$[^2H_2]$-GA_7	331 > 225	30	20	5.89

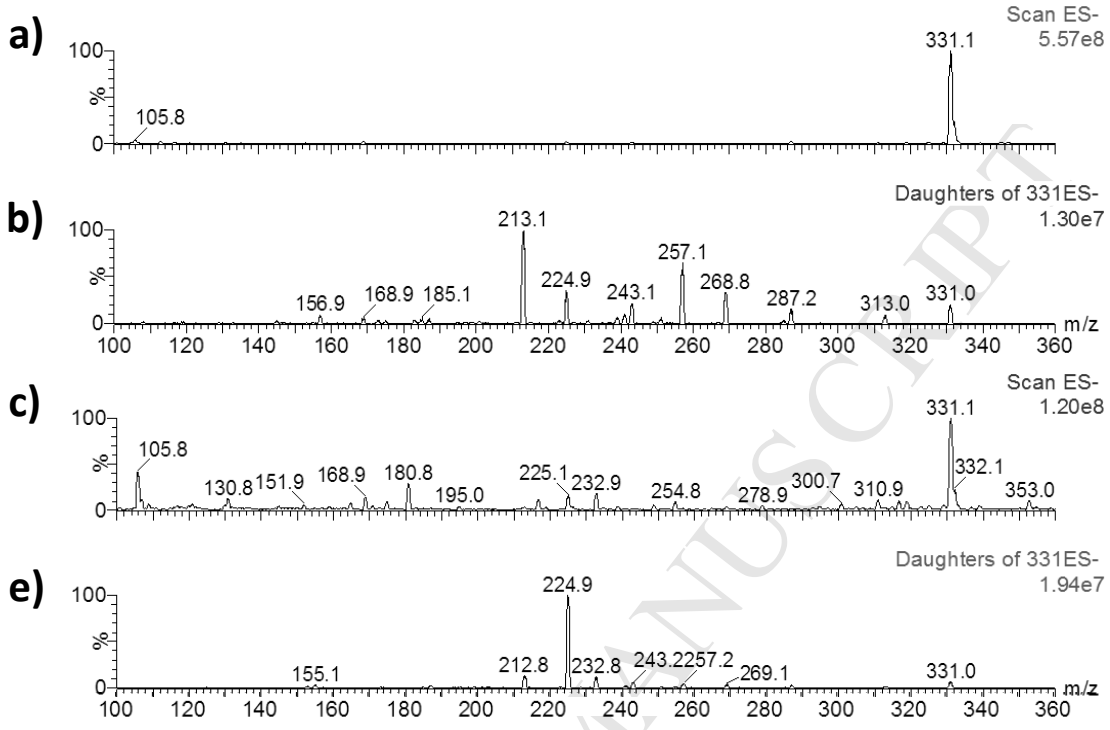


Figure 2

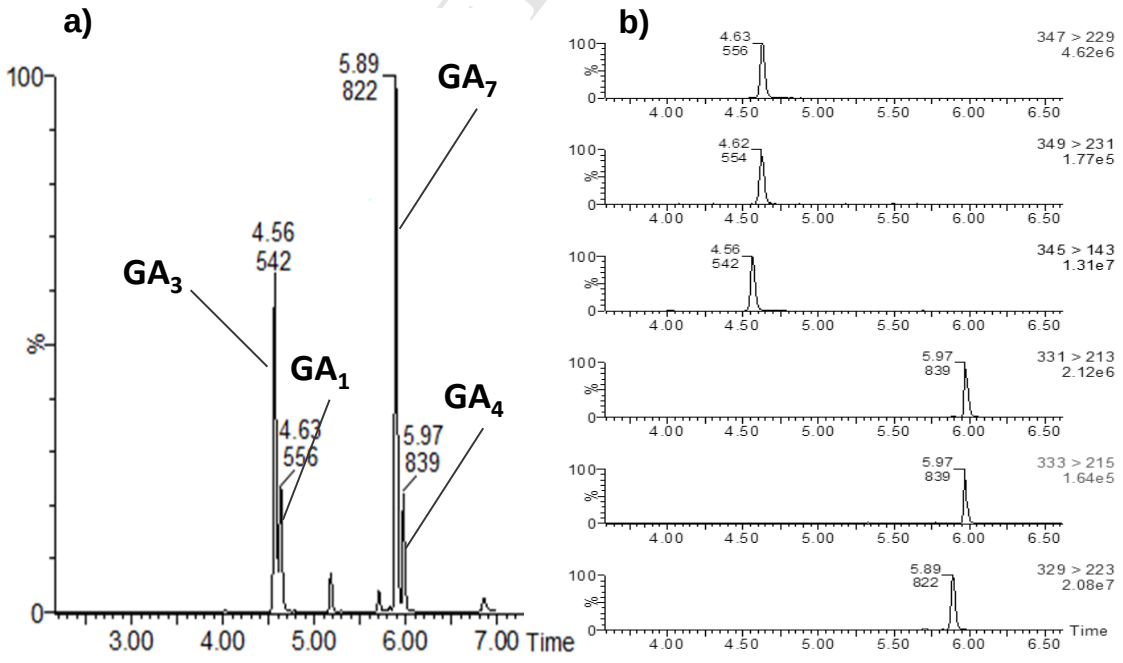


Figure 3

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 C_{18}-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 GA_7, were higher using water in the extraction than using any organic
356 solvent mixture. For GA_7, on the contrary, higher peak areas were achieved
357 using Isop:AA or MeOH:H_2O:AA (Figure 4). Moreover, peak area of [$^2H_2$]-GA_4 in
358 samples extracted in Isop:AA showed similar values to those found in samples
359 extracted with water. As expected, GA_3 was not detected in mandarin fruitlet
360 samples [5]. From these results, a preliminary estimation of GA concentrations
361 could be performed: GA_1 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 GA_4, 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, GA_7 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:H_2O: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.

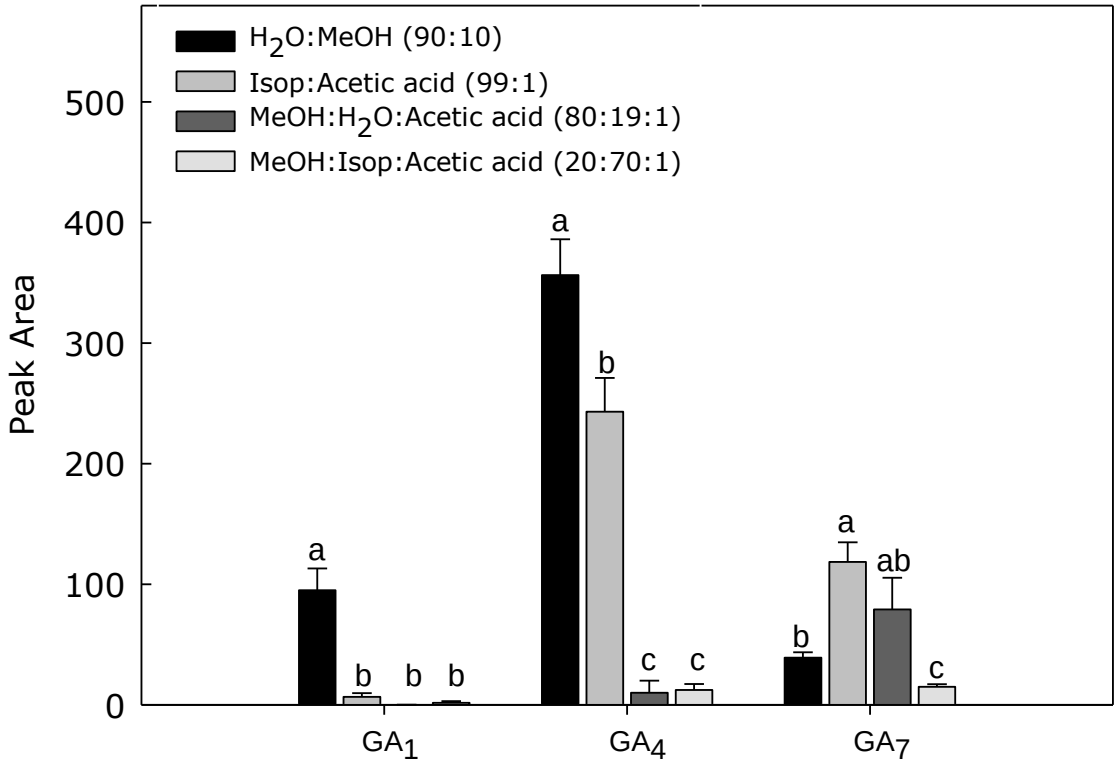
403 **Table 2**. Analysis of gibberellins in developing citrus fruitlets using different extraction
404 methodologies.

Hormone	GA_1	GA_4	GA_7
		ng.g^{-1} FW	
H_2O (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_2O: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

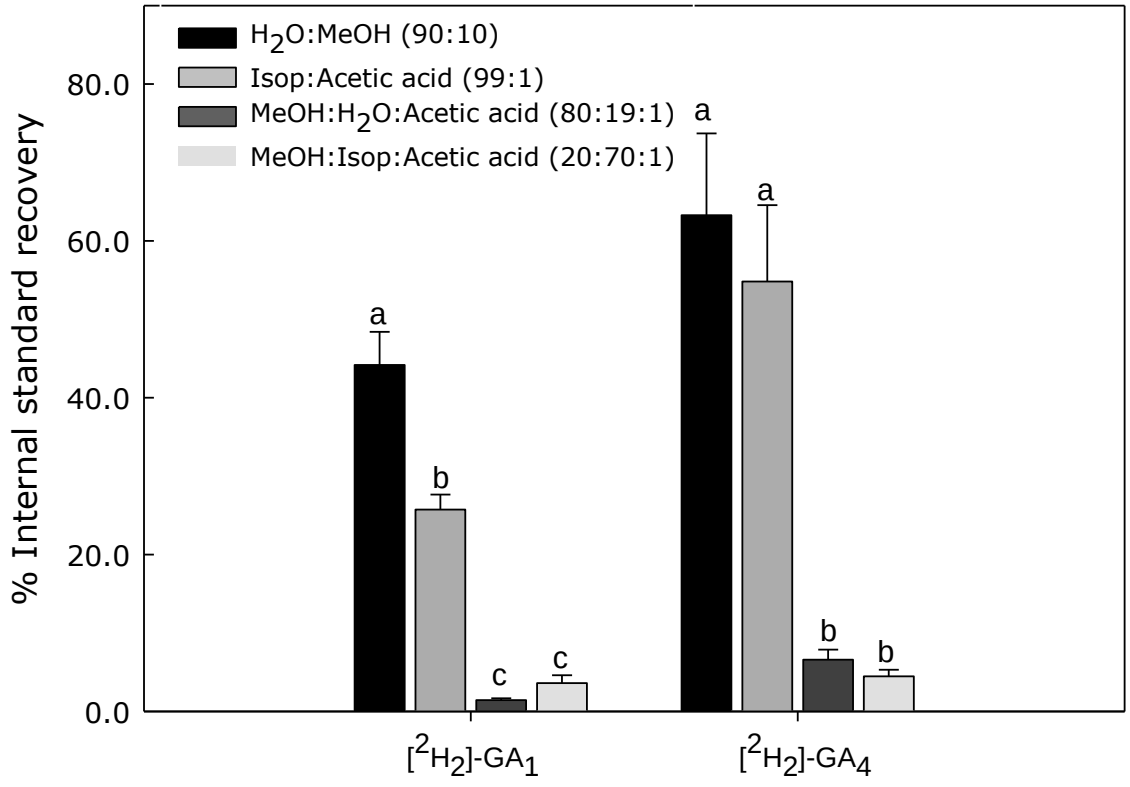
406 H2O: miliQ water; MeOH: methanol; Isop: isopropanol; AA: acetic acid; nd; non-
407 detectable. Data are means ± standard errors and letters indicate statistical differences
408 among methods for each analyte (n=3; p≤0.05).

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



430

431 **Figure 4**



432

433 **Figure 5**

3.3 Internal standard selection

The addition of IS with identical chemical properties as the target analyte(s) compensates for analyte losses during extraction and purification, as well as metabolite stability, ion suppression and run-to-run variations [11,16]. Commercially available labeled compounds contain deuterium, ^{13}C or another stable isotope and identical amounts are added to every sample before extraction. Isotope-labelled compounds show identical chromatographic behavior as non-labeled analytes but differ in their mass values. In this sense, most of the usually analyzed GA is available as deuterium-labelled standard including the bioactive GA_1, GA_3, GA_4 and GA_7. As mentioned above, $[^2H_2]$-GA_3 and $[^2H_2]$-GA_7 could not be added as IS because these IS interfere chromatographically and spectrometrically (similar retention time and mass transition compared to GA_1 and GA_4, respectively). Instead, $[^2H_2]$-GA_1 and $[^2H_2]$-GA_4 were used as IS [21,32], improving recovery, which is essential for an accurate quantitation of the four bioactive GAs (Figure 6). Moreover, RSD values for these compounds corrected by the IS fell within the limits established (<20%), evidencing the high precision of the method and the recoveries obtained using $[^2H_2]$-GA_1 and $[^2H_2]$-GA_4 as IS. Furthermore, the use of $[^2H_2]$-GA_4 as IS of GA_7 seems to amend the low initial recovery found for this phytohormone (Figure 4).

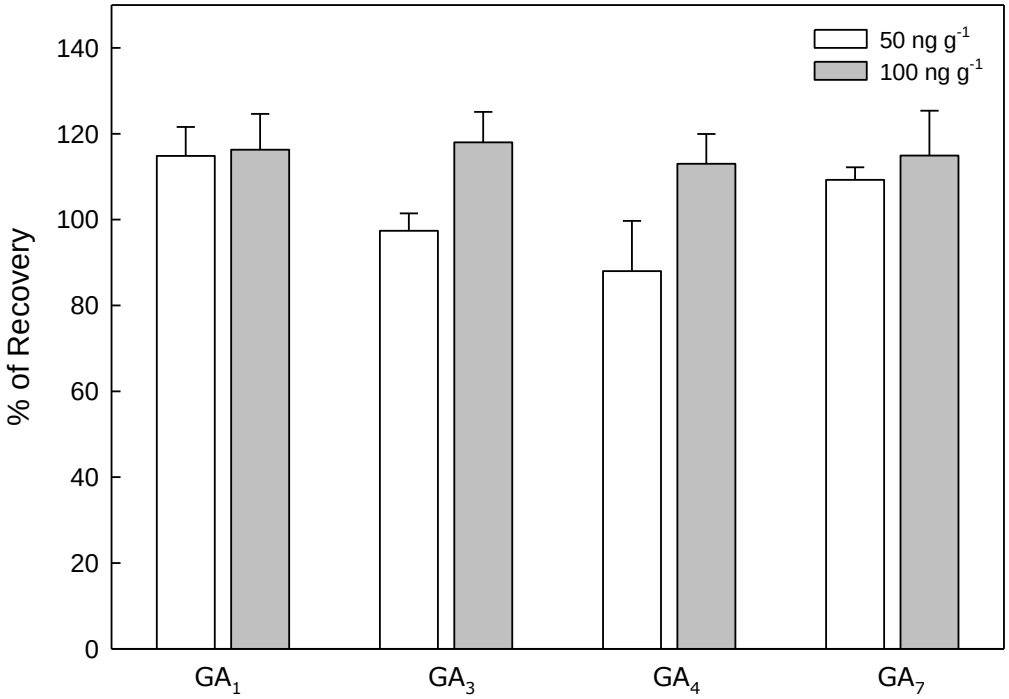


Figure 6

3.4. Method validation and analysis of samples

Method validation was accomplished by calculation of LOB, LOD and LOQ. First, calibration curves (from 0.4 to 200.0 ng mL^{-1}) were analyzed in triplicate, including increasing GA concentrations and keeping IS amount constant ($[^2H_2]$-GA_1 and $[^2H_2]$-GA4). The correlation coefficients (R^2) for the calibration curves were always higher than 0.98 for each GA, 0.9809 for GA_1; 0.9859 for GA_3; 0.9901 for GA_4; and 0.9959 for GA_7 and therefore considered acceptable. Sensitivity parameters (LOB, LOQ and LOD) were calculated from the standard curves as in [23]. LOB values ranged from baseline to 0.4 ng g^{-1}, LOD levels 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 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)	
		ng g^{-1}		Within 24 h	Within 1 week
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 4ºC (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

assessed in three stages of fruitlet development: pre-anthesis (non-pollinated ovaries), anthesis and S2 (10 days after anthesis, 10 DAA; Figure 7). Levels of GA_4 (the main bioactive GA in citrus) and GA_7 were higher on anthesis, reaching 404 and 72 ng g^{-1} DW, respectively. On the other hand, levels of GA_1 were low compared to the other GAs analyzed and similar among the three stages chosen, ranging from 6.4 to 16.6 ng g^{-1} DW. As expected, GA_3 was not detected in these samples as reported before in citrus [5]. The GA levels found with the optimized methodology are consistent with previous reports [5,6,42], although measured levels of GA_4 were substantially higher than those previously reported [5,6]. However, we could not ascertain whether these differences were attributable to environmental factors such as water deficit [8], or previous fruit load that influences the number of flowers and subsequently fruit set ability, processes in which GAs play a key role [1]. During fruitlet development, GA content increases about two-fold during anthesis and then decreases again to pre-anthesis values [6]. In this respect, final fruit set in citrus seems to be linked to the decrease of GA content (GA_4 and GA_7) after the anthesis stage, mainly in seedless and self-incompatible varieties such as clementines [6,42].

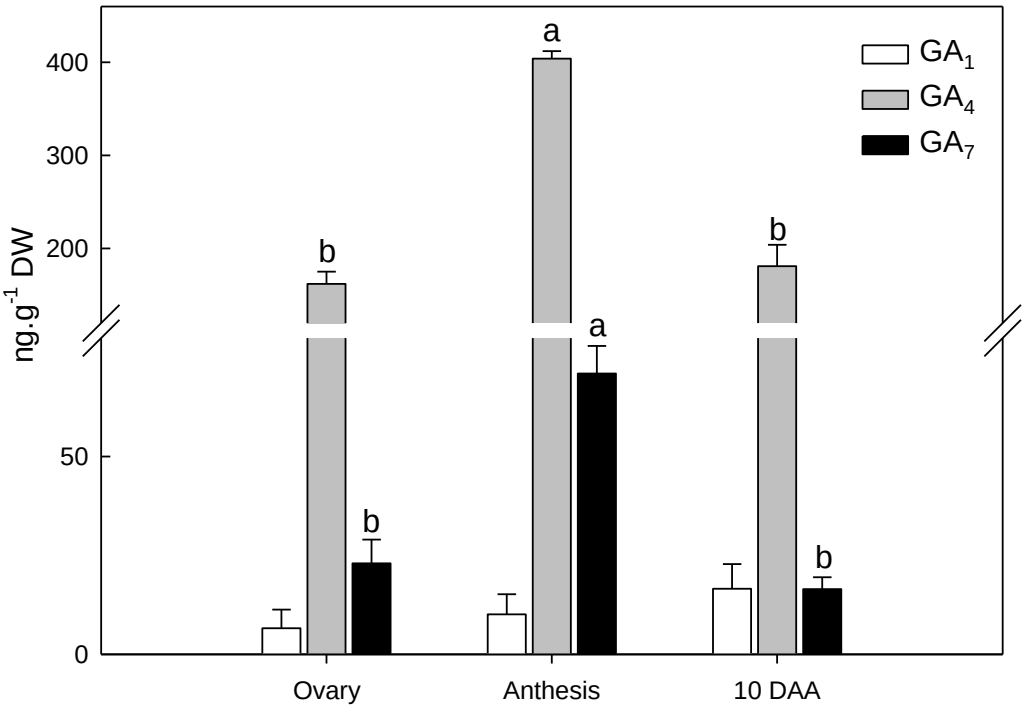


Figure 7

3. Conclusions

In summary, procedures to attain gibberellin determination face several issues such as the high structural similarity among the different components of this family and the extremely low concentrations found in plant tissues. In this work, ultrapure water as extraction solvent for plant phytohormones proved to be more efficient than organic solvents, which allows skipping time-consuming clean-up steps. This was related to the low extraction potential of water that reduces the occurrence of interfering compounds that might induce ion suppression leading to increased sensitivity. Quantitation of endogenous GAs was achieved by spiking samples with deuterium-labeled analogues. In addition, it has been shown that [$^2H_2$]-GA_3 and [$^2H_2$]-GA_7 could interfere with GA_1 and GA_4 determination due to its similar *m*/*z* values and fragmentation

patterns and retention times, thus excluding their use as IS. However, [$^2H_2$]-GA_1 and [$^2H_2$]-GA_4 were efficient to assess the recovery of GA_1 and GA_4 but also for GA_3 and GA_7, respectively. Finally, the method was validated and confirmed in different fruit growth developing stages, proving the robustness of the method.

Acknowledgements

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

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Figure captions

Figure 1. Structure and *m/z* values $[M-H]^-$ of the bioactive gibberellins GA_1; GA_3; GA_4 and GA_7.

Figure 2. Mass spectrum for GA_4 and $[^2H_2]$-GA_7 obtained from direct flow injection experiments. (**a**) Precursor ion of GA_4 found in Full Scan mode. (**b**) Product ions of GA_4 ($[M-H]^-$331) after fragmentation found in Daughter Scan mode. (**c**) Precursor ion of $[^2H_2]$-GA_7 found in Full Scan mode. (**d**) Product ions of the $[^2H_2]$-GA_7 ($[M-H]^-$ 331) after fragmentation found in Daughter Scan mode.

Figure 3. Detection and identification of gibberellins using UPLC-MS/MS. (**a**) Total Ion Current (TIC) chromatogram showing GA_1; GA_3; GA_4 and GA_7 peaks. (**b**) Extracted ion chromatograms of (from top to bottom) GA_1; $[^2H_2]$-GA_1; GA_3; GA_4; $^2H_2$]-GA_4 and GA_7.

Figure 4. Gibberellin peak area values obtained after extracting 200 mg of mandarin fruitlet samples with each method. H_2O: miliQ water; MeOH: methanol; Isop: isopropanol; AA: acetic acid. Data are means, error bars represent standard errors and different letters indicate statistical significance among methods for each analyte (n=3; $p \leq 0.05$).

Figure 5. Recovery of deuterium-labelled gibberellin standards ($[^2H_2]$-GA_1 and $[^2H_2]$-GA_4) spiked to 200 mg of mandarin fruitlet samples and extracted with each method. H_2O: miliQ water; MeOH: methanol; Isop: isopropanol; AA: acetic acid. Data represent mean percentage of peak area for each compound calculated on the basis of blank area values and error bars are standard errors. Different letters indicate statistical differences among extraction methods (n=9; $p \leq 0.05$).

Figure 6. Percentage of recovery for GA_1/GA_3 and GA_4/GA_7 in citrus fruitlet samples spiked at known GA concentrations (50 and 100 ng g^{-1} FW). Concentrations were corrected using $[^2H_2]$-GA_1 and $[^2H_2]$-GA_4 as internal standards for GA_1/GA_3 and

705 GA_4/GA_7, 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.