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Characterization of ginseng saponins using electrospray mass spectrometry and collision-induced dissociation experiments of metal-attachment ions

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Electrospray mass spectrometry (ESMS) and collision-induced dissociation (CID) methodologies have been developed for the structural characterization of ginseng saponins (ginsenosides). Ginsenosides are terpene glycosides containing a triterpene core to which one to four sugars may be attached. They are neutral molecules which readily form molecular metal-attachment ions in positive ion ESMS experiments. In the presence of ammonium hydroxide intense deprotonated ions are generated. Both positive and negative ion ESMS experiments were found to be useful for molecular mass and structure determination of ten ginsenoside standards. Negative ion experiments made possible the determination of the molecular mass of each ginsenoside standard, the mass of the triterpene core and the masses and sequences of the sugar residues. Positive ion ESMS experiments with the alkali metal cations Li^+ or Na^+ and the transition metal cations Co^{2+}, Ni^{2+} and Zn^{2+} were also useful in determining molecular masses. These alkali and transition metal cations form strongly bonded attachment ions with the ginsenosides. As a result, the CID mass spectra of the metal attachment ions show a variety of (structure characteristic) fragmentations. These experiments can be used to determine the identity of the triterpene core, the types and attachment points of sugars to the core and the nature of the *O*-glycosidic linkages in the appended disaccharides. Combining the results from the negative and positive ion experiments provides a promising approach to the structure analysis of this class of natural products.

Introduction

The therapeutic effects of ginseng have long been known in Asia and its use in North America has become increasingly popular in recent years. Demand for American ginseng in Asia has led to its increased plantings in North America.[1] The pharmacologically active components of ginseng are believed to be ginsenosides, a class of distinctive triterpene glycosides, see Scheme 1. In recent years, there has been a large increase in reports on their biological activity.[2–12] Some are central nervous system stimulants and hypotensive agents;[2–4] for example, ginsenoside Rf inhibited voltage-dependent Ca^{2+} channels in sensory neurons.[5–7] The growth rate of human ovarian cancer expressed in mice was reduced after feeding the mice with ginsenoside Rh_2,[8] while ginsenosides Rb_1 and Rg_1 reduced the inflammatory response in asthmatic patients.[9–12]

Of the sixty-four reported ginsenosides,[13] only seven are commercially available, see Scheme 1. The majority of known ginsenosides consist of a triterpene core which can be either protopanaxadiol or protopanaxatriol. Sugars are attached to the triterpenes *via* *O*-glycosidic linkages; diol cores have sugars attached to C3 and C20 while triol cores have sugars attached to C6 and C20, respectively.

The overall goal of the present work is the development of ESMS methods for structural characterization of ginsenosides using small amounts of material and minimal chemical derivatization. Up to 1985, the mass spectrometric techniques available required chemical derivatization prior to analysis, a time-consuming process. These methods included electron impact mass spectrometry of acetate, trimethylsilyl ether and methyl derivatives of ginsenosides,[14] field desorption mass spectrometry,[15] and secondary ion mass spectrometry.[16] Definitive structural assignments have relied primarily on nuclear magnetic resonance spectroscopy[17] which requires a considerably larger amount of analyte than any mass spectrometric approach.

In 1995, van Breemen *et al*. demonstrated that ginsenoside sodium-attachment ions could be formed and analyzed by liquid chromatography-mass spectrometry (LC-MS).[18] Compared to traditional methods of analysis, we found this approach attractive since it does not require a time-consuming derivatization prior to analysis. Reports on the mass spectrometric analysis of structurally related saponins provided further useful information,[19,20] confirming that the fragmentation patterns of triterpene glycosides are determined by their sugar moieties. From the vast body of literature on the mass spectrometry of sugars, refs. 21–34 are of immediate interest to this study. The studies reported in these papers demonstrate that underivatized sugars yield excellent ESMS spectra in the presence of metal ions. In three of these studies[26,27,29] it is shown that CID experiments of sugar metal-attachment ions with transition metal ions[26,27] as well as Li^+ and Na^+,[29] provide information on the nature of the *O*-glycosidic linkage. In particular, the method reported by Asam and Glish[29] appeared to hold considerable promise for deriving useful information on the nature of *O*-glycosidic linkages in the disaccharides from ginsenosides.

Very recently, Wang *et al.*[35] proposed an LC-MS method in which protonated ginsenosides are subjected to CID experiments. This approach provides information on the types of sugars, the *O*-glycosidic linkages and the triterpene cores. Metal-attachment ions with sodium and potassium were also briefly examined in this study. However, these species were not further investigated, possibly because their CID mass spectra showed fewer fragment ions than the protonated species.

Based upon the pioneering studies of van Breemen *et al.*[18] and those in refs. 26, 27 and 29, we report an ESMS based

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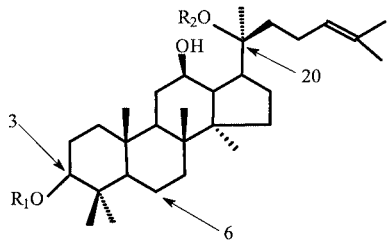
approach to the structural characterization of ginsenosides complementary to that of Wang *et al.*[35] Using ten ginsenoside standards as model compounds, we find our method to be useful for molecular mass and structure determination, isomer differentiation and determination of *O*-glycosidic linkages in ginsenosides. The approach involves both positive and negative ion ESMS and CID experiments.

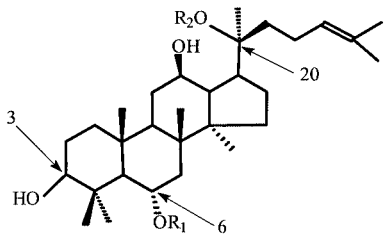
The longer term goal of this work is the development of a routine LC-MS methodology for the structure analysis and quality control of ginsenosides in complex mixtures (root extracts) and the refinement of structure determination methods for isolated ginsenosides.

Experimental

Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 were purchased from Indofine Chemical Company Inc. (Somerville, NJ, USA), while ginsenosides Rb_3 and Rg_3 and gypenoside XVII were obtained from Agriculture Canada (Delhi, Ontario, Canada). Glucose disaccharides having α-1,2-, α-1,6-and β-1,6-*O*-glycosidic linkages were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Caledon Laboratories (Georgetown, Canada) and water was obtained from a Milli-Q water purification system (Millipore, Mississauga, Canada). A.C.S. reagent grade metal salts were purchased from Aldrich (Milwaukee, WI, USA). Li^+, Na^+, K^+ and Rb^+ were introduced as their acetate salts while Cs^+ was introduced as its iodide salt. Transition metal cations (Co^{2+}, Ni^{2+} and Zn^{2+}) were introduced as their chloride salts.



	R1	R2	MW
Protopanaxadiol	H	H	460
Ginsenoside			
Rb_1	Glc1-O-2Glc-	Glc1-O-6Glc-	1108
Rb_2	Glc1-O-2Glc-	Ara1-O-6Glc-	1078
Rb_3	Glc1-O-2Glc-	Xyl1-O-6Glc-	1078
Rc	Glc1-O-6Glc-	Ara(f)1-O-6-Glc-	1078
Rd	Glc1-O-2Glc-	Glc-	946
Gypenoside XVII	Glc-	Glc1-O-6Glc-	946
Rg_3	Glc1-O-2Glc-	H	784



	R1	R2	MW
Protopanaxatriol	H	H	476
Ginsenoside			
Re	Rhm1-O-2Glc-	Glc-	946
Rf	Glc1-O-2Glc-	H	800
Rg_1	Glc-	Glc-	800

Scheme 1 Structures of the ginsenoside standards used. Glc = glucose; Ara = arabinose; Xyl = xylose; Rhm = rhamnose; f = furanose (unless otherwise specified the sugars are in their pyranose form).

Mass spectrometry experiments were performed on a Micromass Quattro LC triple quadrupole mass spectrometer (Manchester, UK) equipped with a 'pepper-pot'. The solvent delivery system was a Brownlee Labs Microgradient System syringe pump which introduced the mobile phase at a flow rate of 5 μL min^{-1}. The mobile phase was a 1 + 1 solution of water and acetonitrile. Solutions containing 40 μM of a ginsenoside and 65 μM of a metal salt were introduced by infusion in positive ion experiments, whereas negative ion experiments were performed by infusion of solutions containing 100 μM ginsenoside with 0.1% NH_4OH. The yield of metal-attachment ions was highest at a cone voltage of 120 V. In CID experiments on the alkali metal attachment ions, argon (at an indicated pressure of 2×10^{-3} mbar) was used as the collision gas at a collision energy of 50 eV. CID experiments on the transition metal attachment ions were performed using a collision energy of 30 eV.

Results and discussion

The structures of the ten ginsenoside standards used in this work are listed in Scheme 1. While the triterpene cores have either three or four hydroxyl groups, sugars are attached to the triterpene at only two positions, C3 and C20 in the case of protopanaxadiol-based ginsenosides and C6 and C20 in the case of the protopanaxatriol-based ginsenosides. Variations in the types of sugars, their sequence and type of glycosidic linkages can lead to a variety of structures, including isomeric ginsenosides. Carbon 20, a common attachment point for both triterpene series, is a tertiary centre while C3 and C6 are secondary carbons; it was anticipated that fragmentation under positive ion conditions would occur more readily at C20 than at C3 or C6, due to the greater stability of tertiary cations (at C20) compared to the corresponding secondary species at either C3 or C6.

At the outset of this work we realized that in order to propose a structure for a ginsenoside, various mass spectrometric techniques would be necessary to obtain some or all of the following data: (1) the molecular mass of the ginsenoside; (2) the type of triterpene core; (3) the number and types of sugars attached to the core; (4) the points of attachment of the sugars; (5) the sequence of sugar residues attached to the core; and (6) the stereochemistry of each sugar–sugar linkage. The combination of triterpenes, sugar moieties and glycosidic linkages in ginsenosides affords structures with molecular ions and fragment ions that may (or may not) be unique or common to a number of ginsenosides. For example, differentiation between the triterpene cores (protopanaxadiol and protopanaxatriol) is often important because their mass difference is only 16 Da; this mass difference is reflected in two otherwise identical ginsenosides, ginsenoside Rg_3 (diol core, MW = 784 Da, Scheme 1) and ginsenoside Rf (triol core, MW = 800 Da, Scheme 1). If a deoxyhexose such as rhamnose is substituted for a hexose such as glucose, the resulting mass difference is also 16 Da. Thus, the diol-based ginsenoside Rd with three glucose sugars has a molecular mass, 946 Da, (and elemental composition) identical to that of the triol-based ginsenoside Re, which contains two glucose sugars and one rhamnose sugar.

Negative ion experiments: infusion of ginsenosides in the presence of NH_4OH

Infusion of a solution containing a ginsenoside in the presence of 0.1% NH_4OH, yielded spectra containing a wealth of data that provided information on the molecular mass, the triterpene core, the nature of the sugars and their sequence in each ginsenoside. First, all ginsenosides show intense $[M - H]^-$ ions, confirming their molecular masses as exemplified by the

spectra of ginsenosides Rc and Re in Fig. 1. Second, each ginsenoside shows a fragment ion at either *m/z* 459 or at *m/z* 475, clearly identifying the triterpene core as protopanaxadiol [Fig. 1(a)] or protopanaxatriol [Fig. 1(b)], respectively. Third, fragment ion masses in the full scan mass spectrum provide structure characteristic information which was verified by MS-MS experiments. For example, ginsenoside Rc has four sugar residues attached to a diol core, see Scheme 1, and it gives a strong $[Rc - H]^-$ ion at *m/z* 1077 [Fig. 1(a)]. Ions at *m/z* 945 and 915 arise from losses of a 5-carbon sugar and a 6-carbon sugar, respectively, indicating that these sugars are likely the terminal sugars of each of the two disaccharides. Loss of sugar moieties continues sequentially until only the triterpene core remains; see *m/z* 459 in Fig. 1(a). These observations are in line with negative ion fast-atom bombardment (FAB) MS-MS results on saponins from black bean.[20]

Positive ion experiments: infusion of ginsenosides in the presence of metal ions

Preliminary experiments probed the formation of ginsenoside metal-attachment ions with the alkali metal ions (Li^+ to Cs^+). Infusion ESMS experiments showed that all ginsenoside standards contained endogenous amounts of sodium and potassium salts in the lower percentage range, with sodium predominating. Infusing solutions containing a cation:ginsenoside molar ratio of 1.5 : 1 ensured that the added alkali metal ion was in excess of the endogenous Na^+ and K^+ ions. This step allowed a better assessment of adduct ion formation with the added metal ions.

A series of ES experiments with ginsenoside Rc (MW = 1078 Da) serves to illustrate observations typical for all ginsenoside standards. In the presence of Li^+, the lithiated molecular ion $[Rc + Li^+]^+$ was observed at *m/z* 1085; see Fig. 2(a). This mass spectrum also shows molecular metal-attachment ions with Na^+ and K^+ at *m/z* 1101 and *m/z* 1117, respectively, due to the sodium and potassium salts in the standard. CID experiments performed on these ginsenoside metal-attachment ions revealed a preferred fragmentation pathway. Scheme 2 shows a stylized diagram of a ginsenoside structure, together with the full structure of ginsenoside Rc, which shows the principal dissociations that the molecular metal-attachment ions undergo. The cleavage reactions occur predominantly at the glycosidic linkage at C20 of the triterpene core and further at C3 (diol core) or C6 (triol core); all molecular ions and fragment ions are metallated ions. Fragment ions **a** and **a*** result from bond cleavages of glycosidic linkages at the C3 or C6 glycosidic linkage. Fragment ion **a** is a metallated saccharide species while fragment ion **a*** is a metallated triterpene–saccharide species. The key fragment ions **b** and **b*** result from cleavage at the C20 glycosidic linkage; fragment ion **b** represents the metallated saccharide while

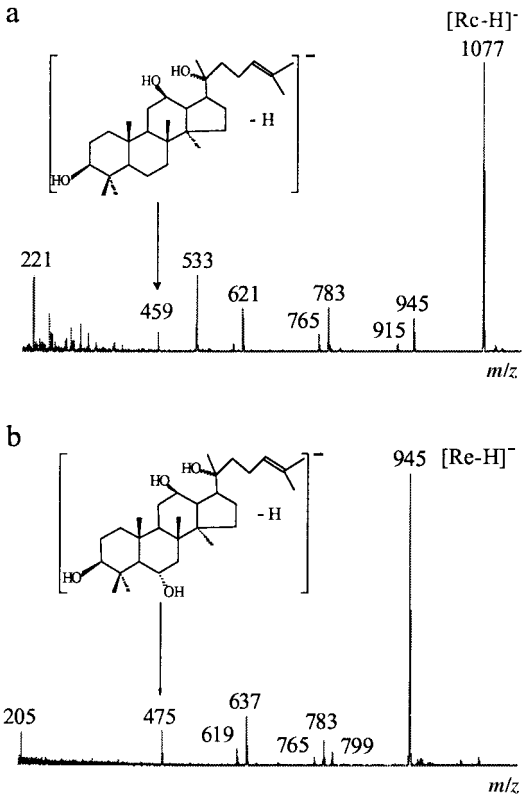


Fig. 1 Negative ion ES mass spectra of ginsenoside Rc (a), and ginsenoside Re (b).

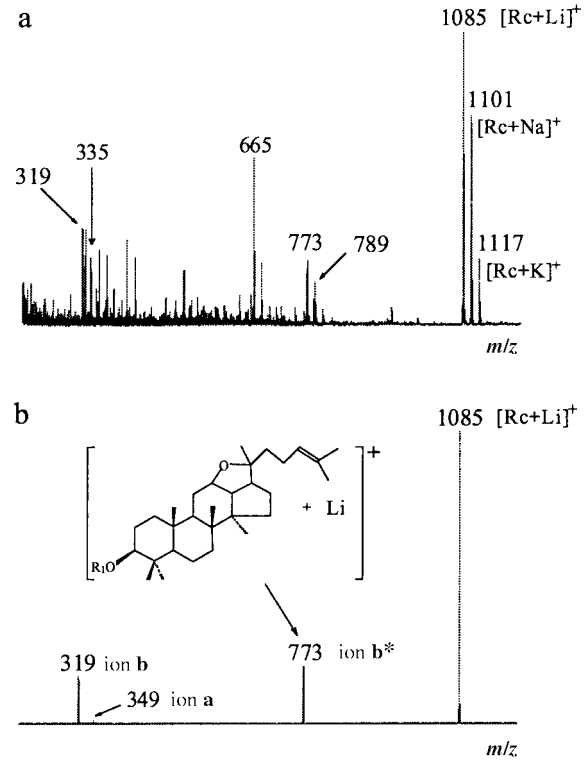
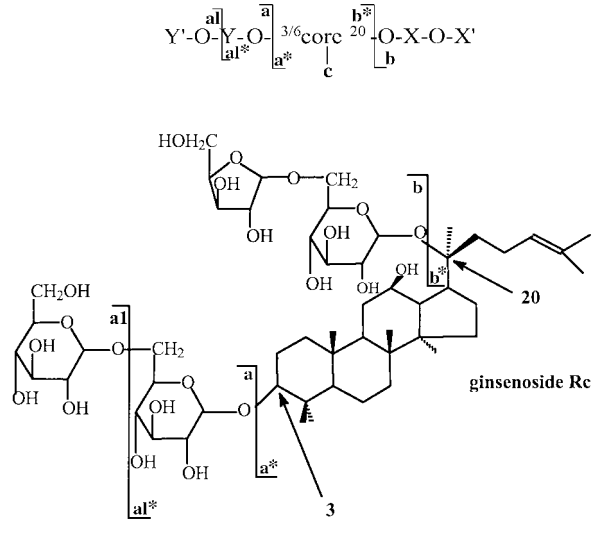


Fig. 2 Positive ion ES mass spectrum of ginsenoside Rc and Li^+ (a) and the CID mass spectrum of the $[Rc + Li^+]^+$ ion at *m/z* 1085 (b).



Scheme 2 Main fragmentation pathways in the CID mass spectra of ginsenoside metal-attachment ions (see text and Table 1).

fragment **b*** is a metallated triterpene–saccharide species. The **a*** and **b*** ions can also lose the saccharide(s) attached to the triterpene core to yield a metallated triterpene species, denoted as ion **c** in Scheme 2.

A CID experiment on the $[Rc + Li]^+$ ion yielded a simple spectrum with two major fragment ions, at *m/z* 319 and 773; see Fig. 2(b). These ions likely correspond to the lithiated disaccharide and core-disaccharide fragment ions resulting from bond cleavage at C20. Using the nomenclature in Scheme 2, the *m/z* 319 ion is a **b** type ion while the *m/z* 773 ion is a **b*** type ion. Infusion of ginsenoside Rc in the presence of Na^+ or K^+ yielded $[Rc + Na]^+$ or $[Rc + K]^+$ adduct ions. Upon collision these attachment ions generated the metallated disaccharide and core-disaccharide fragment ions **b** and **b***. Experiments with Rb^+ or Cs^+ also yielded intense attachment ions but their MS-MS spectra only showed Rb^+ and Cs^+ cations, at *m/z* 85 and *m/z* 133, respectively. The ginsenosides examined all showed a pronounced degree of fragmentation in the presence of Li^+ and Na^+, less so in the presence of K^+ while little or no fragmentation occurs in the presence of Rb^+ and Cs^+. This trend suggests that the interaction of a ginsenoside with an alkali metal cation decreases with an increase in the size of the ion. Kohler and Leary[26] noted a similar trend in a study of carbohydrates.

In the presence of Co^{2+}, Ni^{2+} or Zn^{2+} ions, the ginsenosides do not generate molecular metal-attachment ions but rather $[M - H^+ + metal^{2+}]^+$ ions. Thus, adduct ion formation with transition metal cations involves deprotonation, presumably of a hydroxyl group of a saccharide unit. The resulting alkoxide site provides a strong electrostatic interaction for the transition metal ion which is not available to the alkali metal ions. Increasing the internal energy of these ions in a CID experiment results in a large number of fragmentations.

Ginsenoside Rc is used to illustrate the trends: its ES mass spectrum in the presence of Co^{2+} is shown in Fig. 3(a). The spectrum displays an intense $[M - H^+ + metal^{2+}]^+$ ion at *m/z* 1136 while the peak at *m/z* 1173 represents an adduct ion with $CoCl^+$. Fig. 3(b) shows the CID mass spectrum of the $[Rc - H^+ + Co^{2+}]^+$ ion which shows a higher degree of dissociation than any alkali metal ion, even Li^+; compare Fig. 2(b).

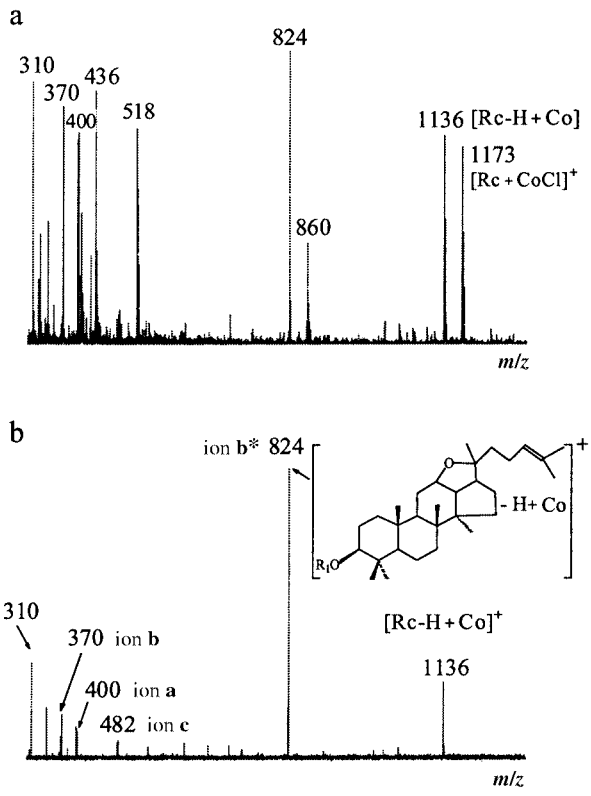


Fig. 3 Positive ion ES mass spectrum of ginsenoside Rc and Co^{2+} (a) and the CID mass spectrum of the $[Rc - H^+ + Co^{2+}]^+$ ion at *m/z* 1136 (b).

The normalized fragment ion abundances observed in MS-MS experiments on the metallated molecular ions of the ten ginsenoside standards are reported in Table 1. Table 1(a) lists the data for those ginsenosides containing four or three saccharides while Table 1(b) deals with ginsenosides containing two saccharides. The data only refer to the metal ions Li^+, Na^+, K^+, Co^{2+} and Ni^{2+}: adducts with Rb^+ and Cs^+ showed little or no fragmentation, while those with Zn^{2+} fragmented similarly to Co^{2+}.

Table 1(a) Normalized fragment ion abundances derived from the CID mass spectra of ginsenoside metal-attachment ions. (a blank space indicates a relative intensity ⩽ 1%, while — indicates that the ion in question cannot be formed)

Ginsenoside	Metal ion	**a**	**a***	**b**	**b***	**c**
Rc	Li^+	12	13	100		
	Na^+	2	67	100	50	
	K^+			100	19	
	Co^{2+}		5	15	100	5
	Ni^{2+}		100	22	91	
Rb_2	Li^+	64	26	100	84	
	Na^+	4	4	100	51	
	K^+	3	4	100	47	
	Co^{2+}		6	40	100	1
	Ni^{2+}		30	60	100	58
Rb_3	Li^+	6	41	85	100	
	Na^+	5	7	100	90	
	K^+			100	23	
	Co^{2+}		15	11	100	15
	Ni^{2+}		100	30		
Rb_1	Li^+	100	3	100	19	
	Na^+	100		100	20	
	K^+	100		100	20	
	Co^{2+}	31	5	31	100	2
	Ni^{2+}	100		100	78	22
Rd	Li^+	16	5	16	100	
	Na^+	17	8	17	100	
	K^+	25	11	25	100	
	Co^{2+}		16		100	13
	Ni^{2+}		25		100	21
Gypenoside XVII	Li^+	—	9	100		
	Na^+	—		100		
	K^+	—		100		
	Co^{2+}	—		100	23	
	Ni^{2+}	—		100	70	
Re	Li^+	9	68	9	100	14
	Na^+		25	3	100	3
	K^+		23	67	100	29
	Co^{2+}				37	100
	Ni^{2+}				100	

Table 1(b)

Ginsenoside	Metal ion	**a1**	**a**	**a1***	**b**	**b***	**c**
Rg_1	Li^+	—	100	—	100	40	40
	Na^+	—	100	—	100	46	3
	K^+	—	76	—	76	100	31
	Co^{2+}	—		—		100	6
	Ni^{2+}	—		—		100	
Rf	Li^+	9	100		—	—	9
	Na^+	5	100		—	—	3
	K^+				—	—	
	Co^{2+}		100	60	—	—	95
	Ni^{2+}				—	—	
Rg_3	Li^+	45	11	100	—	—	2
	Na^+	77	18	100	—	—	
	K^+	100	45	87	—	—	
	Co^{2+}	10		100	—	—	10
	Ni^{2+}			100	—	—	

The first part of Table 1(a) lists data for ginsenosides Rc, Rb_1, Rb_2 and Rb_3 which contain diol cores and four sugars in the form of two disaccharide units attached at C3 and C20. All sugars in ginsenoside Rb_1 (MW = 1108) are glucose while ginsenosides Rb_2, Rb_3 and Rc (MW = 1078) each have a 5-carbon sugar in the terminal position of the disaccharide attached to C20. Since the masses of the disaccharide units attached to C20 in the latter three ginsenosides are 30 Da less than the glucose disaccharide in ginsenoside Rb_1, differentiation of these ginsenosides from ginsenoside Rb_1 is straightforward [Table 1(a)]. The predominant fragmentation route for all four metallated ginsenosides was bond cleavage at C20, a tertiary centre, to afford **b** and **b*** ions; fragmentation at C3 to afford ions **a** and **a*** was less abundant [Table 1(a)]. Ginsenoside Rb_1 has identical disaccharide units at C3 and C20, therefore fragment ion **a** is indistinguishable from fragment ion **b**. The smaller alkali metal cations afforded more intense **b** ions, implying a greater association with the departing disaccharide. However, adduct ions with transition metals gave more intense **b*** ions. Hard acids like these transition metal ions tend to associate strongly with hard bases like the hydroxyl group. Therefore, transition metal ions prefer to be associated with the portion of the molecule containing more hydroxyl groups; in these cases it is the triterpene–saccharide portion.

The ginsenosides containing three sugars showed fragmentation patterns very similar to those with four sugars; that is, the major fragment ions were **b** and **b*** type ions resulting from cleavage at C20, see Table 1(a) (bottom part). These three ginsenosides have the same molecular mass and each contains a monosaccharide unit and a disaccharide unit (Scheme 1). MS-MS data for these isomeric ginsenosides showed that fragmentation at C20 predominates and that metal cations tend to be retained preferentially on disaccharide units rather than on monosaccharide units. These observations allowed for easy differentiation of ginsenoside Rd and gypenoside XVII. Both are diol-based ginsenosides with a disaccharide at C20 (gypenoside XVII) and a monosaccharide at C20 (ginsenoside Rd). The major fragment ion in the CID mass spectrum of lithiated gypenoside XVII was type **b** while for ginsenoside Rd it was type **b***. Ginsenoside Re is triol-based with a similar arrangement of sugars around the triterpene core to ginsenoside Rd. The **b*** fragment ion was predominant in the CID mass spectrum of ginsenoside Re. This is consistent with the proposal that metal cations prefer to be associated with fragments containing a disaccharide.

Three ginsenoside standards contained only two sugars, see Table 1(b). Ginsenoside Rg_1 with a single sugar attached to both C6 and C20 showed predominant fragment ions of the **a**/**b** and **a***/**b*** type, indicative of cleavage at C3 or C20. In light of the previous discussion it is probable that the ions observed result from bond cleavage at C20 but this cannot be confirmed. The remaining ginsenosides (Rf and Rg_3) are unusual in that they contain disaccharides at C3 or C6 and no sugar substituent at C20; thus fragment ions **b** and **b*** cannot be formed. Only **a** type fragment ions are observed [Table 1(b)].

In summary, ginsenosides with three and four sugar units fragment in a readily interpretable fashion. Based on these observations, the assignment of a basic structure of an unknown ginsenoside with three or four sugars should be straightforward. Ginsenosides with two (or fewer) sugars have less predictable fragmentation pathways and their structural assignments may be more challenging.

Identification of the triterpene core

The diol and triol triterpene cores were identified by both negative and positive ion ESMS experiments. Full scan negative ion ESMS experiments showed ions corresponding to the two core types. Ginsenosides containing diol cores showed an ion at *m/z* 459 [Fig. 1(a)], while ginsenosides containing triol cores showed an ion at *m/z* 475 [Fig. 1(b)]. CID experiments confirmed that the *m/z* 459 and *m/z* 475 ions are generated from their respective $[M - H]^-$ precursor ions (results not shown). Negative ion ESMS data from the ten ginsenoside standards provided unambiguous identification of the triterpene core.

From the results presented in Table 1, it follows that metallated core ions, **c** type ions, are generated in MS-MS experiments on the various metal-attachment ions but their intensity varies considerably and is highly dependent on both the ginsenoside structure and the nature of the metal ion. One trend is that the triol-containing ginsenosides yield more intense metallated core ions than their diol-containing counterparts. The results tabulated in Table 1 further indicate that the nature of the triterpene core could be established by *combining* the information from the Li, Co and Ni experiments but the negative ion data clearly provide a better diagnostic tool.

Determination of the *O*-glycosidic linkage

One major challenge is the determination of the nature of the glycosidic linkages between the sugar units. Most of the ginsenosides contain disaccharides with 1,2- and 1,6-*O*-glycosidic linkages.[13] Recent studies of saccharides aimed to establish the nature of the linkage using Li^+ and Na^+[29] or transition metal cations[27] prompted us to explore these approaches in the present work. We infused Li^+ with three glucose disaccharide standards having α-1,6-, β-1,6- and α-1,2-*O*-glycosidic linkages and obtained results very similar to those of Asam and Glish;[29] see the first three entries in Table 2. CID experiments on the lithiated α-1,2-linked glucose disaccharide molecular ion showed loss of a single neutral loss of mass 120. In contrast, the CID mass spectra of the α- and β-1,6-linked glucose disaccharides were characterized by neutral losses of 60, 90 and 120 Da, the loss of 60 yielding the base peak. However, the CID mass spectra of the α- and β- anomers are not sufficiently different to permit an unambiguous differentiation between the α- and β- anomers of an unknown saccharide.

The disaccharides present in the various ginsenosides were isolated for structural analysis *via* in-source CID.[36] The relative fragment ion intensities for the three disaccharide reference compounds and those derived from the eight ginsenoside standards that contain disaccharides, are presented in Table 2. The glycosidic linkages listed (left column in Table 2) for the

Table 2 Normalized neutral loss abundances for glucose disaccharide standards and disaccharides derived from ginsenoside lithium-attachment ions; α = hydroxyl group on C1 is below the plane; 5C6C = disaccharide unit containing a five carbon sugar and a six carbon sugar; 6C6C = disaccharide unit containing two six carbon sugars; 1,2-linked = sugars in the disaccharide are linked via a 1,2-*O*-glycosidic linkage; a blank space indicates a relative intensity ⩽ 1%

Compound		Abundance of neutral losses		
		120 Da	90 Da	60 Da
α-1,2-Glucose disaccharide		100		
α-1,6-Glucose disaccharide		31	91	100
β-1,6-Glucose disaccharide		50	68	100
Ginsenoside Rg_3	6C6C (1,2-linked)	100		
Ginsenoside Rf	6C6C (1,2-linked)	100		
Ginsenoside Re	6C6C (1,2-linked)	100		
Ginsenoside Rd	6C6C (1,2-linked)	100		
Gypenoside XVII	6C6C (1,6-linked)	43	75	100
Ginsenoside Rb_2	5C6C (1,6-linked)	18	48	100
	6C6C (1,2-linked)	100		
Ginsenoside Rb_3	5C6C (1,6-linked)	21	50	100
	6C6C(1,2-linked)	26	52	100
Ginsenoside Rc	5C6C (1,6-linked)	26	54	100
	6C6C (1,6-linked)	100		30

ginsenosides are taken from the literature.[13] The first three entries in Table 2 show the normalized neutral loss abundances of the three glucose disaccharide standards. The remaining entries are the normalized neutral loss abundances for disaccharide species derived from ginsenosides containing disaccharide units. The pattern of neutral losses in the ginsenoside-derived disaccharides is very similar to those of the glucose disaccharide standards. Nine of the eleven *O*-glycosidic linkages in the ginsenosides examined are correctly identified using this approach. However, two glucose–glucose disaccharides—the 6C6C entries for ginsenosides Rb_3 and Rc—yielded neutral loss data which are not consistent with the literature assignments.[13] This method has a high probability of success in differentiating 1,2-linked from 1,6-linked disaccharides in these complex molecules but analysis of more reference compounds is required to establish its full potential.

Differentiation of isomers

The differentiation of isomeric substances is a good test of the usefulness of this methodology. Among the available standards were three isomeric ginsenosides with molecular mass 946 Da (Rd, Re and gypenoside XVII), three with molecular mass 1078 Da (Rb_2, Rb_3 and Rc) and two with molecular mass 800 Da (Rf and Rg_1); see Scheme 1. Ginsenoside Re, one of the 946 Da isomers, has a triol core and so is easily differentiated from ginsenoside Rd and gypenoside XVII using negative and positive ion ESMS approaches. The positive ion CID mass spectrum of the lithiated molecular ion of ginsenoside Re clearly showed the triol core [*m/z* 447, Fig. 4(a)] whereas the lithiated molecular ions of the other two isomers did not afford [core + Li^+]$^+$ ions (Table 1). Ginsenoside Rd and gypenoside XVII differ in the arrangements of the sugars attached to the core. Gypenoside XVII, with a glucose disaccharide at C20, showed preferential bond cleavage at C20 and the formation of a lithiated disaccharide ion [**b** type ion, *m/z* 349, Fig. 4(b)]. On the other hand, the isomeric ginsenoside Rd with a single glucose at C20 showed a major fragment ion at *m/z* 773 [**b*** type ion, Fig. 4(c)], corresponding to the loss of a glucose molecule.

The 1078 Da isomers could not be differentiated by our ESMS methods. This is perhaps not too surprising, considering that their structures are closely similar. The only difference between ginsenosides Rb_2 and Rc, see Scheme 1, is the conformation of the terminal pentose in the C20 disaccharide; arabinose has the pyranose form in ginsenoside Rb_2 and the furanose form in ginsenoside Rc. In ginsenoside Rb_3 the terminal pentose in the C20 disaccharide is xylose, not arabinose. On the other hand, the 800 Da ginsenosides Rf and Rg_1 were easily differentiated because Rg_1 has a glucose at C20 whereas Rf has no sugar at C20; loss of glucose from ginsenoside Rg_1 is a predominant pathway. Overall, the combination of ESMS methods is useful for differentiating ginsenosides belonging to two of three groups of isomers.

Conclusions

Under ESMS conditions ginsenosides readily form molecular attachment ions with small alkali metal and transition metal ions. These adduct ions provided molecular mass information which is consistent with results from negative ion experiments. Structural information was obtained from MS-MS experiments on the ginsenoside metal-attachment ions which includes the nature of the triterpene core, the types of sugars (pentose or hexose) attached to the ginsenoside and the types of attachment points of the sugars to the core (secondary *vs*. tertiary centres). The nature of the *O*-glycosidic linkage present in the disaccharides could be obtained from CID experiments on the Li^+ adducts of source-generated disaccharides from the ginsenosides.

Negative ion experiments allowed for the determination of the molecular mass and the type of triterpene core of each ginsenoside. Furthermore, negative ion mass spectra allowed determination of the sequence of the sugar moieties. The results presented show that structural characterization of ginsenosides by these mass spectrometric techniques is feasible and that these approaches are amenable to future applications in the LC-MS and LC-MS-MS analyses of complex mixtures of ginsenosides such as in root extracts.

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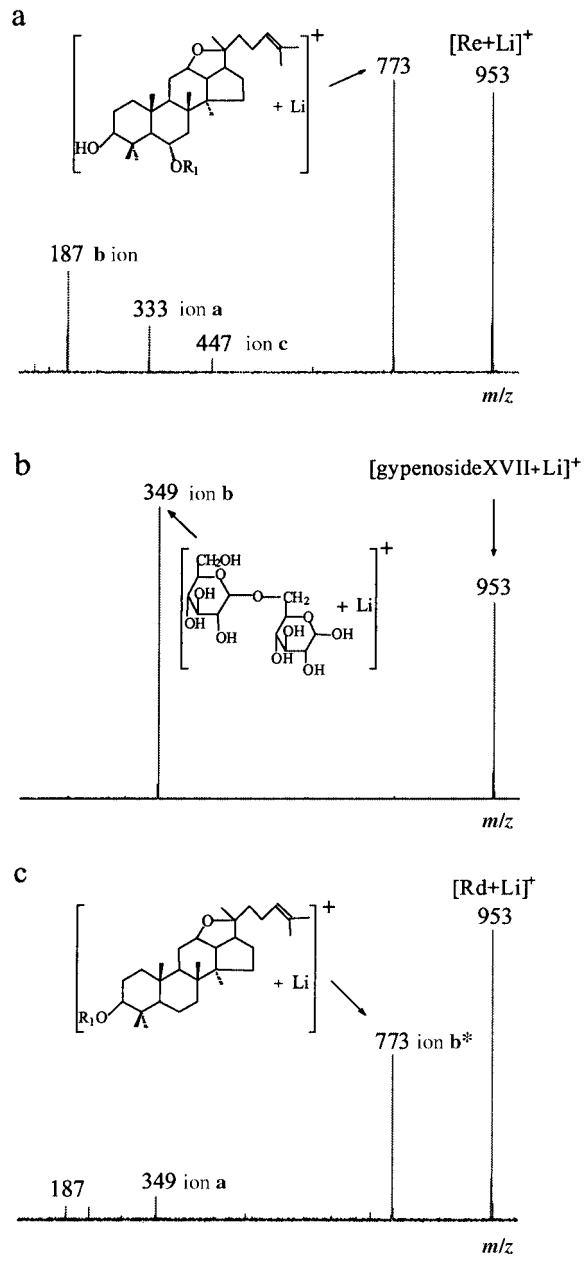

Fig. 4 CID mass spectra of the *m/z* 953 ion generated by Li^+ attachment to: ginsenoside Re (a), gypenoside XVII (b) and ginsenoside Rd (c).

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