High-performance liquid chromatography–thermospray mass spectrometry of gibberellins

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ABSTRACT

Seven tetracyclic monocarboxylic acid gibberellins (GA_1, GA_3, GA_4, GA_5, GA_7, GA_9 and GA_{30}) and the methyl ester derivatives of GA_1, GA_3, GA_4, GA_7 and GA_9 were analyzed using combined high-performance liquid chromatography–thermospray mass spectrometry or direct injection thermospray mass spectrometry. The seven free acid GAs were resolved using a 25-min water–acetonitrile (0.1 M ammonium acetate) gradient mobile phase and a 5 μm, 150 mm × 4.6 mm, ODS column. Positive-ion thermospray mass spectra of these compounds typically showed intense $[M + NH_4]^+$ ions and few, if any, fragment ions. In the negative-ion mode (filament on) the free acid GAs showed $[M - H]^-$ or $[M + HCO_2]^-$ ions as the base peaks. Negative-ion spectra of the methyl ester GAs showed the $[M + HCO_2]^-$ ions, however, the base peaks were $[M]^{-\cdot}$ or $[M + HCO_2H]^-$ ions attributed to electron capture processes. Thermospray tandem mass spectra were obtained for GA_1, GA_3 and GA_{30} from the collisionally activated dissociation (CAD) of their $[M + NH_4]^+$ ions. The CAD mass spectra show differences which allow the differentiation of the isomers GA_3 and GA_{30} without the chromatographic separation. The daughter ions resulting from fragmentation processes corresponded to varying losses of CO, CO_2, ammonia and water.

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INTRODUCTION

Gibberellins (GAs) are a group of naturally occurring plant hormones responsible for a variety of biological activities, such as growth promotion, and are generally thought to be ubiquitously present in plants [1]. Numerous GAs have been analyzed, typically in low concentrations, from biological sources including spruce [2], begonia leaves [3], apricot seeds [4], peas [5], fungi [6] and soil [7]. The analysis of specific GAs in biological samples routinely involves the use of high-performance liquid chroma-

tography (HPLC), primarily as an isolation and purification step, prior to analysis by gas chromatography GC—mass spectrometry (MS). Methods for GA separations utilizing normal- and reversed-phase HPLC have been reported [8–11]. Although GC–MS is a sensitive method for the determination of GAs, the procedure requires two derivatization steps typically via methylation and silylation reactions. On-column permethylation of GAs has been reported as one alternative to the previous procedures [12]; however, the MS analysis is still of a structurally altered GA. Ideally, a method for GA analysis involving no structural alterations is preferred.

The development of combined HPLC and thermospray (TSP) MS technology has allowed new opportunities for the mass spectral analysis of compounds which cannot be analyzed by GC MS without prior derivatization [13]. Limited data concerning the HPLC–MS analysis of GAs have been reported in the literature [14–16]. This report describes the analysis of a set of seven free acid GAs and five methyl ester GA derivatives by HPLC–TSP-MS, HPLC–TSP-MS–MS, or direct injection TSP–MS.

EXPERIMENTAL

Chemicals

Gibberellic acid (GA_3) and the methyl esters of GA_1, GA_3, GA_4, GA_7 and GA_9 were obtained from Sigma (St. Louis, MO, USA). The free acid gibberellins GA_1, GA_4, GA_5, GA_7, GA_9 and GA_{30} were the generous gift of Dr. Noboru Murofushi, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. The compounds were dissolved in methanol and an aliquot diluted with 0.1 M ammonium acetate. The organic solvents used were of HPLC grade and all other chemicals were of the highest purity available. Deionized water (18 $M\Omega$/cm) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

High-performance liquid chromatography

Two HPLC systems were utilized during this study.

System 1 employed a 250 mm × 4.6 mm I.D., 5 μm ODS column (Beckman Instruments/Altex Scientific Operations, San Ramon, CA, USA). The

mobile phase was aqueous 0.1 M ammonium acetate–acetonitrile (85:15) maintained at a flow-rate of 1.25 ml/min by an Isco LC-5000 syringe pump (Isco, Lincoln, NE, USA). Typically, 50–100-μl volumes were injected on-column utilizing a Model 7125 injector (Rheodyne, Cotati, CA, USA).

System 2 consisted of a SP8700XR programmer and pumping system (Spectra-Physics, Santa Clara, CA, USA) and a 5 μm (150 mm × 4.6 mm I.D.) Spherisorb ODS-2 column (Phase Separations, Norwalk, CT, USA). Gradient eluents were ratios of 0.1 M ammonium acetate (adjusted to pH 4 with formic acid) and either acetonitrile or methanol as the organic modifier. The flow-rate was maintained at 1.0 ml/min and sample injections were made using a Rheodyne Model 7125 injector.

Direct injection thermospray mass spectrometry

The liquid carrier for direct injection TSP-MS analysis was 0.1 M aqueous ammonium acetate–methanol (90:10, v/v) and was maintained at a flow-rate of 1.25 ml/min using the Isco LC-5000 syringe pump. Typically, 100-μl volumes were injected utilizing a Rheodyne Model 7125 injector.

Thermospray mass spectrometry

A Finnigan MAT TSQ 70 triple stage quadrupole mass spectrometer equipped with a Finnigan MAT thermospray interface and source (San Jose, CA, USA) was used. The vaporizer and block temperatures were optimized to maximize the ion intensity of the $[M + NH_4]^+$ ion. Typically, the vaporizer was adjusted to 105°C and the ion source block was set at 220°C. Negative-ion spectra were obtained using the filament-on mode. When operated in the tandem MS mode the collision gas was argon set at approximately 0.5 mTorr and the collision energy was set at 25 eV.

RESULTS AND DISCUSSION

Combined HPLC–TSP-MS was used to analyze the seven monocarboxylic acid gibberellins (GAs) shown in Fig. 1. The chromatographic characteristics and the positive-ion TSP-MS results are shown in Table I. The retention order observed using these chromatographic systems correlates with that reported elsewhere [11] and is consistent with the chemical structures of the compounds.
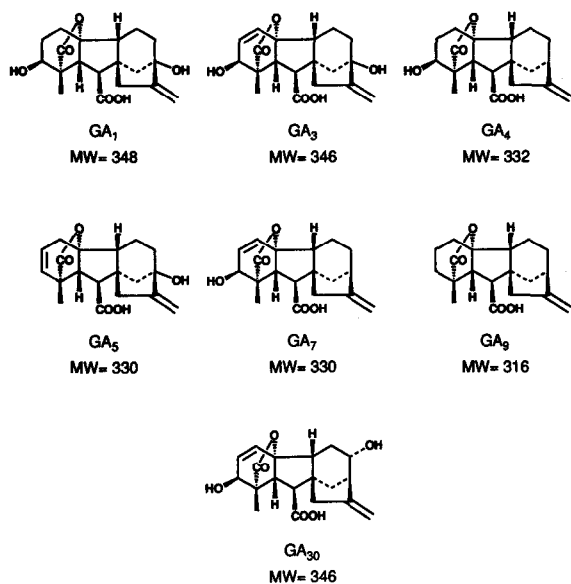
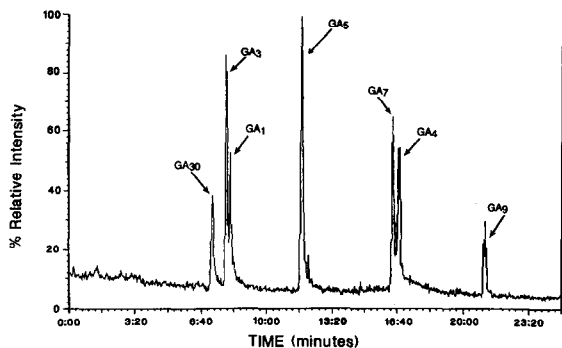Fig. 1. Chemical structures of the gibberellins analyzed during this study. MW = Molecular weight.

Fig. 2. Gradient HPLC–thermospray mass chromatogram of seven free acid gibberellins. HPLC system 2, mobile phase: gradient from acetonitrile–0.1 M ammonium acetate (5:95) to acetonitrile–0.1 M ammonium acetate (10:90) in 0.5 min, then to acetonitrile–0.1 M ammonium acetate (50:50) in 17 min, flow-rate: 1.0 ml/min, thermospray souce: 250°C, thermospray interface temperature programming from 88°C to 85°C in 15 min. Time in min:s.

Fig. 2 shows an HPLC–thermospray mass chromatogram of the seven free acid GAs analyzed. The chromatographic conditions used are given in the figure caption. The amount of each GA contained in the injection was in the range of 0.5–1.0 μg. Although gradient programming frequently results in varying thermospray conditions and probe temperature programming is needed to preserve optimal

TABLE I

SUMMARY OF THE RESULTS FROM THE HPLC–TSP-MS ANALYSIS OF GIBBERELLINS

Compound	MW	Retention time (min)[a]		Major ions observed	
		System 1	System 2	$[M + NH_4]^+$ (%)[b]	Others (%)[b]
GA$_1$	348	1.3	8.2	366(100)	
GA$_3$	346	1.3	7.9	364(100)	302(6)
GA$_4$	332	7.0	16.8	350(100)	
GA$_5$	330	1.8	11.8	348(100)	
GA$_7$	330	6.3	16.4	348(100)	286(41)
GA$_9$	316	16.0	21.1	334(100)	317(5)
GA$_{30}$	346	1.3	7.2	364(100)	302(26)
GA$_1$-methyl	362	d.i.[c]	d.i.[c]	380(100)	
GA$_3$-methyl	360	d.i.[c]	d.i.[c]	378(100)	
GA$_4$-methyl	346	d.i.[c]	d.i.[c]	364(100)	
GA$_7$-methyl	344	d.i.[c]	d.i.[c]	362(100)	
GA$_9$-methyl	330	d.i.[c]	d.i.[c]	348(100)	331(70), 299(10)

[a] HPLC retention times: system 1 is described in the Experimental section, system 2 is the gradient HPLC system described in the legend to Fig. 2.

[b] Spectral data shown were obtained using HPLC system 1. The relative intensity of the ion is listed in the parentheses after the m/z of the ion.

[c] The TSP-MS analysis was peformed via direct injection of the sample.
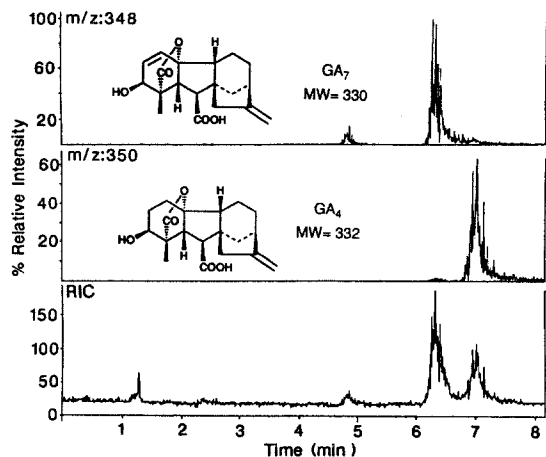
Fig. 3. Isocratic HPLC–thermospray mass chromatogram of GA$_4$ and GA$_7$ in admixture obtained using HPLC system 1.

response, acceptable separation of these compounds within a reasonable analysis time (22 min) required using a gradient HPLC system. Baseline separation between GA$_4$ and GA$_7$ was observed using HPLC system 1 (Fig. 3), however, no resolution between the more polar GA$_3$, GA$_1$ and GA$_{30}$ was achieved (Table I).

As shown in Fig. 4 and Table I, the positive-ion thermospray mass spectra of the free acid GAs
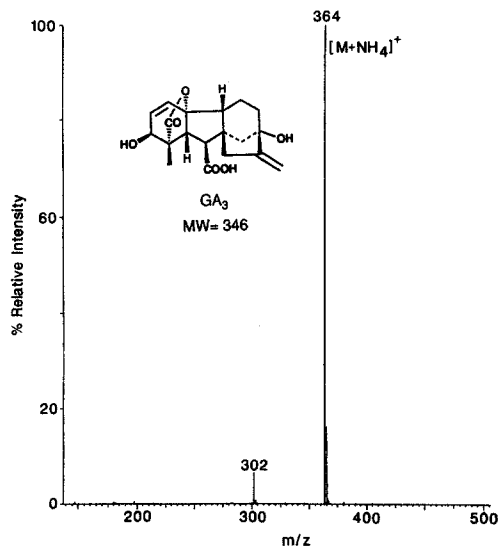

Fig. 4. HPLC–thermospray mass spectrum of GA$_3$ obtained using HPLC system 1.

showed an intense [M + NH$_4$]$^+$ ion as the base peak with few, if any, fragment ions. Although the data set is limited, two observations can be made regarding the structure of the GAs and the ions detected when the compounds were analyzed by TSP-MS. First, of the set of GAs analyzed by TSP-MS, GA$_3$, GA$_7$ and GA$_{30}$ have a double bond across the C-3 and C-4 carbons and a hydroxyl group at the C-2 carbon (see Fig. 1). It is shown in Table I that the thermospray mass spectra for these three GAs included an [M + NH$_4$ − 62]$^+$ ion in addition to the [M + NH$_4$]$^+$ ion. This [M + NH$_4$ − 62]$^+$ ion might be attributed to the [M + NH$_4$ − CO$_2$ − H$_2$O]$^+$ ion. There appears to be a correlation between the presence of the C-3–C-4 double bond near the −COO− bridge and the removal of CO$_2$ in combination with a loss of water resulting in a total loss of 62 daltons from the [M + NH$_4$]$^+$ ion. Secondly, in Table I it is noted that GA$_9$ is the only GA of the sample set analyzed to show an [M + H]$^+$ ion for both the free acid and the methylated species. As seen in Fig. 1, GA$_9$ is the only GA analyzed that does not have a double bond or a hydroxyl moiety. This suggests that even in the absence of these groups the proton affinity of GA$_9$ is sufficient to produce an [M + H]$^+$ ion. The abundance of the [M + NH$_4$]$^+$ adducts and the absence of a [M + H]$^+$ ion seen in the TSP-MS spectra of the GAs which have double bonds and hydroxyl groups could be explained by stabilization of the adducts with ammonia associated with the presence of the double bond and hydroxyl groups.

Fig. 5 shows the thermospray response curve obtained by injecting various amounts (0.1 to 2.0 μg) of GA$_3$ and the methyl ester GA$_3$ derivative. The thermospray response data for GA$_3$ are shown as the peak area under the fragment ion peak seen at m/z 302, the base peak at m/z 364, and the combined peak areas of both. Each gave a linear thermospray response as evidenced by correlation coefficients of 0.995, 0.996 and 0.996 respectively. The linearity observed for the [M + NH$_4$]$^+$ ion at m/z 378 for the methyl ester of GA$_3$ is also shown in Fig. 5. In addition to the thermospray response being more sensitive for this ion it gave the most linear response of the ions analyzed (correlation coefficient = 0.999). These data suggest that HPLC–TSP-MS could be used to detect these GAs down to levels of about 100 ng.
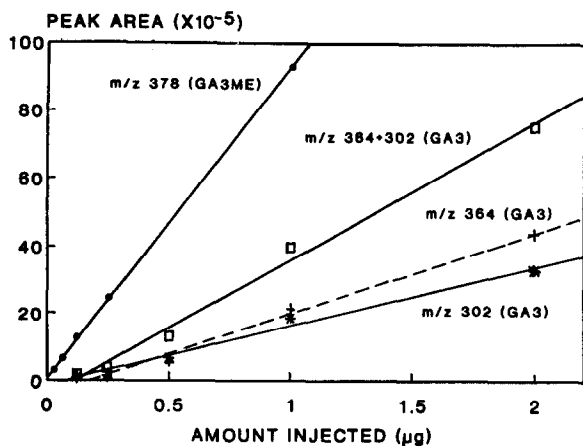
Fig. 5. Graph showing the thermospray response (peak area) vs. the amount (μg) of GA_3 and its methyl ester injected. HPLC system 2, mobile phase: isocratic, for GA_3 acetonitrile–0.1 M ammonium acetate (15:85), for GA_3-methyl ester acetonitrile–0.1 M ammonium acetate (20:80), flow-rate 1.0 ml/min, thermospray source: 250°C, thermospray interface: 87°C for GA_3, 85°C for GA_3-methyl ester (GA_3ME).
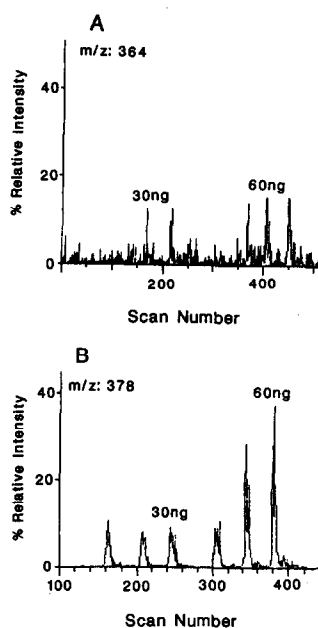

Fig. 6. Selected ion current profiles of consecutive injections of 30 ng and 60 ng each of GA_3 (A) and GA_3-methyl ester (B). HPLC system 2, mobile phase: isocratic, for GA_3 acetonitrile–0.1 M ammonium acetate (15:85), for GA_3-methyl ester acetonitrile–0.1 M ammonium acetate (20:80), flow-rate: 1.0 ml/min, thermospray source: 250°C, thermospray interface: 87°C for GA_3, 85°C for GA_3-methyl ester.

For comparative purposes, thermospray mass spectra of five methyl ester GA compounds were also obtained. As shown in Table I, the positive-ion thermospray mass spectra resulting from the direct injection analysis of five methyl ester GA derivatives also showed predominantly the $[M + NH_4]^+$ ion as the base peak with few fragment ions. Fig. 6 compares consecutive injections of 30 and 60 ng GA_3 (Fig. 6A) and GA_3-methyl ester (Fig. 6B) using an isocratic HPLC system. This figure clearly shows the increased sensitivity of the thermospray detector for the methyl ester GA_3 derivative over that of the free acid. Detection-oriented derivatization resulting in increased thermospray response for various compounds has been reported [17–19]. Although the objective of this study was to investigate the utility of HPLC–TSP-MS to directly analyze the free acid GAs, these data suggest that the increased thermospray sensitivity gained by methylation of the free acid function may be incorporated for GA analysis in biological samples requiring higher detection sensitivity. This can be readily accomplished using diazomethane. We have previously described a simplified procedure for the preparation of diazomethane and subsequent sample derivatization [20].

Negative-ion thermospray data (filament-on mode) are presented in Table II. To our knowledge this is the first report of the negative-ion thermospray analysis of GAs. As shown in Table II, most of the free acid GAs showed the $[M - H]^-$ or the $[M + HCO_2]^-$ ion as the base peak or as a major peak in their negative-ion mass spectrum. The methyl ester GAs did not display an $[M - H]^-$ ion, but showed either the $[M]^{\cdot-}$ or the $[M + HCO_2]^-$ ion as the base peak in their negative-ion thermospray mass spectra. These data demonstrate that TSP-MS in the negative-ion mode can also be used for the analysis of either free acid GAs or the methyl ester derivatives. However, positive-ion detection of these compounds was about 10 times more sensitive and did not require use of the filament-on mode.

HPLC–TSP-MS–MS spectra were obtained for the isomeric GAs GA_3 and GA_{30}. As shown in Table I GA_1, GA_3 and GA_{30} co-eluted when analyzed using HPLC system 1. Figs. 7 and 8 show the collisionally activated dissociation (CAD) daughter ion mass spectra of the $[M + NH_4]^+$ ions at m/z 364 for GA_3 and GA_{30}, respectively. These mass spectra

TABLE II

NEGATIVE-ION TSP-MS SPECTRA OF GIBBERELLINS

Filament-on mode. The spectra were obtained using the following conditions: HPLC system 2, mobile phase: isocratic, for GA_3 acetonitrile–0.1 M ammonium acetate (15:85), for GA_3-methyl ester acetonitrile–0.1 M ammonium acetate (20:80), flow-rate 1.0 ml/min, thermospray source: 250°C, thermospray interface: 87°C for GA_3, 85°C for GA_3-methyl ester. Me = Methyl.

[Assign]$^-$[b]	Relative abundance[a]											
	GA_1	GA_3	GA_4	GA_5	GA_7	GA_9	GA_{30}	GA_1Me	GA_3Me	GA_4Me	GA_7Me	GA_9Me
M + CH$_3$COO	11	–	4	–	2	–	–	–	–	–	–	–
M + HCO$_2$	100	30	100	45	100	100	40	100	5	13	–	–
M$^#$	–	33	–	–	37	–	63	–	60	100	37	–
M – H	97	44	62	100	63	95	43	–	100	2	100	–
M + HCO$_2$H$^#$	–	–	–	–	28	–	–	–	–	–	–	–
M + HCO$_2$ – 44	–	38	–	–	5	–	22	–	42	–	25	–
M + HCO$_2$ – 62	–	100	–	–	6	–	100	–	–	–	–	–
M – H – 44	–	10	–	–	–	–	16	–	–	–	–	–
M – H – 62	–	65	–	–	–	–	54	–	–	–	–	–

[a] – denotes that no signal was seen or the relative intensity of the signal was <1%.

[b] # denotes an ion radical probably formed by electron-capture processes.
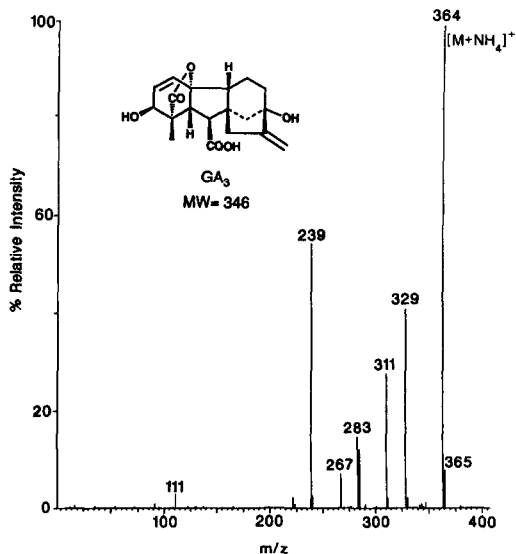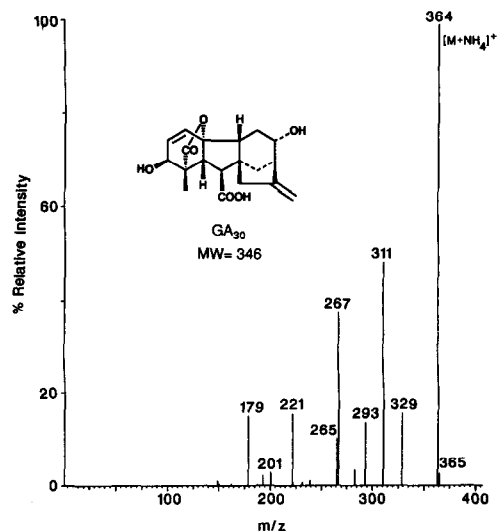
Fig. 7. Daughter ion spectrum obtained from collision activated dissociation of the $[M + NH_4]^+$ ion at m/z 364 of GA_3. HPLC system 1.
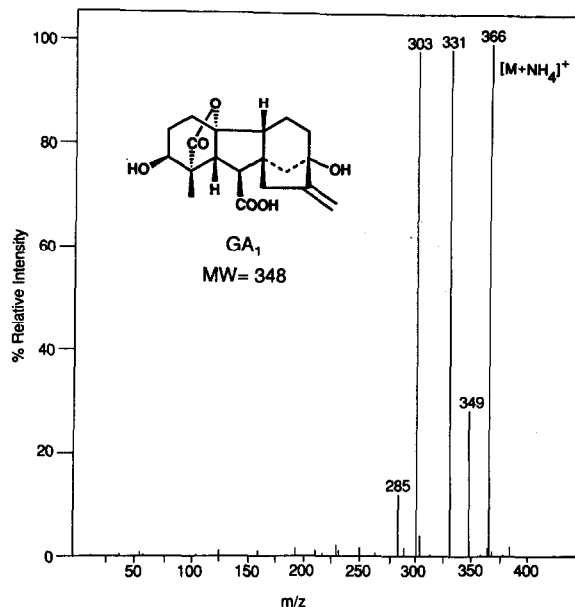

Fig. 9. Daughter ion spectrum obtained from collision activated dissociation of the $[M + NH_4]^+$ ion at m/z 366 of GA_1. HPLC system 1.

show the potential of tandem MS to differentiate these isomers without chromatographic separation. Comparison of the CAD daughter ion mass spectrum of GA_3 with that of GA_{30}, shows that the

Fig. 8. Daughter ion spectrum obtained from collision activated dissociation of the $[M + NH_4]^+$ ion at m/z 364 of GA_{30}. HPLC system 1.

principal difference is the relative intensities of the daughter ions at m/z 239, m/z 265 and m/z 293. In the CAD mass spectrum GA_3, m/z 239 is a prominent daughter ion, while daughter ions at m/z 265 and m/z 293 are not observed. In the CAD mass spectrum for GA_{30}, m/z 239 is a minor daughter ion, while daughter ions at m/z 265 and m/z 293 are readily observed. The differences seen in the daugther ion spectra could be explained by different fragmentation pathways involving the loss of a water molecule which is observed for GA_{30} but not for GA_3. The importance of these CAD data is that it demonstrates that TSP-MS–MS can be used to differentiate these two isomers under HPLC conditions where they co-elute and have indistinguishable thermospray mass spectra.

Because GA_1 co-eluted with GA_3 and GA_{30} when analysed using HPLC system 1 CAD daughter ion analysis was also performed on GA_1. Fig. 9 shows the CAD daughter ion mass spectrum of m/z 366 from GA_1. This CAD daughter ion mass spectrum showed daughter ions at m/z 349, m/z 331, m/z 303, and m/z 285. These ions can be attributed to the loss of NH_3, followed by losses of H_2O, CO and

H_2O. Thus both the thermospray mass spectrum and the TSP-MS–MS CAD daughter ion mass spectrum of GA_1 can be used to distinguish it from either GA_3 or GA_{30}.

CONCLUSIONS

This study demonstrates TSP-MS, HPLC–TSP-MS and tandem MS to be useful for the analysis and characterization of gibberellin compounds that have one carboxylic acid group as well as their methyl ester derivatives. Elimination of the need for derivatization is one advantage of HPLC–TSP-MS over GC–MS analysis, however, the enhanced thermospray sensitivity observed with the methylated GAs suggest methylation may be advantageous for the HPLC–TSP-MS analysis of low-level GAs in biological samples. These techniques may also be applicable for the analysis of other gibberellin compounds including those containing two or more acid groups and their conjugates.

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