Characterization of abscisic acid and metabolites by combined liquid chromatography–mass spectrometry with ion-spray and plasma-spray ionization techniques☆

Lawrence R. Hogge

Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan S7N 0W9 (Canada)

Garth D. Abrams

Prairie Malt Ltd., P.O. Box 1150, Biggar, Saskatchewan S0K 0M0 (Canada)

Suzanne R. Abrams

Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan S7N 0W9 (Canada)

Pierre Thibault

Marine Biosciences Institute, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1 (Canada)

Steven Pleasance

Sciex Division of MDS Health Group Ltd., 55 Glen Cameron Road, Thornhill, Ontario L3T 1P2 (Canada)

(First received May 5th, 1992; revised manuscript received June 16th, 1992)

ABSTRACT

Liquid chromatographic–mass spectrometric methods have been developed for the characterization of abscisic acid (ABA) and metabolites in plant cell cultures using plasma-spray and ion-spray ionization techniques. Both techniques provide useful mass spectral data for ABA and its acidic metabolites. The most intense ions in these spectra are represented by $[M + H]^+$ and $[M + H - H_2O]^+$. The limit of detection for ABA, using plasma-spray and ion-spray was 5 and 9 ng, respectively, for full scans and 0.5 and 1 ng, respectively, for selected-ion recording of m/z 265, the protonated molecular ion. Ion-spray also provided intense protonated molecular ions for the neutral ABA metabolites whereas plasma-spray did not. Spectra acquired using both techniques showed intense ions indicating the presence of the ABA moiety in these neutral conjugates.

Correspondence to: Lawrence R. Hogge, Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan S7N 0W9, Canada.
☆ NRC Publication No. 33 536.

INTRODUCTION

The plant hormone abscisic acid (ABA, **1**) (see Fig. 1) is implicated in regulating many developmental processes in higher plants and in triggering
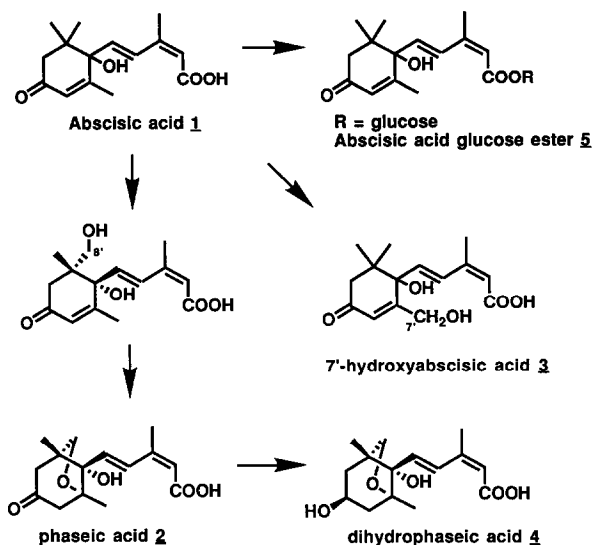
Fig. 1. Structures of ABA and ABA metabolites (products of oxidation of ring methyl groups and/or conjugation with sugar molecules).

plant responses to environmental stresses [1]. Numerous analytical methods have been developed to measure levels of endogenous ABA found in plant tissues, and newer physico-chemicals methods have been recently reviewed by Parry and Horgan [2]. Gas chromatography–mass spectrometry (GC–MS) of the methyl ester derivative with selected ion monitoring (SIM) and the use of deuterated internal standards has proved to be a powerful method for quantifying ABA [2]. Immunochemical methods, verified by GC–MS, have become popular with plant physiologists studying ABA responses in plants [3].

The currently known principal metabolites of ABA in plants are products of oxidation of ring methyl groups and/or conjugation to sugar molecules (Fig. 1). Of the acidic metabolites, phaseic acid (PA, 2), like ABA, is known to be active in inhibiting barley embryo germination [4] while the roles of other metabolites, 7′-hydroxyabscisic acid (7′-OH-ABA, 3) [5] and dihydrophaseic acid (DPA, 4) have been less well studied [1,6].

As the role of these metabolites in the developmental process of plants is of interest to an increasing number of plant physiologists, there is a need for analytical methods providing information on the entire ABA metabolic profile within a single ex-

periment. Direct analysis of crude plant extracts has been accomplished using desorption chemical ionization (DCI) and secondary-ion MS in conjunction with linked scanning at constant B/E, where B is the magnetic field strength and E is the electrostatic sector voltage [7]. The detection limit for these compounds using DCI is very low but linked scanning is very difficult to perform because of the short-lived nature of the ion current [7]. Secondary-ion MS or fast atom bombardment (FAB) is more amenable to linked-scanning MS–MS because this technique does possess a much longer ion current life time. Unfortunately, detection limits for secondary-ion MS or FAB are much higher than for DCI, often requiring more than a microgram of analyte [8].

High-performance liquid chromatography (HPLC) has been most valuable for the separation and quantitation of ABA and ABA metabolites [2,9]. This technique provides the advantage of analyzing directly the ABA-related compounds without derivatization and therefore fewer manipulations. This is an important considerization, as many ABA metabolites (including ABA conjugates) are prone to decomposition or isomerization [10,11].

However, there is a need for a better method of detection and characterization of the HPLC eluates. Until now, detection of compounds has been either by UV or, in cases in which labelled ABA has been supplied to plant material, by radioactivity detection. The combination of HPLC to separate compounds of interest, coupled with MS for structure identification, has not yet been reported for analysis of ABA and metabolites [2].

We have undertaken to develop HPLC–MS for our studies on metabolism of supplied ABA and related compounds by plant cell cultures. Our current studies have involved two different ionization techniques, plasma-spray and ion-spray, on instruments fitted with HPLC–MS interfaces. We have determined the sensitivities of both ionization techniques for the detection of ABA as well as the feasibility of characterizing both acidic and neutral metabolites of ABA using these methods of analysis. We now report the results of our investigations.

EXPERIMENTAL

ABA and ABA metabolite mixtures
Racemic ABA was purchased from Sigma

(A-2784). A stock solution containing 0.05 μg/ml ABA in methanol was prepared for the purpose of determining the detection limit and quantitative response for each mode of ionization.

Mixtures of unconsumed ABA and its acidic metabolites were obtained by ethyl acetate extraction of acidified filtrates from bromegrass cell suspension cultures fed racemic ABA, followed by back extraction of the acidic substances into aqueous $NaHCO_3$, as previously described [12]. Neutral metabolites were obtained from the ethyl acetate solution by washing with saturated aqueous NaCl, drying with Na_2SO_4, filtration, and evaporation of the solvent from the filtrate under reduced pressure.

Mass spectrometry

Plasma-spray LC–MS. LC separation was performed using a Waters system consisting of two pumps, Models 590 and 510, controlled by a Model 680 gradient controller. Sample injections of 20 μl were made on a Vydac 218TP54 C_{18} column (150 mm × 4 mm I.D.) using a linear gradient elution [10–40% acetonitrile in aqueous 0.1% trifluoroacetic acid (TFA) over 20 min] at a flow-rate of 0.8 ml/min.

The LC system was interfaced to a VG Analytical 70-250 SEQ hybrid mass spectrometer equipped for plasma-spray analysis. The probe temperature was 220°C and the source temperature 250°C Spectra were obtained by scanning from m/z 500 to 100 (resolution of 1000) at a rate of 3 s per decade. SIM experiments were performed by monitoring m/z 265.1442 using a dwell time of 80 ms and a settling time of 20 ms at a mass resolution of 4000.

Ion-spray LC–MS. LC separation was performed using a Hewlett-Packard 1090 high-pressure liquid chromatograph equipped with a diode-array detector. Sample injections of 5 μl were made on a Vydac 218TP52 C_{18} column (150 mm × 2 mm I.D.) using the gradient elution mentioned above at a flow rate of 0.2 ml/min.

The LC system was interfaced to a Sciex API/111 triple quadrupole mass spectrometer equipped with an ion-spray source. Spectra were obtained by scanning from either m/z 100 to 300 in 3 s (ABA full-scan calibration curve) or m/z 100 to 500 in 3 s (acidic and neutral extracts). For SIM experiments, m/z 265 was monitored using a dwell time of 200 ms.

RESULTS AND DISCUSSION

Detection of ABA by HPLC–MS

Racemic ABA was used to assess the usefulness of both plasma-spray and ion-spray for the characterization of natural ABA and its metabolites in plant cell cultures. Fig. 2A and B shows representative spectra obtained for ABA (approximately 200 ng sample size) using plasma-spray and ion-spray, respectively. These spectra are very similar with intense ions at m/z 265 (the protonated molecular ion) and the base ion at m/z 247 (the loss of water from the protonated molecular ion). However, the plasma-spray spectrum contains ions at m/z 306 and 288 that are not present in the ion-spray spectrum. These are adduct ions due to the addition of acetonitrile (from the mobile phase) to the intense ions mentioned above (m/z 265 and 247). On the other hand, the ion-spray spectrum contains an adduct ion at m/z 282 which is not present in the plasma-spray spectrum. The nature of this ion is not yet fully understood, but appears to be equivalent to the addition of an NH_4^+ ion which could be formed during the ion evaporation process either from trace amounts of ammonia in the mobile phase or in the air. Similar observations have been noted in the analysis of other compounds containing carboxylic acid moieties such as okadaic acid [13].

Limit of detection for ABA

The limit of detection for ABA using both ionization techniques (plasma-spray and ion-spray) was determined by analyzing known amounts under isocratic LC–MS conditions. The results obtained for full scan acquisitions are shown in Fig. 3, the plot of peak heights (arbitrary units) for the ABA protonated molecular ion (m/z 265) *versus* amount of ABA injected for the plasma-spray and ion-spray LC–MS systems. For plasma-spray the limit of detection was 5 ng (signal-to-noise ratio of approximately 8) with a linear response that ranged to 1000 ng while with ion-spray the limit of detection was 9 ng with a similar range of linear response.

Fig. 2C and D show the respective mass spectrum obtained from plasma-spray and Ion spray at the lower detection limit. The expected major ions at m/z 265 ([MH]$^+$) and m/z 247 ([MH − H_2O]$^+$) are present in both spectra. However, it should be
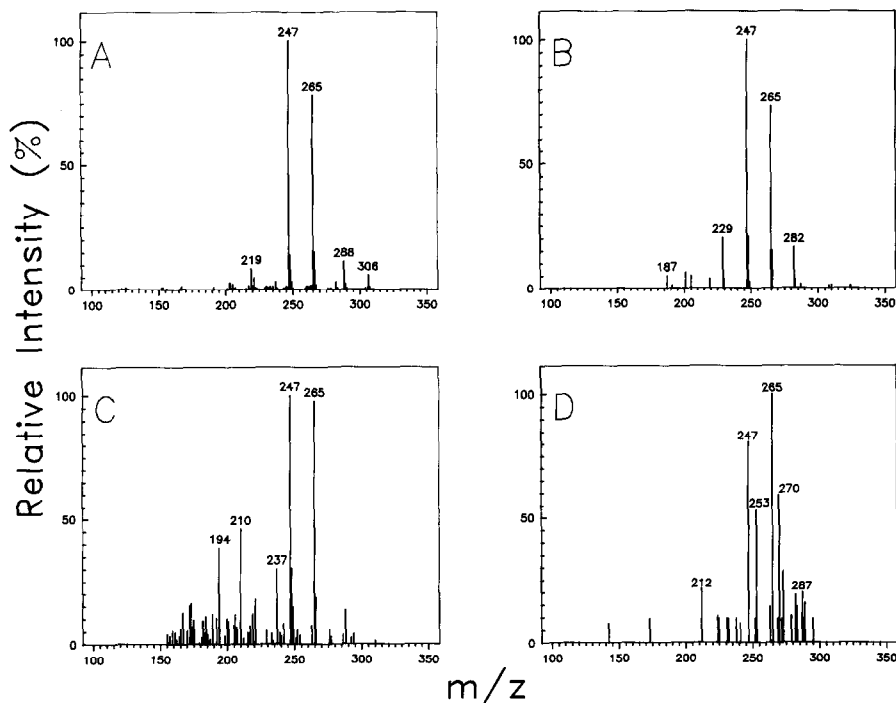
Fig. 2. Plasma-spray (A) and ion-spray (B) mass spectra of approximately 200 ng of ABA. (C) Plasma-spray limit of detection mass spectrum for ABA (5 ng) and (D) ion-spray limit of detection mass spectrum for ABA (9 ng).

noted that not all the background ions could be subtracted in these spectra (see Fig. 2A and B).

Detection limits were also determined using the SIM mode. Fig. 4 shows the calibration curve for both techniques, a plot of peak heights of the protonated molecular ion (m/z 265) versus the amount of ABA injected. Ion-spray SIM experiments were carried out at unit mass resolution. However, because the plasma-spray was installed on a double-focusing magnetic system, which has the advantage of operating with higher mass resolution and therefore providing a greater degree of specificity, a mass resolution of 4000 was used for these experiments. As shown, the limit of detection was 500 pg for plasma-spray and 1 ng for ion-spray (signal-to-noise ratio of 5). The linear response for the amount of ABA injected was similar to that obtained with the full scan experiments.

It should be noted that although plasma-spray appears to have slightly lower detection levels under these experimental conditions, the results should not be seen as proof that one of the above techniques is more sensitive than the other as the chro-

matographic conditions used were quite different. For example, the different injection volumes, column sizes and flow rates used would produce differ-
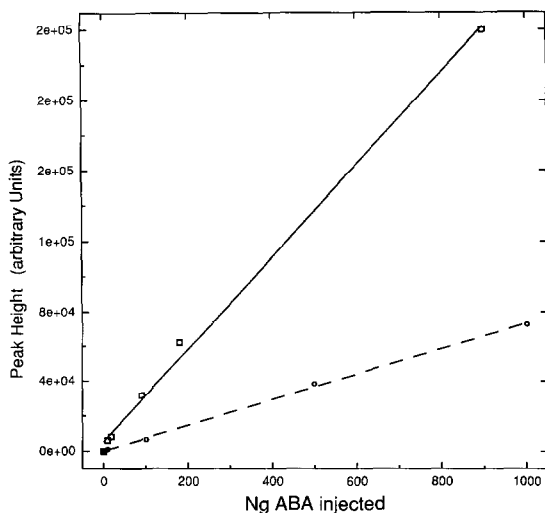

Fig. 3. Plot of peak heights for the ABA protonated molecular ion (full-scan acquisitions) versus amounts (ng) of ABA injected on the LC column. □ = Ion-spray; ○ = plasma-spray.
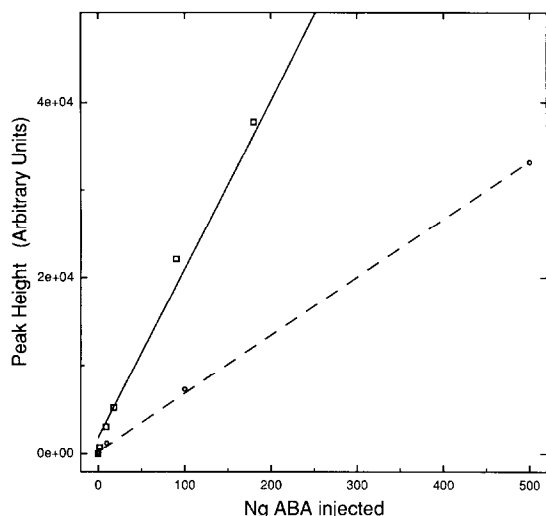
Fig. 4. Plot of peak heights for the ABA protonated molecular ion (selected ion recording) *versus* amount (ng) of ABA injected on the LC column. $\square$ = Ion-spray; $\bigcirc$ = plasma-spray.

ent concentration levels of the analyte even though the absolute amounts injected were the same. As plasma-spray and ion-spray have different flow constraints, direct comparisons of concentration levels are difficult. However, the results do show the practical limits of detection for each of the techniques using experimental conditions that are typical for that particular interface.

LC–MS of acidic metabolites isolated from plant cell cultures fed racemic ABA

Plasma-spray and ion-spray LC–MS was used to confirm the identity of the major ABA metabolites produced in the ABA-fed bromegrass tissue cultures and to determine the usefulness of these techniques for the characterization of other ABA metabolites or ABA analogue metabolites for future plant tissue culture feeding experiments. In previous work in this laboratory, the major ABA metabolites as well as ABA in this culture extract were identified as DPA (**4**), PA (**2**), 7'-OH-ABA (**3**) and ABA (**1**) [5] (see Fig. 1). Fig. 5 shows the LC chromatogram obtained with UV detection as well as the limited ion chromatograms obtained from plasma-spray and ion-spray LC–MS with the major components identified in each chromatogram. The ion chromatograms show similar profiles to the UV

trace. Although the ion-spray chromatogram appears more attractive from a chromatographic point of view, it should be noted that the amount of sample injected was over three times that injected in the plasma-spray experiment.

Mass spectral data (plasma-spray and ion-spray) for the four major components are summarized in Table I, a listing of the masses and intensities for the diagnostically important ions along with their proposed assignments. These data show that the four major acidic components have very intense ions similar to those of ABA. They include the protonated molecular ion, solvent adduct ions, and ions due to the loss of water for each of the components. It should be noted that the protonated molecular ion for DPA is of low intensity (less than 1%) in the ion-spray spectrum. However, the spectrum does exhibit an adduct ion ($[M + NH_4]^+$), confirming the molecular mass. Furthermore, the most intense ion in the spectrum corresponds to the loss of water from DPA which contains both a secondary and tertiary hydroxyl group. In contrast, the plasma-spray-generated protonated molecular ion for PA has a much lower intensity than the protonated molecular ion obtained from the ion-spray analysis (12% *versus to* 74%).

PA and 7'-OH-ABA are isomeric and give similar fragmentation patterns in both ionization modes. Although the intensities of the protonated molecular ions of the specific isomers (relative to the base peak) do vary significantly, particularly in the plasma-spray spectra, it is more reliable to distinguish the isomers using their specific chromatographic elution times.

The plasma-spray spectra for ABA and 7'-OH-ABA and the ion-spray spectrum of ABA also contain an ion at m/z 219 with intensities of 8.5%, 52.5% and 3.9%, respectively. This ion may be the result of the loss of formic acid from the protonated ABA molecular ion or in the case of 7'-OH-ABA, the loss of water and carbon dioxide. The ion-spray spectrum of 7'-OH-ABA also contains an intense ion at m/z 233 (56.6%) not observed in the plasma-spray spectrum. This ion is likely formed as a result of the loss of neutral molecules of methanal and water from the protonated molecular ion.
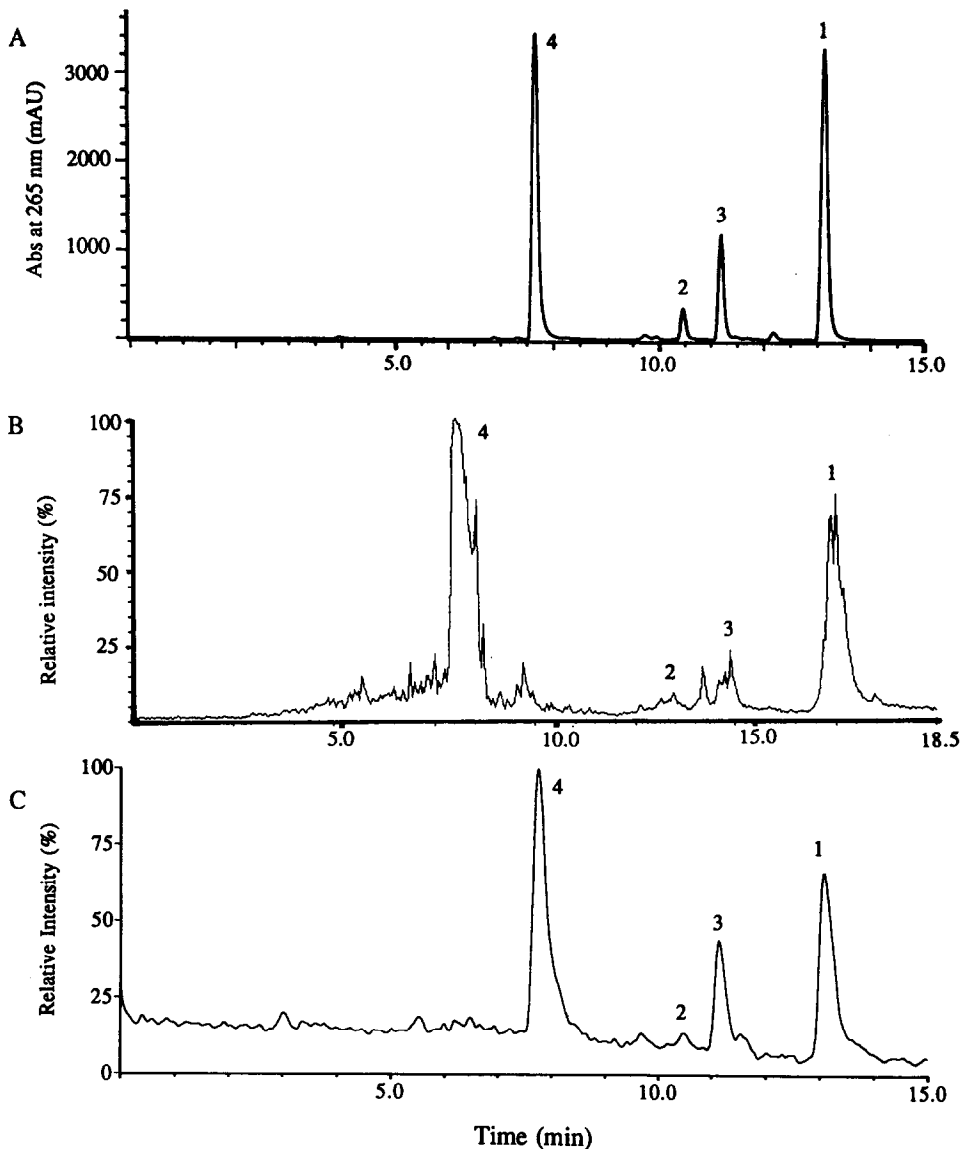
Fig. 5. Analysis of the acidic fraction of ABA-treated bromegrass plant cell extract. (A) LC–UV chromatogram, 5 μl injection; (B) LC–MS with plasma-spray, 3 μg injection, reconstructed ion chronmatogram (RIC) for m/z 250–430; (C) LC–MS with ion-spray, 10 μg injection, RIC for m/z 250–430. Separation conditions: (A, C) Vydac 218TP52 HPLC column, flow-rate 0.20 ml/min, linear gradient from 10 to 40% acctonitrile in aqueous 0.1% TFA in 20 min; (B) Vydac 218TP54 HPLC column, flow-rate 0.80 ml/min linear gradient as in A and C, peaks: **4** = DPA; **2** = PA; **3** = 7'-OH-ABA; **1** = ABA.

LC–MS of neutral metabolites isolated from plant cell cultures fed ABA

The analysis of the neutral metabolites showed different results for the two ionization techniques. Fig. 6 shows the LC chromatogram obtained with UV detection as well as the limited ion chromatograms obtained from the plasma-spray and ion-spray LC–MS analyses. These chromatograms indicate there is one major neutral metabolite. This metabolite was identified as the glucose ester of

TABLE I

DIAGNOSTIC IONS FROM PLASMA-SPRAY AND ION-SPRAY ANALYSIS OF ACIDIC ABA METABOLITES

Component	Proposed structure (percent relative intensity)							
	Plasma-spray				Ion-spray			
	$[MH]^+$	$[MH + CH_3CN]^+$	$[MH - H_2O]^+$	$[MH - H_2O + CH_3CN]^+$	$[MH]^+$	$[M + NH_4]^+$	$[MH - H_2*]^+$	$[MH - 2H_2O]^+$
DPA	283 (4.89)	324 (8.06)	265 (100)	306 (0.97)	283 (<0.5)	300 (8.44)	265 (100)	247 (8.00)
PA	281 (11.6)	322 (1.48)	263 (100)	304 (5.64)	281 (74.1)	298 (85.4)	263 (100)	245 (0.0)
7'-OH-ABA	281 (44.1)	322 (0.0)	263 (100)	304 (3.34)	281 (55.6)	298 (35.9)	263 (100)	245 (33.8)
ABA	265 (78.3)	306 (5.81)	247 (100)	288 (11.3)	265 (74.5)	282 (17.4)	247 (100)	229 (19.9)

TABLE II

DIAGNOSTIC IONS FOR PLASMA-SPRAY AND ION-SPRAY LC–MS ANALYSIS OF NEUTRAL ABA METABOLITE

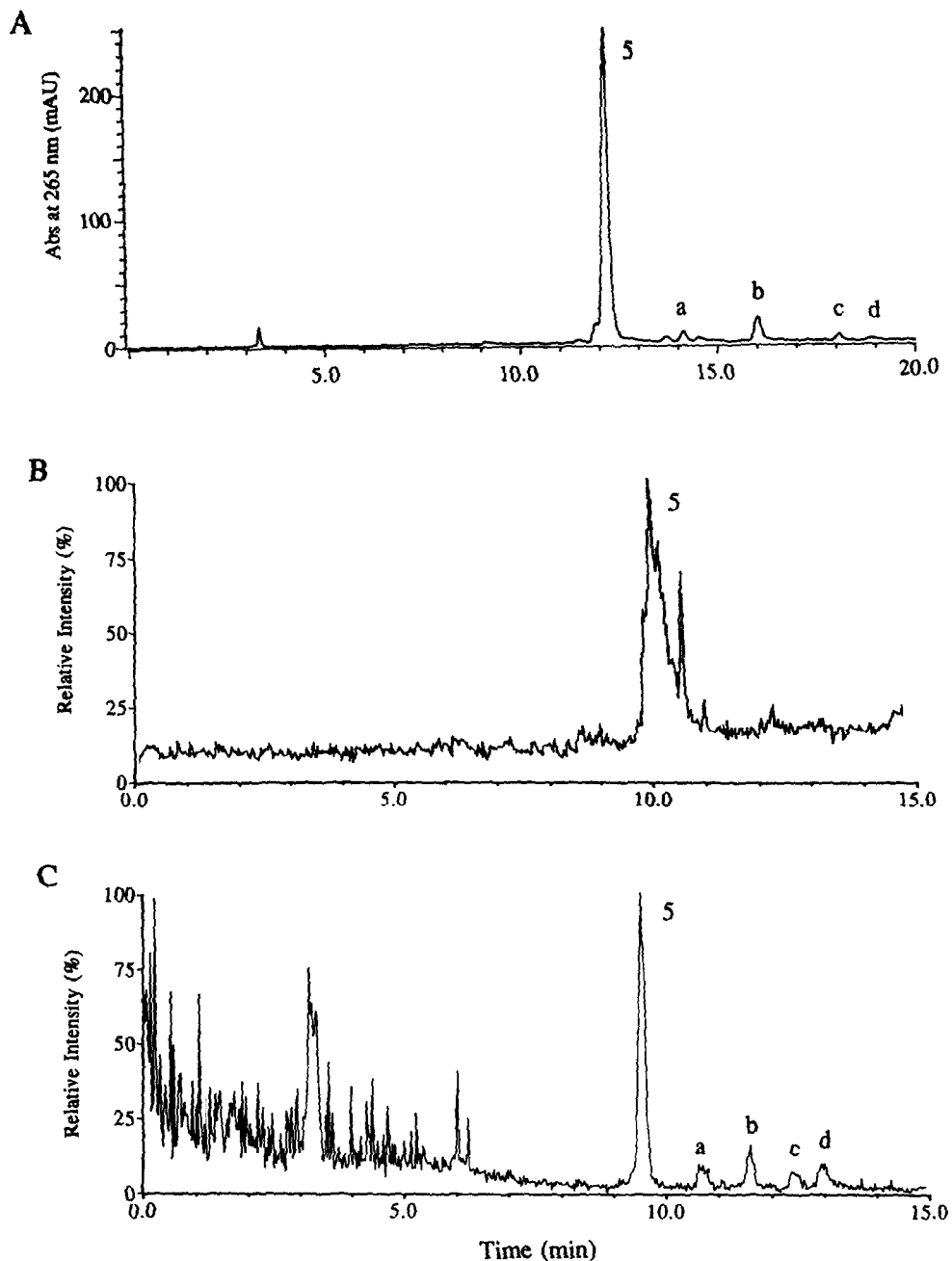| Component	Proposed structure (percent relative intensity)							
	Plasma-spray				Ion-spray			
	$[MH]^+$	$[MH + CH_3CN]^+$	$[MH - glycone]^+$	$[MH - glycone - H_2O]^+$	$[MH]^+$	$[M + NH_4]^+$	$[MH - glycone]^+$	$[MH - glycone - H_2O]^+$
ABA glucose ester	427 (0.0)	468 (0.0)	265 (63.8)	247 (100)	427 (99.4)	444 (18.3)	265 (100)	247 (41.2)

Fig. 6. Analysis of the neutral fraction of ABA-treated bromegrass plant cell extract. (A) LC–UV chromatogram, 1 μg injection; (B) LC–MS with plasma-spray, 0.5 μg injection, RIC for m/z 200–450; (C) LC–MS with ion-spray, 1 μg injection, RIC for m/z 200–450. Separation conditions: (A, C) Vydac 218TP52 HPLC column, flow-rate 0.20 ml/min, linear gradient from 10 to 35% acetonitrile in aqueous 0.1% TFA in 20 min; (B) Vydac 218TP54 HPLC column, flow-rate 0.80 ml/min, linear gradient as in A and C. Peaks: **5** = ABA glucose ester; a, b, c and d = ABA conjugates.

ABA by comparing its LC elution time, UV spectrum and mass spectrum to an authentic standard of ABA glucose ester (**5**) synthesized in this laboratory. Details of the confirmation of the identity of this glucose ester which is known to exist in plants [10] will be given in a future publication. As with the acidic fraction (Fig. 5), the ion-spray chromatogram appears chromatographically more appealing. This may be in part due to the amount of sample injected (1 μg *versus* 0.5 μg in the plasma-spray experiment). However, there are more significant differences in the mass spectral data. Table II lists the diagnostically important ions produced from each ionization technique for the glucose ester. There are no protonated or other adduct ions observed in the plasma-spray analysis while very intense protonated molecular ions (99%) as well as $[M + NH_4]^+$ adduct ions are observed in the ion-spray analysis.

In addition, the ion-spray analysis indicates that other neutral ABA conjugates are present in minor amounts (see Fig. 6C, components a, b, c and d). Although the structures of these minor components have not yet been determined, the mass spectral data suggest that all contain an intact ABA moiety and two (components a and b) likely have a molecular mass of 410 daltons, 16 units less than the ABA glucose ester.

CONCLUSION

LC–MS using either plasma-spray or ion-spray is a practical method for the separation and characterization of ABA and ABA metabolites extracted from plant tissue cultures. Information obtained from the elution times and mass spectral data allow for qualitative analysis of these components. Calibration curves done with full scan acquisition and selected ion recording showed a linear response for up to 1000 ng of ABA injected on the column. The preparation and use of deuterated internal standards should therefore make quantitative analysis possible. With the LC–MS instrumentation and chromatographic conditions used in this study, detection levels were slightly lower for ABA and its acidic metabolites using plasma-spray, while ion-spray provided more useful mass spectral data (intense protonated molecular ions) for the ABA glucose ester and other neutral ABA conjugates.

REFERENCES

1 J. A. A Zeevaart and R. A. Creelman, *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 39 (1988) 439.
2 A. D. Parry and R. Horgan, in W. J. Davis and H. G. Jones (Editors), *Abscisic Acid Physiology and Biochemistry*, Bios Scientific, Cambridge, 1991, p. 5.
3 M. K. Walker-Simmons and S. R. Abrams, in W. J. Davis and H. G. Jones (Editors), *Abscisic Acid Physiology and Biochemistry*, Bios Scientific, Cambridge, 1991, p. 53.
4 R. D. Hill, D. Durnin, L. A. K. Nelson, G.D. Abrams, L. V. Gusta and S. R. Abrams, *Plant Physiol.*, in press.
5 C. R. Hampson, M. J. T. Reaney, G. D. Abrams, S. R. Abrams and L. V. Gusta, *Phytochemistry*, 31 (1992) 2645.
6 L. A. K. Nelson, A. C. Shaw and S. R. Abrams, *Tetrahedron*, 47 (1991) 3259.
7 N. Takeda, K. Harada, M. Suzuki, A. Tatematsu, N. Hirai and K. Koshimizu, *Agric. Biol. Chem.*, 51 (1987) 2351.
8 J.R. Chapman, in *Practical Organic Mass Spectrometry*, John Wiley, Chichester, pp.122–129.
9 R. A. Creelman and J. A. D Zeevaart, in H. F. Linskens and J. F. Jackson (Editors), *Modern Methods of Plant Analysis*, Vol. 5, Springer-Verlag, Berlin, 1987 pp. 39–51.
10 B. V. Millborrow and R. Mallaby, *J. Exp. Bot.*, 26 (1975) 741.
11 S. J. Neill, R. Horgan and J. K. Heald, *Planta*, 157 (1983) 371.
12 S. R. Abrams, M. J. T. Reaney, G. D. Abrams, T. Mazurek, A. C. Shaw and L. V. Gusta, *Phytochemistry*, (1989) 2885.
13 P. Thibault, S. Pleasance and P. G. Sim, unpublished results.