FALSE SUGAR SEQUENCE PHENOMENON AND STRUCTURAL ANALYSIS OF α-HEDERIN AND HEDERACOSIDE C THROUGH ELECTROSPRAY IONIZATION AND MULTI-STAGE TANDEM MASS SPECTROMETRY

FENÓMENO DE FALSA SECUENCIA DE AZÚCARES Y ANÁLISIS ESTRUCTURAL DE α-HEDERINA Y HEDERACÓSIDO C EMPLEANDO IONIZACIÓN POR ELECTROSPRAY Y ESPECTROMETRÍA DE MASAS EN TÁNDEM

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ABSTRACT

Saponins are glycosides regularly found in plants, and they are frequently analyzed by means of electrospray ion sources and tandem mass spectrometry (ESI/MS^n), not only to obtain an overall molecular weight through the high sensitivity of mass spectrometry, but also to obtain their structural information. The use of modern analytic techniques (such as ESI/MS^n) aids the discovery and quality control assessment of new molecules found in botanicals; however, some rearrangements have been described for oligosaccharides that produce an incorrect elucidation of the molecules. For the first time, evidence for an internal sugar loss on the protonated fragmentation of α-hederin has been reported. This phenomenon can be avoided by using alkali metal ion adducts, such as lithium and sodium cations, to confirm the real sugar sequence when a structural identification is performed. In this study, the structural characterization of α-hederin and hederacoside C is conducted by comparing positive and negative modes. Different fragmentation patterns occur, and such patterns are useful in the structural analysis of saponins when they are present on different matrixes.

Keywords: Internal sugar loss, triterpenic saponins, electrospray, ionization source, mass spectrometry.

RESUMEN

Las saponinas son glicósidos encontrados frecuentemente en plantas y son analizados habitualmente por espectrometría de masas en tándem y fuentes de ionización por electrospray (ESI/MS^n), no solo por su alta sensibilidad sino además por la información estructural que es obtenida. Tanto el descubrimiento de nuevas entidades químicas como el control de calidad de productos naturales involucran el uso de técnicas analíticas modernas como ESI/MS^n. Sin embargo, algunos re-arreglos de azúcares en los glicósidos pueden conducir a realizar falsas interpretaciones de las secuencias de los mismos. La pérdida de un azúcar interno de la porción oligosacárida de α-Hederina es evidenciada por primera vez. Este fenómeno puede ser evitado si se favorece la formación de aductos con iones metálicos como litio y sodio, con el fin de obtener la secuencia real de azúcares de un glicósido. La caracterización estructural

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de α-Hederina y hederacósido C es realizada comparando los espectros obtenidos tanto en modo de ionización positiva como negativa, evidenciando diferentes mecanismos de fragmentación los cuales proporcionan información útil en el análisis estructural.

Palabras clave: pérdida de azúcar interno, saponinas triterpénicas, electrospray, sistema de ionización, espectrometría de masas.

INTRODUCTION

The use of tandem mass spectrometry in the structural analysis of glycosides is prevalent in several laboratories. The most important application of this methodology is the identification of sugar sequences and differentiation of the types of glycoside linkages. However, under several specific conditions, the information obtained can be incorrectly interpreted. Kovacik *et al.*, 1995 (1) previously described this phenomenon on oligosaccharides and postulated three possible reasons. The most intriguing reason involves the elimination of an internal monosaccharide residue in a rearrangement process that preferentially takes place in 1→2 glycoside linkages with α-anomer monosaccharides. Nonetheless, due to the formation of oligosaccharide metal adducts, the tandem mass spectra of these molecules only show ions type B and Y without the presence of Y★, which represents the nomenclature for internal residue loss. For this reason, the authors recommended that further research should be done about other factors such as the linkage, configuration, substitution and nature of the eliminated residue, and the adjacent residue in order to clarify the real sequence. Thus, further investigation demonstrated that this process appears to be independent of anomericity, and that it takes place not only in 1→2, but also in 1→6 glycoside linkages (2, 3). This rearrangement can be detected in ammonium adducts as described by Ernst, 1997 (4) in sialyl Lewis oligosaccharides. An α-fucose residue underwent rearrangement, producing a higher amount of abundant Y* type ions compared with the β-fucose anomer, although both glycoside linkages were different. Anthracyclines (5) and flavonoids (6, 7) were the first glycoconjugates analyzed that presented internal glucose losses in protonated spectra. This fact was also observed for some steroidal and tritepenoid glycosides (8). Moreover, internal glucose loss for pentacyclic triterpene saponins is also described in this research work.

Pentacyclic triterpene saponins are molecules widely distributed in plants, and they are also chemical markers for chemotaxonomic or herbal quality control purposes. These molecules are present in plants such as *Sapindus saponaria*, *Phytolacca americana*, *Tribulus terrestris* and *Chenopodium quinoa*; and in some cases, these types of structures have been associated with toxicological effects and even death cases, in which high levels of saponins were found, as it occurs in the case of *Hedera helix* (9). An accurate structural characterization is important when identifying new saponins, because an internal sugar loss can arise, which results in the erroneous interpretation of the data. A complete structural analysis of hederacoside C and α-hederin as markers of *Hedera helix* was accomplished using fragmentation reactions. By focusing on the MS spectra of α-hederin, it was possible to deduce a rearrangement process on this molecule, which favors a false sugar sequence. In this study, the internal sugar loss of pentacyclic triterpene saponin is reported for the first time as a means for structural analysis of saponins in plant extracts or other types of matrixes, such as commercialized natural products.

MATERIALS AND METHODS

Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Sodium acetate, formic acid and acetic acid were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany). Lithium chloride and sodium acetate (AnalaR Normapur® grade) were purchased from Merck (Darmstadt, Germany). Hederacoside C and α-hederin, which were used for mass spectrometric degradation reactions with a purity greater than 98%, were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany).

Sample preparation

For MS purposes, compounds were prepared at 10 μg mL^{-1} and injected by infusion for structural analysis. The following concentrations were used: sodium acetate, 1x10^{-5} M; formic acid, 0.1%; acetic acid, 5 mM; and chloride salts containing sodium and lithium, 1x10^{-7} M.

Mass Spectrometry

System control and data evaluation were performed using the Agilent LC/MSD Chemstation software package. ESI/MS was performed by means of the LC/MSD ion trap (Agilent, Waldbronn, Germany). The ESI/MS/MS was performed by infusion on an MSD ion trap. The ESI/MS/MS conditions were the following: nitrogen was used as a nebulizer/drying gas at 15 psi at 5 L min^{-1}; drying temperature was 350°C; capillary voltage was set at $\pm$ 4.5 kV; and the flow rate was set to 300 μL h^{-1}. Spectra were obtained over an m/z range of 100-1500. The fragmentation amplitude was changed from 0.5 to 1.5 to increase the collision energy for obtaining a higher degree of degradation.

RESULTS AND DISCUSSIONS

Structural analysis of α-hederin

The fragmentation scheme and nomenclature used is based on the ones described by Costello *et al.*, 1988 (10).

Negative mode

ESI mass spectra in negative mode evidenced deprotonated species that can be useful for the sugar sequence analysis because of their low fragmentation. Due to the neutral charge of the sugar array, the molecule shows a low ionization abundance in this acquisition mode. In figure 1A, the TIC spectra of α-hederin shows a deprotonated molecule [M-H]$^-$ ion at an m/z value of 749. In figure 1B, the MS^2 spectra of the [M-H]$^-$ ion presents three major intensity ions with m/z values of 603, 585 and 471. The ion with an m/z value of 585 is a consequence of the dehydration suffered by the 603 ion. Therefore, 585 is a Z type ion, 603 is a Y type ion and 471 is assigned to deprotonated species, corresponding to the hederagenin aglycone. The prior analysis showed a deoxyhexose followed by a pentose ring (see figure 1B).

Positive mode

On the protonated spectra of α-hederin, an internal sugar residue loss phenomenon was observed. This ion is named Y* and it was attributed to protonated α-1→2 and α-1→6 linkages on tri and tetrasaccharides (1). Although this process was mainly described for [M+H – 162 amu]$^+$ losses (6), the results indicate an unusual [M+H – 132]$^+$ ion loss in high abundance, obtaining a false sugar sequence. This phenomenon was clearly evidenced in figure 3A and it was also showed by Gaillard *et al.*, 2003 (9), in the cases when structural analysis was not carried out (9). The m/z 619 ion and its dehydration ion (m/z 601) in the protonated MS^2 spectrum show a loss of pentose residue (132 amu), followed by a rhamnose residue (146 amu) up to the m/z 473 ion (the protonated hederagenin aglycone). However, the m/z 605 ion allows deducing the real sequence with rhamnose losses, but in a lower abundance compared with the Y* ion. This "false" sugar sequence can be solved as the sodium adduct, for which this reaction has not been observed. A proposed reaction pathway is shown in figure 2, where the internal loss of the deoxypentose sugar of α-hederin is generated.

In figure 3A, a protonated spectrum of α-hederin was detectable at m/z 547. This is an X type of ion across the sugar ring assigned to a $^{1,3}X_0\beta$. However, due to the low collision energy generated in protonated adducts, no additional cross ring cleavages were detected, therefore, no more structural information could be obtained from this spectrum.

In figure 3B, the spectrum was taken with formic acid and sodium acetate. The sodium adduct shows cross ring cleavages assigned as C_2H_4O loss (-44 Da), which are characterized by a rearrangement that results in a stable oxo-group (by oxidation of an alcohol), forming a stable γ-hydroxyester (11). An MS^2 analysis of the protonated ion produced the ion ($^{0,4}X_{1\beta}$) with an m/z of 729 with the highest abundance from rhamnose sugar, accompanied by the m/z 627 ion, which represents a glycosidic cleavage (Y$_{1\beta}$). And the m/z 583 ion, which represents the loss of C_2H_4O (-44 Da). The C_2H_4O (-44 Da) lost is a cross ring cleavage, but this time from a pentose ring ($^{3,5}X_{0\beta}$), generating a stable γ-hydroxyester. In table 1, the same fragmentations that were mentioned above are observed, but without cross ring cleavages in the rhamnose sugar when a lithium adduct is used. The MS^3 spectrum was obtained from the $m\backslash z$ 729 ion, showing a 146 amu loss (data not showed), from which it can be inferred that the m/z 583 ion ($^{3,5}X_0\beta$) can be produced not only through the loss of C_2H_4O of the 627 ion, but also through the loss of the 729 ion.
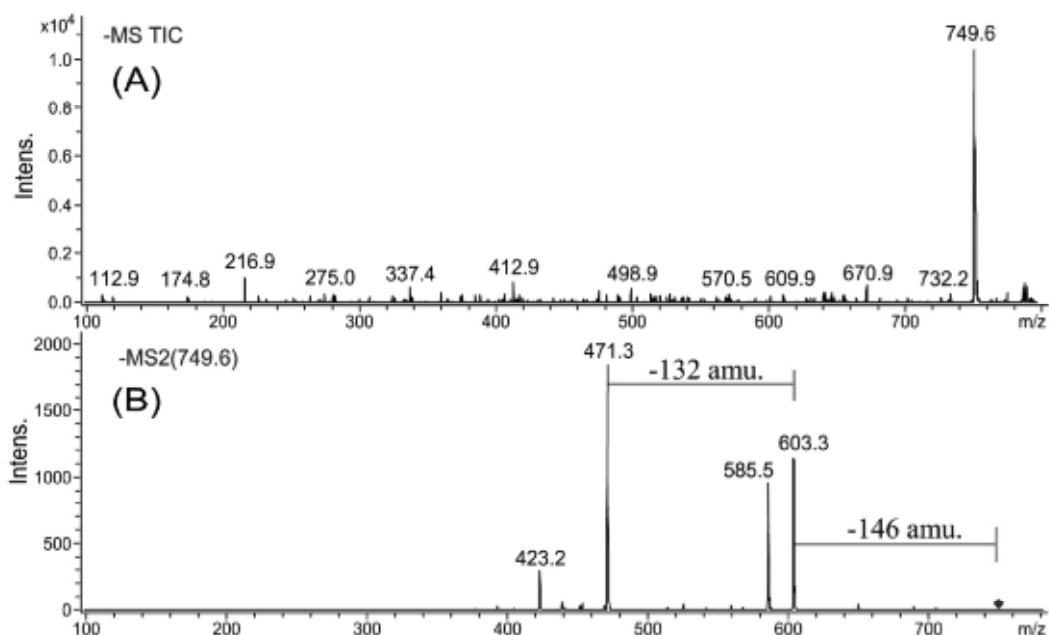
Figure 1. Negative ions ESI-MS^n spectra: **A**. TIC of α-hederin (750.95 Da) in negative mode; **B**. MS^2 of 749 ion.
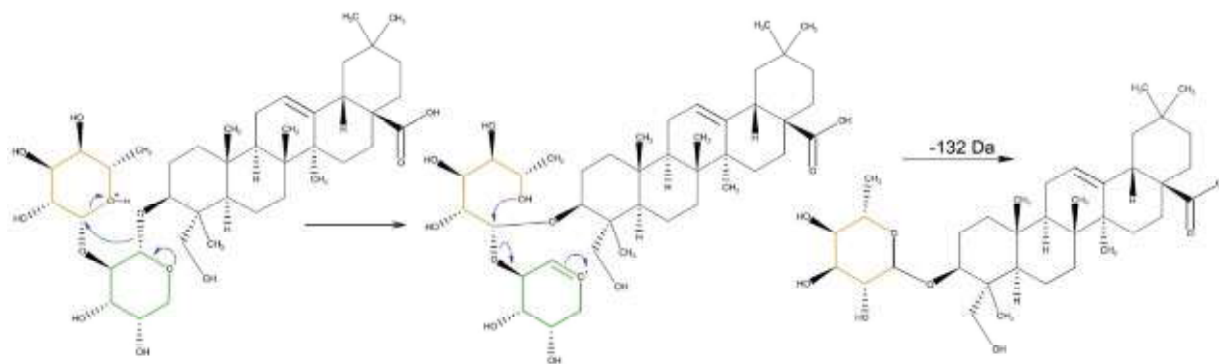

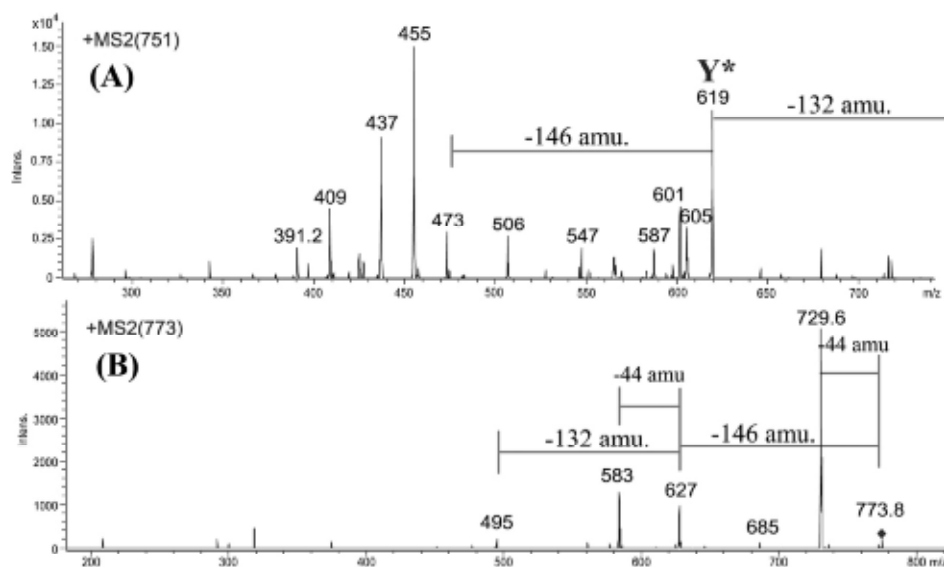**Figure 2.** Internal sugar loss in α-hederin. A reaction mechanism proposed to occur on the β-sugar chain (C3).

Figure 3. A. MS^2 of protonated α-hederin (*m/z* 751). B. MS^2 sodium adduct of α-hederin.

Table 1. Fragment ions (*m/z*) observed in MS^n spectra of [M-H]$^-$, [M+H]$^+$, [M+Li]$^+$ and [M+Na]$^+$ precursor ions of saponins. The protonated species of hederacoside C were not obtainable.

			C28			C3					Sugar moiety					
		m/z	$Y_{2\alpha}$	$Y_{0\alpha}$	CRC	$Y_{1\beta}$	$Z_{1\beta}$	$Y_{0\beta}$	Y^*	CRC	C_3	B_3	Y_2	Z_2	C_2	CRC^a
α-hederin	[M-H]	749.6				603.3	585.5	471.3								
	[M+Na]	773				627		495		729.6 $^{0,4}X_{1\beta}$ 583 $^{3,5}X_{0\beta}$					319	
	[M+Li]	757				611		479		567 $^{3,5}X_{0\beta}$						
	[M+H]	751.6				605	587	473	619	547 $^{1,3}X_{0\beta}$						
Hederacoside C	[M-H]	1120.5		749.8		603						470				
	[M+Na]	1244	1098	773	1140 $^{0,2}X_{3\alpha}$	627		495		729.6 $^{0,4}X_{1\beta}$	493		347	331	349	243 $^{3,5}A_{1\alpha}$ 289 $^{2,4}X_{1\alpha}$ 391 $^{0,4}A_{2\alpha}$ 405 $^{3,5}A_{2\alpha}$
	[M+Li]	1227		757		611				567 $^{3,5}X_{0\beta}$	476.9	458.9	331	315	333	

^{a} CRC: Cross ring cleavage.

Structural analysis of hederacoside C

Negative mode

The ESI mass spectra in negative mode showed a deprotonated species [M-H]$^-$ *m/z* at 1220.5. The MS^2 spectrum shows three ions: 749.8, 603, and 469.6. The sum of the ions 749.8 and 469.6 equals the complete molecule (1220.5), so the *m/z* 469.6 is a B type ion and 750 is a Y type ion, which is the most abundant ion due to the especially weak ester glycosidic linkage, but it can be retained when the charge is at the reducing terminus. Likewise, in the prior analysis of α-hederin, it was possible to confirm the sugar sequence.

Positive mode

Due to the structural differences between α-hederin and hederacoside C, protonated species were not obtainable. Depending on the functional groups, some molecules are easier to obtain a charged species than others. In this case, the neutral character of this sugar residue induces problems in the generation of ionic species (12). Nevertheless, the sodium adduct can be obtained even with very low amounts of sodium. Additionally, the sodium ion can provide more information due to the generation of more fragmentations. In figure 4, the ESI mass spectra of hederacoside C showed a [M+Na]$^+$ ion at *m/z* 1244. The MS^2 spectra clearly demonstrated that the most abundant ion is the *m/z* 773 ion, which can be assigned to the cleavage of the low bond energy of the ester sugar linkage (13, 14). However, it is possible to analyze the first sugar break at *m/z* 1098, which indicates that the last sugar residue is deoxyhexose, followed by a hexose residue with an *m/z* of 935. To obtain more structural information,

it is possible to increase the collision energy with higher values of fragmentation amplitude, and to change the width value of the spectrum when the stability of the precursor ions are demonstrably low. When the spectra were obtained at 0.5 V amplitude value, the *m/z* 1244 ion was not completely fragmented, and less information could be obtained. When the fragmentation amplitude was set at 1.5 V, the precursor ion 1244 was completely fragmented, producing more fragment ions (data not shown). However, a great amount of ion production does not permit a structural analysis; and a balance between an amount of fragmentation (where the information that can be obtained) and upper ion production (which makes spectra uninterpretable) is necessary.

The two more abundant ions in hederacoside C fragmentation were the *m/z* 773 ion, assigned to sodiated α-hederin, and the *m/z* 493 ion, which corresponds to sodiated α-sugar moiety, owing to the cleavage of the ester bond at C28. This charge is easy to transfer to the oligosaccharide chain. The MS^3 results of 493 are shown in figure 4. The two more abundant ions are those that can retain the charge on the reducing termini. The 349 and 347 ions were attributed to ions type C_2 and Y_2, respectively. As it can be observed in figure 5, the cross ring cleavage occurring at the *m/z* 391 and 405 ions, along with the *m/z* 349 ion, provided information about the 1→6 linkage between two hexoses. In the same way, it was possible to note that 289 and 243 ions provided information about 1→4 linkages between the second hexose and deoxyhexose sugar. In the lithium adducts, not enough structural information could be obtained. Furthermore, table 1 shows that the cross ring cleavages seen in the sodium adducts were not observed in the sugar moiety.
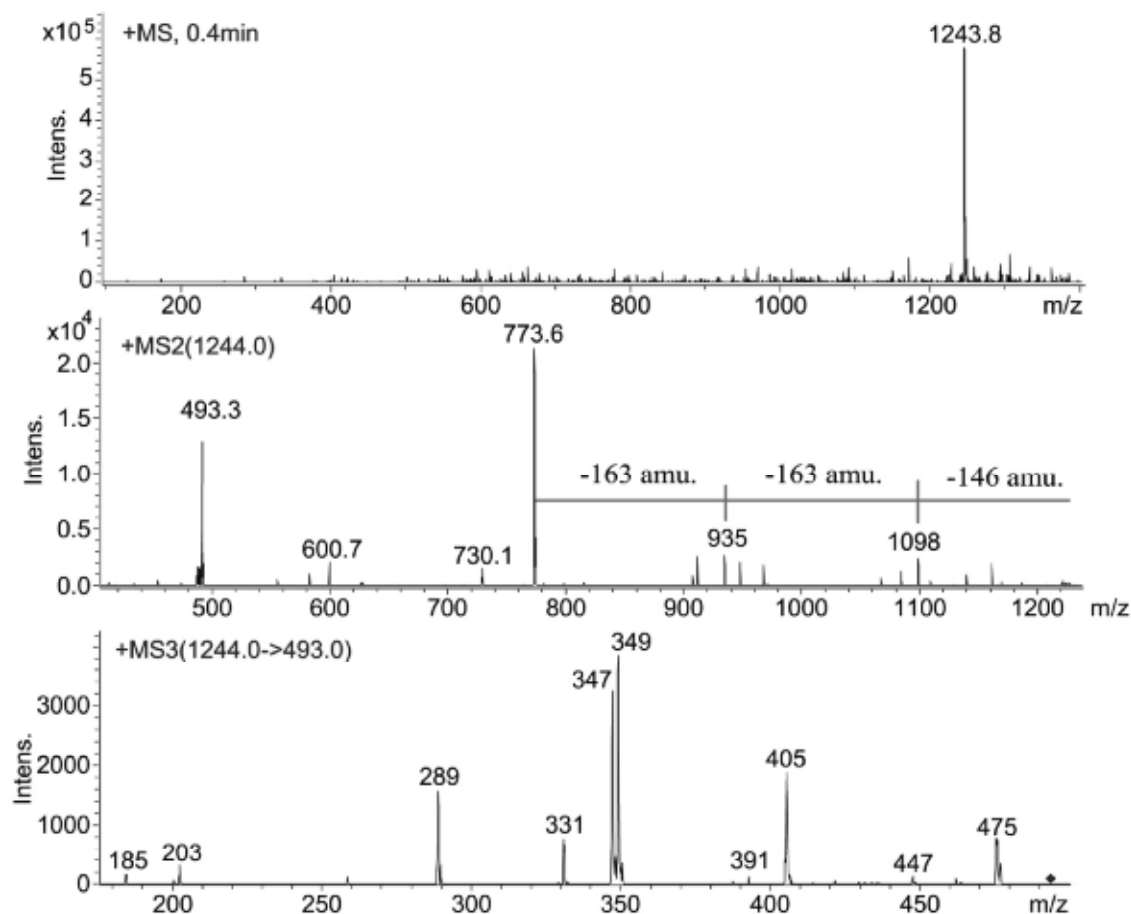
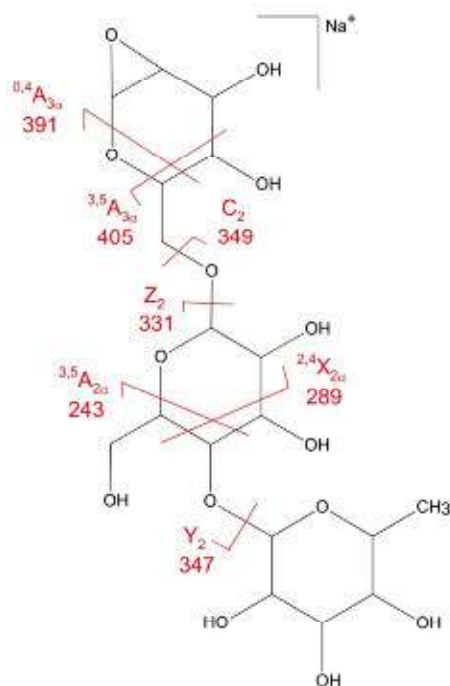**Figure 4**. Fragment ions of the [M+Na]+ adduct of hederacoside C.

Figure 5. Schematic drawing of the sodiated m/z 493 ion MS^3 fragmentation. The α-sugar moiety of hederacoside C.

Clearly, controlling adduct formation and fragmentation energies is a practical tool that can provide much usable structural information for the elucidation of structurally related compounds (15). In natural products, it is common to isolate molecules that share the same nucleus, and it is important for an analyst to obtain useful information through a high-sensitivity methodology when just a few micrograms or even nanograms of a purified fraction or purified compound can be used.

CONCLUSIONS

Under ESI-MS^n conditions, hederacoside C and α-hederin presented characteristic fragmentations, and they were practical for the structural analysis of these types of compounds. Hence, due to the α-1→2 linkage present on the C3 carbon, the molecule α-hederin showed an internal sugar residue loss in the protonated mode. This fact must be taken into account every time plants containing these triterpene saponins are analyzed through ESI-MS^n

with protonated species. However, hederacoside C did not present this problem because it favors sodium adducts due to the longer sugar chain of this molecule. It can be corroborated that the ester bond at C28 presents a preferential cleavage via the higher abundance of the $Y_0\alpha$ ion on the MS^2 of the hederacoside C spectra. In conclusion, the afore mentioned analysis can be applied to the rapid identification of these triterpene saponins in plant extracts as a further quality control method.

ACKNOWLEDGEMENTS

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