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Electrospray and APCI analysis of polyhydroxyalkaloids using positive and negative collision induced dissociation experiments in a quadrupole ion trap†

Martin J. Egan,[ab] Geoffrey C. Kite,[a] Elaine A. Porter,[a] Monique S. J. Simmonds[ab] and Siân Howells[b]

[a] *Biological Interactions, Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey, UK TW9 3AB*
[b] *Chemical and Life Sciences, University of Greenwich, Wellington St., Woolwich, London, UK SE18 6PF*

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Atmospheric pressure chemical ionisation (APCI) and electrospray (ES) are compared as ion sources in the analysis of polyhydroxyalkaloids (PHAs) by liquid chromatography mass spectrometry (LC-MS) and collision induced dissociation (CID) product ion spectra, from tandem mass spectrometry (MS-MS) experiments in a quadrupole ion trap, are reported for 12 naturally occurring PHAs. APCI was found to be a more useful source than ES, as APCI could be used to generate deprotonated molecule ions in negative mode and for some isomeric PHAs the negative CID product ion spectra were more diagnostic than the positive product ion spectra. On-column detection limits were also approximately 32 times lower by positive APCI than ES. The work provides data that will facilitate screening and characterisation of this group of important natural products in plant and fungal extracts.

Introduction

Naturally-occurring polyhydroxyated piperidine, pyrrolidine, indolizidine, pyrrolizidine and *nor*-tropane alkaloids (polyhydroxyalkaloids, PHAs) are of pharmaceutical and agrochemical interest due to their ability to inhibit glycosidases[1] and interfere with sugar receptors.[2] Their lack of a chromophore and hydrophilic nature caused them to evade discovery in plants until 1974.[3] Since then 68 PHAs have been isolated from natural sources and there is interest in improving methods of analysis to search for new types and investigate their taxonomic distribution in plant families.[4]

Initially, analyses of PHA mixtures were carried out by high voltage paper electrophoresis and thin layer chromatography (TLC) followed by ninhydrin staining.[5] The compounds fail to react (except at high concentrations) with Dragendorff's, iodoplatinate or other reagents traditionally used to detect alkaloids,[6] but stains such as the pyrrole-specific Ehrlich's reagent,[7] sodium prusside–sodium carbonate–acetaldehyde,[8] and chlorine–*o*-tolidine[9] have been used to detect specific PHA-subclasses. Gas chromatography mass spectrometry (GC-MS) of trimethylsilyl (TMS) ethers of PHAs has resulted in more detailed analyses[10,11] but the results obtained for PHA-monoglycosides can be unsatisfactory due to incomplete derivatisation and the TMS ethers of PHA-diglycosides are too involatile for GC.[12] Although isolated PHAs have been analysed by MS, using electron impact[13] (EI), chemical ionisation[14] (CI) or fast atom bombardment[15] (FAB) ionisation, analysis of PHA mixtures by liquid chromatography mass spectrometry (LC-MS) has received little attention in the literature, mainly due to a lack of suitable LC methods.[16] It was not until 1990 that preliminary LC-MS analyses of five PHAs in semi-purified fractions of the Morton Bay chestnut (*Castanospermum australe*) was achieved using thermospray MS,[17] but the method was not taken up as a routine technique. However, with the advent of reliable commercial LC-MS instruments, particularly the quadrupole ion trap with multi-stage MS (MS^n) capability,[18] there is now new scope for the analysis of PHAs by LC-MS. It should be noted that whilst MS^n in a quadrupole ion trap is a form of tandem MS, the experiments are tandem in time not in space, as in traditional tandem experiments.[19,20] The practical aspects of ion trap MS have been the subjects of a number of reviews.[21–25]

Direct analysis of crudely purified plant extracts by atmospheric pressure chemical ionisation (APCI) MS followed by MS^n experiments in a quadrupole ion trap has been employed as a rapid means of detecting and partially characterising PHAs in plant extracts.[26] Subsequently, more detailed analysis has been achieved with the development of MS-compatible liquid chromatography (LC) methods for separating the PHAs in an extract of bluebell (*Hyacinthoides non-scripta*).[12] It was concluded that LC-APCI-MS^n complemented GC-MS in PHA analysis since it produced unambiguous molecular weight data and also better detection and characterisation of PHA-glycosides by providing information on the types of component aglycone and sugar residues.

The main challenge in the analysis of PHAs is to discriminate between the numerous isomeric and epimeric forms. Published LC separation methods for these very polar and charged molecules remain unsatisfactory.[16] Thus there is the need to investigate whether certain MS-MS experiments, for example, collision induced dissociation (CID), to provide product ion spectra, can be used to achieve identifications in PHA mixtures that are not completely resolved chromatographically. Reproducibility of CID product ion spectra will be particularly critical in this respect and recently it was suggested that electrospray (ES) shows better long-term reproducibility of product ion spectra than APCI.[27] Thus, in the present paper we investigate and compare the applicability of APCI with that of ES for LC-MS analysis of PHAs and assess whether CID product ion spectra obtained for a variety of PHAs using different MS

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techniques permits the discrimination of isomeric forms. The two types of CID permitted on a quadrupole ion trap instrument are non-mass selected CID to the total ion beam post source ('source CID') and mass selected CID in the trap.

Experimental

PHAs

The following PHAs (Fig. 1) were analysed as pure or semi-pure isolates from plants grown at the Royal Botanic Gardens, Kew: 2*R*,5*R*-bis(hydroxymethyl)-3*R*,4*R*-dihydroxypyrrolidine (DMDP), 1-deoxynojirimycin (DNJ), 1-deoxymannojirimycin (DMJ), α-homonojirimycin (HNJ), 3,5-di-*epi*-α-homonojirimycin (di-*epi*-HNJ), 2,5-dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol (homo-DMDP), 1,4-dideoxy-1,4-imino-D-arabitol (DAB-1), 2*R*-hydroxymethyl-3*S*-hydroxypyrrolidine (CYB-3), casuarine, swainsonine, castanospermine and alexine. Acetonitrile (HPLC grade) was purchased from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK) and analytical grade water was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Liquid chromatography

Pure material for MS experiments was provided by on-line separation using normal phase high performance liquid chromatography (Waters 600 pumps with 600E system controller: Waters, Milford, MA, USA). Filled 20 μl loop (Rheodyne) injections of a 0.01 mg ml^{-1} PHA solution in 90:10 acetonitrile–water were made onto a 150 mm × 4.6 mm, 7 μm Adsorbosphere XL Carbohydrate AX column (Alltech Associates, Carnforth, UK). Chromatography was achieved using a linear mobile phase gradient of 90:10–80:20 acetonitrile–water over 20 min at 1 ml min^{-1} (for ES) or 1.5 ml min^{-1} (for APCI). The limits of detection for the LC-MS method were obtained for castanospermine and DMDP by analysing 20 μl loop injections of a ×2 dilution series beginning at 125 ng ml^{-1}.

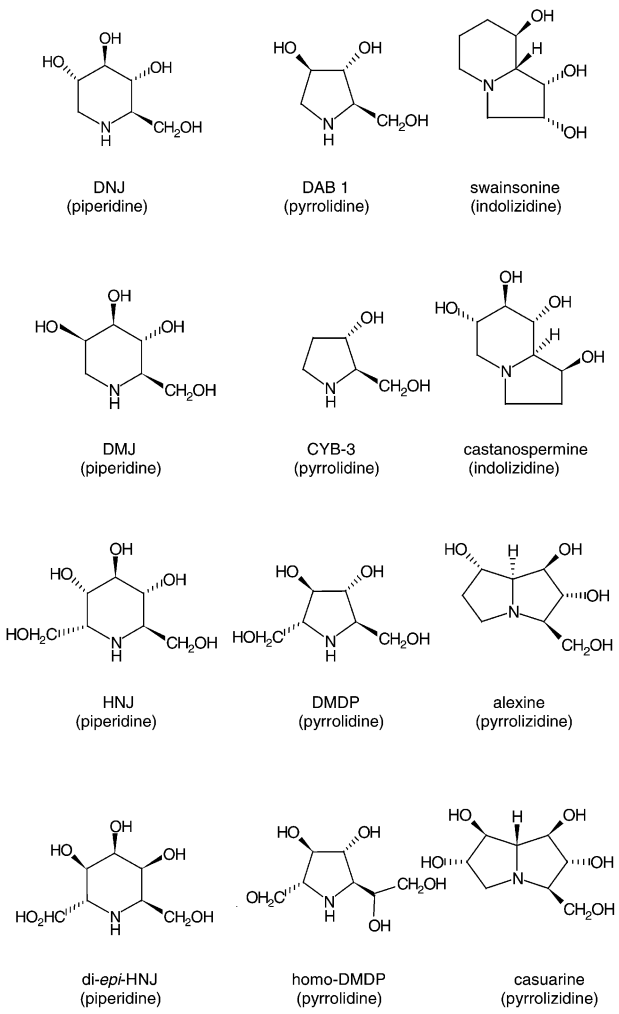

Fig. 1 Structures of polyhydroxyalkaloids.

Mass spectrometry

Mass spectra were acquired with a quadrupole ion trap instrument (Finnigan LCQ™: ThermoQuest-Finnigan MAT, San Jose, CA, USA) with ionisation by either APCI or ES. For APCI the vaporiser temperature was 450 °C, the needle discharge current was 5 μA and the heated capillary was maintained at 150 °C with a voltage of ±29 V. For ES the source spray voltage was ±4.2 kV and the heated capillary was maintained at 220 °C with a voltage of ±22 V. The nitrogen gas sheath and auxiliary flow pressures were 80 and 20 psi, respectively, for both sources; nitrogen was supplied at 100 psi by a nitrogen generator (Whatman, Maidstone, UK). Instrument calibration was carried out with a standard mixture of L-methionyl-arginyl-phenylalanyl-alanine acetate, caffeine (Sigma Chemical Co., St. Louis, MO, USA) and Ultramark 1621 (Lancaster Synthesis Inc., Windham, NH, USA). The mass spectrometer was tuned for the protonated DMDP ion at *m/z* 164 using the instrument software (Navigator 2.1) to optimise the voltages across the heated capillary and octapole lenses whilst DMDP was infused at 10 μl min^{-1} into a flow of 1 ml min^{-1} 50% aqueous methanol to the source.

Positive and negative ion spectra were recorded in separate LC-MS analyses. In positive mode the mass spectrometer was set to record, in three successive scans repeated throughout the analysis, the first order mass spectrum in the range *m/z* 110–1000, the CID product ion spectrum of the $[M + H]^+$ ion isolated in the trap (*i.e.*, an MS-MS experiment), and the spectrum from CID of the total ion beam post source in the octapole lenses (here referred to as source CID). The lower scan limit for first order spectra was restricted to *m/z* 110 due to the presence of background ions at *m/z* 98 and *m/z* 108 from column bleed. Relative collision energies for positive ion APCI spectra were 15% for the MS-MS experiment (0–100% corresponds to 0–5 V peak-to-peak of resonance excitation rf voltage) and 25% for dissociation undertaken post source (0.1–100% corresponds to 0–100 V dc of octapole offset voltage). For positive ion ES the relative collision energies were 18% for MS-MS and 30% for source CID. Negative ion spectra could only be obtained with APCI and only the first order mass spectrum (*m/z* 110–1000) and the CID product ion spectrum of the $[M - H]^-$ ion by MS-MS (relative collision energy 12%) were recorded as the deprotonated molecule was not sufficiently intense to undertake CID in the total ion beam. The ion isolation width in all MS-MS experiments was 3 Da. Throughout the analyses the number of ions present in the ion trap at any one time was regulated by the automatic gain control to 1.0×10^8 ions for first order and source CID experiments and 2.0×10^7 ions for MS-MS with a maximum ion accumulation time of 400 ms. Helium was used as the damping gas at a pressure of 1.2 Torr.

Results and discussion

Comparison of APCI and ES

For all the PHAs examined, positive APCI yielded a protonated molecule ion ($[M + H]^+$) as the most abundant ionisation

product (Table 1). Fragment ions were also produced but these did not exceed 10% relative abundance except for swainsonine in which an $[(M + H) - 18]^+$ ion was present at about 22% abundance. Therefore, APCI was suitable for determining the relative molecular masses of PHAs to within one 1 Da. For an LC-MS-MS analysis it was also suitable for performing automatic MS-MS of the $[M + H]^+$ ion by employing the data-dependent facility of the mass spectrometer, which selects the base ion for isolation and fragmentation. This is very useful when analysing samples with no prior knowledge of the PHAs they contain. The LC-APCI-MS chromatogram for the separation of swainsonine, castanospermine, DMJ, DNJ and HNJ is presented in Fig. 2.

ES did not yield a protonated molecule ion for DAB-1 and CYB-3 under the mobile phase conditions used and for the other PHAs it was less efficient at producing protonated molecule ions than APCI, making it less sensitive. In LC-MS analyses with APCI, peaks in the base ion chromatogram for DMDP and castanospermine could be detected at 625 pg with a signal-to-noise (S/N) ratio of 4 for each. Using extracted ion chromatograms for the $[M + H]^+$ ion allowed 78 pg DMDP (S/N 4) and 19 pg castanospermine (S/N 3) to be detected. For ES, 2.5 ng DMDP on column could only be detected in the extracted ion chromatogram while on column detection limits (S/N 3) for castanospermine were 2.5 ng (base ion chromatogram) and 625 pg (extracted ion chromatogram). Greater sensitivity may be obtained by ES using an acidic medium to promote protonation of the alkaloids, but acidified mobile phases were incompatible with the stationary phase of the chromatography column used, although post column infusion of acid could be employed.

The greater S/N ratio for the $[M + H]^+$ ion in the total ion beam produced by APCI compared with ES resulted in APCI source CID spectra having less interference from background ions than those obtained with ES, at the concentration of PHA analysed. The quality of product ion spectra from mass selected CID was independent of the ionisation process and ionisation efficiency did not affect the product ion spectra obtained with ES in MS-MS experiments due to prior parent ion isolation. The relative ion intensities in individual product ion spectra of DNJ obtained during compound elution from the HPLC revealed that spectra obtained following ES or APCI were similar (Table 2). Both spectra also showed similar variability, thus there does not appear to be any advantage in using ES in terms of spectral consistency.

The most useful advantage of APCI over ES, however, was that APCI generated deprotonated molecule ions ($[M - H]^-$) in negative ion mode for all the PHAs examined except DAB-1 and CYB-3, whereas ES did not do so for any of the PHAs. Although deprotonated molecule ions were produced at low efficiency, as would be expected for basic molecules, it was sufficient to allow negative product ion spectra to be recorded by MS-MS, but generally not by post source fragmentation. Automatic MS-MS could not be relied upon to produce the negative product ion spectrum of the deprotonated molecule ion since cluster ions (*e.g.* $[2M - H]^-$) and adducts, which were sometimes more intense than the $[M - H]^-$ ion, were also produced. Therefore, the *m/z* value of the precursor ion had to be specified manually. This has also been reported for hydroxypipecolic acids, a group of related compounds.[28] Although more reliable automatic MS-MS could be obtained by restricting the

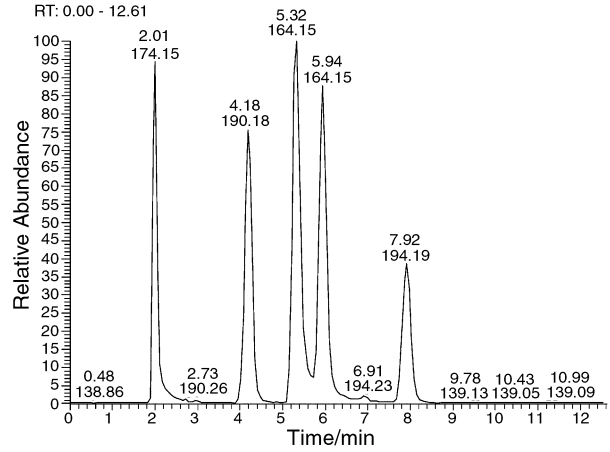

Fig. 2 LC-MS base ion chromatogram from the separation of swainsonine (2.01 min), castanospermine (4.18 min), DMJ (5.32 min), DNJ (5.94 min) and HNJ (7.92 min) on an Adsorbosphere XL Carbohydrate AX column with a gradient elution of 1.5 ml min^{-1} with 90 : 10–80 : 20 acetonitrile : water over 20 min using an APCI ion source. (Peaks are labeled with the retention times in min and the base ion.)

Table 2 Relative intensities of product ions from successive product ion spectra of DNJ obtained by CID of the $[M + H]^+$ ion (*m/z* 164) produced by either APCI or ES of the compound as it eluted from an HPLC column; ion isolation width 3 Da and relative collision energy 15% for both sources

Source	Product ion (*m/z*)	Relative intensity (mean ± SEM)
APCI	146	100.0 ± 0.00
	128	18.8 ± 0.66
	110	16.6 ± 0.33
	60	10.3 ± 0.61
	80	4.4 ± 0.91
ES	146	100.0 ± 0.00
	128	16.3 ± 1.95
	110	17.5 ± 2.07
	60	9.5 ± 1.01
	80	4.0 ± 0.43

Table 1 Retention times and full spectra mass ions for APCI and ES of PHAs. Retention times were recorded at a flow rate of 1.5 ml min^{-1} under the conditions stated in the Experimental section

		Mass spectrum, *m/z* (% base peak APCI/ES)			
Compound	Retention time/min	$[M + H]^+$	$[(M + H) - 18]^+$	$[(M + H) - 36]^+$	$[(M + H) - 54]^+$
CYB-3	Unretained	118 (100/–)			
DAB-1	Unretained	134 (100/–)	116 (1/–)		
Swainsonine	2.0	174 (100/100)	156 (22/15)	138 (6/–)	130 (1/–)
Castanospermine	4.2	190 (100/100)	172 (10/7)	154 (1/4)	
DMDP	5.1	164 (100/100)	146 (1/3)	128 (1/–)	110 (1/–)
DMJ	5.3	164 (100/100)	146 (2/9)	128 (0.6/–)	
DNJ	5.9	164 (100/100)	146 (7/7)	128 (2/–)	110 (2/–)
Alexine	5.9	190 (100/100)	172 (1/–)		
Homo-DMDP	6.9	194 (100/100)	176 (3/2)	158 (1/–)	
Di-*epi*-HNJ	7.0	194 (100/100)	176 (6/5)	158 (1/–)	140 (0.8/–)
HNJ	7.9	194 (100/100)	176 (7/10)	158 (1/4)	140 (1/–)
Casuarine	9.3	206 (100/100)	188 (1/–)		

upper limit of the scan range (to eliminate the detection of cluster ions), in practice negative product ion spectra were just as easily and more reliably obtained with manual programming of the instrument by specifying the *m/z* values expected, rather than recorded, for the $[M - H]^-$ ions of interest. With an 'unknown' sample, the expected $[M - H]^-$ *m/z* values for peaks of interest could be calculated from the results of prior analysis by positive APCI without the need to perform an extra analysis to obtain the first order negative ion spectra.

CID product ion spectra of PHAs

The positive product ion spectra of $[M + H]^+$ ions of PHAs, obtained by either MS-MS or source CID, were characterised by multiple losses of hydroxy groups, each as a water molecule (Table 3). Other product ions were difficult to assign although the fragments at $[(M + H) - 74]^+$ and $[(M + H) - 104]^+$ observed in some spectra could result from ring cleavage as indicated in Fig. 3. The pyrrolidines examined (DMDP and homo-DMDP) mostly yielded the same array of product ions as their isomeric piperidines (DMJ and DNJ, and HNJ and di-*epi*-HNJ, respectively) but could be distinguished from them by their different ion intensities. Source CID spectra were less useful in this respect as any ion intensity differences were less pronounced. The epimeric piperidines examined, however, could not be distinguished unambiguously from each other by their positive product ion spectra. The pyrrolizidines alexine and casuarine both showed a prominent $[(M + H) - 78]^+$ ion in their product ion spectra and this provided a ready means of distinguishing alexine from the isomeric indolizidine PHA castanospermine, which did not show this ion in its product ion spectrum. Again the source CID spectra were less useful for identification purposes as the $[(M + H) - 78]^+$ ion was generated in the source CID spectrum of castanospermine (Table 3).

The negative APCI product ion spectra of $[M - H]^-$ precursor ions did not show the sequential losses of water seen in the positive product ion spectra (Table 4). For most PHAs a single loss of a water molecule appeared to be associated with simultaneous loss of a hydrogen molecule (H_2) to yield a $[(M - H) - 20]^-$ product ion, although a $[(M - H) - 18]^-$ ion was sometimes also evident at lower abundance. For casuarine and swainsonine the $[(M - H) - 20]^-$ product ion was the base peak. For DMDP, DNJ, DMJ and alexine a $[(M - H) - 32]^-$

Table 3 Product ion spectra from CID of the $[M + H]^+$ ions of PHAs generated by either positive APCI or ES

			Relative intensity of the product ion showing the stated loss from the $[M + H]^+$ parent ion (*m/z* values for the product ion are given in bold italics above each group of isomeric compounds)														
Compound	MS experiment[a]		**0**	**−18**	**−36**	**−54**	**−62**	**−66**	**−72**	**−74**	**−78**	**−82**	**−84**	**−92**	**−95**	**−96**	**−104**	**−114**
		m/z	***118***	***100***	***82***													
CYB-3	APCI-MS²			100	28													
	APCI-source CID		100	52	79													
		m/z	***134***	***116***	***98***	***80***		***68***		***60***								
DAB-1	APCI-MS²			100	81	18		48		8								
	APCI-source CID		100	22	29	23		45		6								
		m/z	***164***	***146***	***128***	***110***		***98***		***90***		***82***	***80***		***69***	***68***	***60***	
DMJ	ES-MS²			100	6	6						0.5	2		6	1	6	
	APCI-MS²			100	7	7		0.2					0.7		1	1	5	
	APCI-source CID		100	81	22	43		6		2		33	40		23	16	17	
DNJ	ES-MS²			100	16	18						3	4		2	1	10	
	APCI-MS²			100	19	17		0.3		0.3		0.2	4		4	1	10	
	APCI-source CID		100	66	27	50		6		1		45	48		24	12	14	
DMDP	ES-MS²			100	65	72		14		8		11	83		3	2	15	
	APCI-MS²			100	57	63		15		9		14	72		1	7	15	
	APCI-source CID		100	15	14	21		3		4		19	50		4	15	15	
		m/z	***174***	***156***	***138***	***120***											***70***	
Swainsonine	ES-MS²			100	2													
	APCI-MS²			100	1													
	APCI-source CID		27	100	59	14											8	
		m/z	***190***	***172***	***154***	***136***		***124***	***118***		***112***			***98***			***86***	
Castanospermine	ES-MS²			100	8	5												
	APCI-MS²			100	6	5												
	APCI-source CID		70	100	34	32			5		7			7			9	
Alexine	ES-MS²			100	29	8		3	4		44			1				
	APCI-MS²			100	22	5		4	4		28			2				
	APCI-source CID		100	23	5	5		2	2		15			2			1	
		m/z	***194***	***176***	***158***	***140***	***132***	***128***	***122***			***112***	***110***			***98***	***90***	***80***
HNJ	ES-MS²			100	32	30	1	6	5			3	5			2	12	6
	APCI-MS²			100	21	18	2	5	4			2	4			2	12	4
	APCI-source CID		100	77	28	32	8	12	20			17	39			9	16	20
Di-*epi*-HNJ	ES-MS²			100	22	14	5	2	2			2	1			0.5	14	2
	APCI-MS²			100	17	12	4	2	1			2	1			0.5	13	1
	APCI-source CID		100	96	30	32	55	10	16			7	24			3	12	10
Homo-DMDP	ES-MS²			100	25	42	16	11	17			4	19			4	15	14
	APCI-MS²			100	25	37	17	10	14			7	16			4	14	10
	APCI-source CID		100	28	11	20	15	11	13			9	28			7	8	16
		m/z	***206***	***188***	***170***	***152***			***134***	***132***	***128***		***122***			***110***	***102***	
Casuarine	ES-MS²			82	100	42			10	57	85		17			2	9	
	APCI-MS²			100	55	36			12	39	48		15			4	6	
	APCI-source CID		100	7	7	5			2	5	14		2			2	2	

[a] MS^2 refers to the CID product ion spectrum of the isolated $[M+H]^+$ ion; source CID refers to the CID spectrum of the ion in the total ion beam post source.

ion was the most abundant product of CID and probably resulted from loss of the hydroxymethyl side chain as methanol. The product ion spectrum of homo-DMDP showed a $[(M - H) - 62]^-$ ion as the base peak, which is in accordance with the loss of the $CHOH–CH_2OH$ side chain, probably as ethyleneglycol. Several of the other product ions observed among the product ion spectra were combinations of these methanol, hydrogen and one or two water losses, while other ions could not be accounted for.

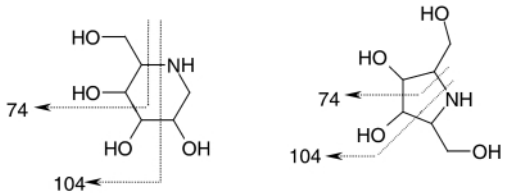

Fig. 3 Possible origin of $[(M + H) - 74]^+$ and $[(M + H) - 104]^+$ ions observed in CID product ion spectra of some PHAs.

The epimeric PHAs, HNJ and di-*epi*-HNJ, which showed very similar positive product ion spectra, could be distinguished clearly from their negative product ion spectra (Fig. 4). The negative product ion spectrum of di-*epi*-HNJ showed a base ion at $[(M - H) - 84]^-$, which is in accordance with the loss of both side chains as two molecules of methanol, a hydroxy group as water and a hydrogen molecule, whereas in the spectrum of HNJ the base ion was due to a water and methanol loss (Fig. 4, Table 4). HNJ also showed the loss just one of its two hydroxymethyl side chains after CID of the protonated parent ion to give the product ion at *m/z* 160, whereas this product ion was absent from the di-*epi*-HNJ spectrum; instead the spectrum of di-*epi*-HNJ showed minor ions at *m/z* 138 and 144 (Table 4). Both HNJ epimers were also clearly distinguished from the isomeric homo-DMDP by negative ion MS-MS since the latter showed a base ion at $[(M - H) - 62]^-$ as stated previously, and no such ion was evident in the HNJ or di-*epi*-HNJ spectra (Table 4).

The isomers castanospermine and alexine also produced diagnostic negative product ion spectra. The negative product ion spectrum of castanospermine showed a base ion at $[(M - H)$

Table 4 MS-MS product ion spectra of the $[M - H]^-$ ions of PHAs generated by negative APCI

		Relative intensity of the product ion showing the stated loss from the $[M - H]^-$ parent ion (*m/z* values for the product ion are given in bold italics above each group of isomeric compounds)																				
Compound		**0**	**−18**	**−20**	**−32**	**−38**	**−48**	**−50**	**−54**	**56**	**−62**	**−66**	**−68**	**−72**	**−76**	**−78**	**82**	**−84**	**−92**	**−94**	**−99**	**−106**
	m/z	***162***	***144***	***142***	***130***	***124***		***112***			***100***	***96***										
DMDP				2	100																	
DMJ			0.7	10	100	2		14			0.1	0.2										
DNJ			4	25	100	2		34			0.2	2										
	m/z	***172***	***154***	***152***		***134***									***96***						***73***	
Swainsonine			11	100		7									5						59	
	m/z	***188***		***168***	***156***	***150***		***138***		***132***					***112***	***110***			***96***	***94***		
Castanospermine				89		11									42	100			10	16		
Alexine				8	100			10		7									2			
	m/z	***192***	***174***	***172***	***160***		***144***	***142***	***138***		***130***	***126***	***124***					***108***	***100***			
HNJ			14	18	32			100	5			21	7					46				
Di-*epi*−HNJ			2	22			19	3	12			38	8					100				
Homo−DMDP			0.5	0.5	19			11			100	1	1						23			
	m/z	***204***	***186***	***184***	***172***	***166***		***154***		***148***	***142***	***138***	***136***	***132***	***128***		***122***		***112***	***110***		***98***
Casuarine			12	100	74	80		21		9	2	2	3	6	12		24		21	7		35

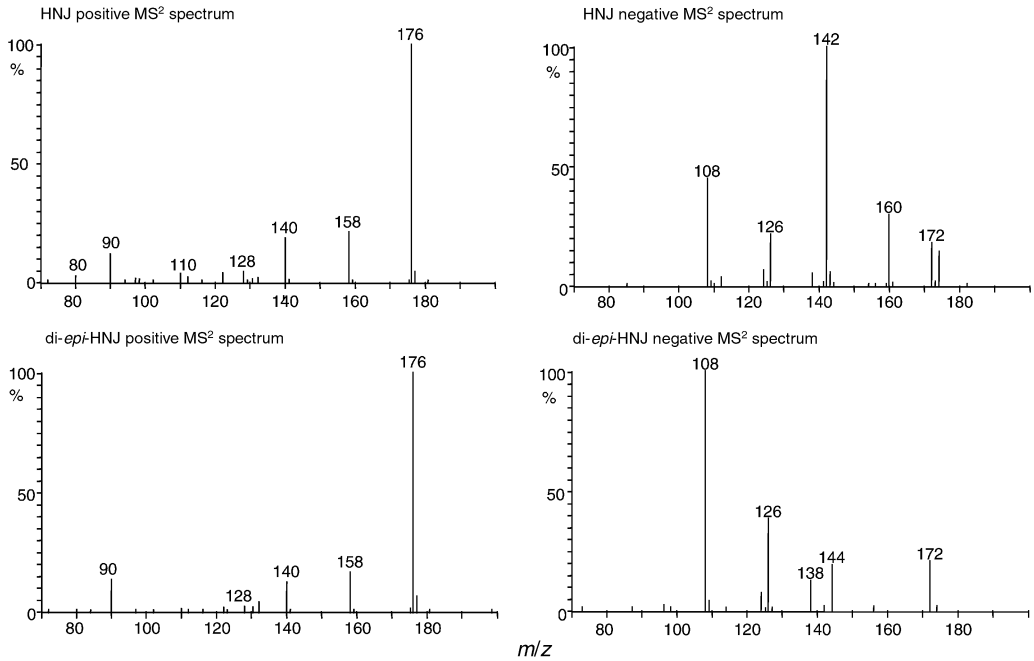

Fig. 4 MS^2 product ion spectra from CID of the $[M + H]^+$ ion at *m/z* 194 and the $[M - H]^-$ ion at *m/z* 192 of HNJ and di-*epi*-HNJ.

− 78]⁻, whereas in the equivalent spectrum from alexine, the [(M − H) − methanol]⁻ ion was the base peak. Unfortunately, the negative product spectra of the piperidine epimers DMJ and DNJ did not show any clear differences with both having the same [(M − H) − methanol]⁻ base ion. DNJ appeared to be slightly more susceptible to the joint loss of water and hydrogen, either from the deprotonated molecule ion or the [(M − H) − methanol]⁻ ion, as evidenced by the greater abundance of these product ions after CID (in the positive product ion spectrum, DNJ also showed more intense product ions due to water losses than DMJ), but these differences would not allow for unambiguous identification by MS experiments alone. However, the two epimers could be resolved chromatographically (Table 1 and Fig. 2).

Conclusions

APCI is clearly a superior ion source to ES in the LC-MS-MS analysis of PHAs under the LC conditions used in this study. Not only did it ionise (in positive mode) all the PHAs examined with greater efficiency than ES, but in negative mode APCI generated deprotonated molecule ions of PHAs bearing more than three hydroxy groups. Thus APCI allows for any differences in either positive or negative product ion spectra of isomeric PHAs to be exploited in an LC-MS-MS analysis. The spectra obtained in the present study suggest that negative product ion spectra can be more diagnostic. For example, the epimers HNJ and di-*epi*-HNJ not only showed different base ions in the negative product ion spectra but also had product ions unique to each spectrum: at *m/z* 160 or 142 for HNJ and *m/z* 144 in di-*epi*-HNJ. Therefore, a mixture of HNJ and di-*epi*-HNJ need not be resolved chromatographically for each to be analysed by product ion monitoring at these *m/z* values.

Even though MS-MS may allow characterisation of some mixtures of epimeric PHAs, improved methods of separation by LC are going to be necessary if the full advantage of the LC-MS analysis used here is to be realised, especially in screening for new compounds. Such LC methods should employ mobile phase that permits deprotonation of PHAs by APCI (*i.e.* acidic additives cannot be used, unless removed post column) since the ability to perform negative ion MS-MS is likely to be advantageous in the analysis of some epimeric types.

More comprehensive examination of the negative CID fragmentation processes of PHA isomers using precursor ion experiments on triple quadrupole or multi-sector instruments may lessen the need for good chromatographic resolution in screening methods. The differences in negative product ion spectra could potentially be used in chemometric programmes employing target tracking to study chromatographic peak purity or in matching spectra of unknowns. The present method, however, provides the potential to apply high throughput technology to screening for these natural products in plants and fungi.

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