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Application of ESI/MS, CID/MS and tandem MS/MS to the fragmentation study of eriodictyol 7-*O*-glucosyl-(1→2)-glucoside and luteolin 7-*O*-glucosyl-(1→2)-glucoside

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Abstract

A mass spectrometric method based on the combined use of positive and negative electrospray ionization, collision-induced dissociation and tandem mass spectrometry has been applied to the structural characterization of the eriodictyol 7-*O*-glucosyl-(1→2)-glucoside and luteolin 7-*O*-glucosyl-(1→2)-glucoside. The low-energy product ion mass spectrum of $[M+H]^+$ and $[M-H]^-$ ions showed extensive fragmentation of the diglucose moiety, loss of the glycan residue, and fragmentation of the aglycon units that permit characterization of the interglycosidic linkage and the substituents in the A- and B-rings. Both glycosides were shown to yield the $^{0,2}X_0{}^{0,2}X_1$ ion which can be considered as characteristic of the 1→2 interglycosidic linkage in the glucoglucoside adducts, since it can not be formed in the case of other interglycosidic types. In the case of the eriodictyol diglucoside the 1, 3 fragmentation of the C-ring was observed before those involving the carbohydrates thus allowing the position determination of the diglucoside moiety on the A-ring. In the negative ion mode only the luteolin diglucoside was shown to undergo collision-induced homolytic and heterolytic cleavages of the *O*-glycosidic bond producing the aglycone radical–anion $[Y_0–H]^{\bullet-}$ and $Y_0{}^-$ product ions, while this was not observed in the case of eriodictyol glycoside. CID MS/MS analysis of the sodiated molecules gave complementary informations for the structural characterization of the studied compounds. The $B_2{}^+$ fragment which is useful for establishing that the terminal carbohydrate unit is linked to another carbohydrate and not directly to the aglycone was obtained as base peak. This result is of analytical value for the differentiation of *O*-diglycosyl and di-*O*-glycosyl flavonoids.

Keywords: Flavonoids; Glucosides; ESI/MS; MS/MS; CID/MS

1. Introduction

Flavonoids are polyphenolic natural products which are recognized as one of the largest and most widespread class of plant constituents occurring throughout the plant kingdom, and are also found in substantial levels in commonly consumed fruits, vegetables and beverages. Flavonoids have recently aroused considerable interest because of their potential beneficial biochemical and antioxidant effects on human health. Most of the experimental results demonstrate that flavonoid compounds have several biological activities including radical scavenging, anti-inflammatory, anti-mutagenic, anti-cancer, anti-HIV, anti-allergic, anti-platelet and anti-oxidant activities [1]. Flavonoids are grouped together into subclasses based on their basic chemical structures; the most common ones being flavones, flavonols, isoflavones, flavanones, anthocyanins and chalcones. Flavonoids can exist as free aglycones but most of them commonly occur as *C*- or *O*-glycosides. Disaccharides are also often found in association with flavonoids and occasionally tri- and even tetrasaccharides.

The structure determination of flavonoids plays an important role in many areas of science. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. The structural characterization of flavonoids is generally performed by a combination of spectroscopic methods, including ultraviolet (UV), nuclear magnetic resonance (NMR) and mass spectrometry (MS). Among these methods

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mass spectrometry (MS) is one of the important physicochemical methods applied to the structure determination of natural products due to its sensitivity, rapidity, and low levels of sample consumption [2–4]. With the development of soft ionization techniques, mass spectrometry has become a powerful analytical tool of flavonoids, a polar, non-volatile, and thermally labile classes of compounds [5–7]. Mass spectrometric methods such as electron ionization EI/MS [8], fast atom bombardment FAB/MS [9], atmospheric pressure chemical ionization APCI/MS [5,10,11] and electrospray ionization ESI/MS [12–14] have proved useful to determine flavonoids in herbs and other foods [8,12,14,15]. The coupling of liquid chromatography/mass spectrometry (LC/MS) with API/MS and ESI/MS/MS techniques has been demonstrated to be a powerful tool for the identification of flavonoids [16–18]. The combination of FAB with collision-induced dissociation (CID) and tandem mass spectrometric techniques has been shown to yield important structural information for the characterization of underivatized flavonoid aglycones and glycosides [9,19]. Furthermore, the low-energy CID MS/MS spectra of various flavonoid aglycones and glycosides in mixture were described in both LC/ESI/MS/MS [14,15] and LC/APCI/MS/MS [7,10]. It has been demonstrated that fragment ions provide important structural information for flavonoids and can be used to establish the distribution of the substituents between the A- and B-rings. A careful study of the fragmentation patterns in CID MS/MS can also be of a particular value in the structural elucidation of *O*- and *C*-glycosides [9].

Since the number of flavonoid compounds is steadily increasing, and in order to further increase the applicability of LC/ESI/MS/MS in the analysis of plant phenolics, it appeared important to investigate the fragmentation pathways of new flavonoids in order to correlate the structures with their fragmentation patterns, thus allowing a rapid identification and analysis of flavonoids. In the present study, LC/MS and MS/MS using ESI were applied for molecular mass and structural information of two flavonoid glycosides, namely eriodictyol 7-*O*-glucosyl-(1→2)-glucoside and luteolin 7-*O*-glucosyl-(1→2)-glucoside. Analyses were first conducted using ESI/MS in the negative and positive ion modes to obtain ionized molecular species. Then, tandem MS/MS spectra were obtained by low energy collision induced dissociation (CID) of the $[M+H]^+$ or $[M-H]^-$ ions, and were interpreted to propose plausible fragmentation pathways for each studied compound.

2. Experimental

2.1. Materials

The two studied flavonoid glycosides were isolated from an aqueous methanolic extract of *G. alypum* aerial parts. Fresh aerial parts were air-dried in shade at room temperature and the dried aerial parts were powdered. Hundred grams of the obtained powder were macerated during 48 h at room temperature with 500 mL of a 3:2 mixture of distilled water–methanol. The crude preparation was filtered and concentrated under reduced pressure to provide a crude extract which was stored at −20 °C until time of use. The resulting aqueous phase was extracted with hexane and the subsequent aqueous phase was subjected to a SPE column. Elution was performed successively by H_2O, MeOH 10%, MeOH 40%, MeOH 50% and MeOH 100%. The fractions were concentrated under reduced pressure, lyophilized, tested for their scavenging activity, and analysed through analytical HPLC. The MeOH 50% fraction, which was shown to be rich in natural antioxidant compounds, was explored first using semipreparative HPLC. After several successive injections, samples corresponding to the same chromatographic peaks were verified by analytical HPLC, concentrated under reduced pressure and lyophilised. This operation gave the studied purified compounds.

2.2. Mass spectrometry

LC/MS analyses were performed with a chromatographic system (Alliance) consisting of a Waters 2695 separations module equipped with an autosampler and a Waters 2487 dual lambda absorbance detector (Waters, Milford, MA, USA). The column was a 150 mm × 2.1 mm Interchrom UP5ODB#15E (Uptisphere 5 μm ODB) with a 10 mm × 2.1 mm precolumn from Interchim (Montluçon, France). Chromatography was carried out in isocratic mode with a 60/40 mixture of acetonitrile (RS-Plus quality for HPLC from Carlo Erba) and water with 0.2% acetic acid. The flow rate was 0.2 mL/min, the analyses were performed with the column and the samples kept at ambient temperature and 5.0–10 μL was injected for each analysis. The effluent from the UV detector was introduced into the mass spectrometer without any splitting of the flow. The HPLC system was coupled directly to a Quattro LC/MS/MS triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a pneumatically assisted electrospray ionisation source (ESI). Data acquisition and processing were performed using a MassLynx NT 3.5 data system. The electrospray source parameters were fixed as follows: electrospray capillary voltage 3.25 kV in positive mode and 3 kV in negative mode, source block temperature 120 °C, desolvation gas temperature 400 °C. Nitrogen was used as drying gas and nebulising gas at flow rates of approximately 50 and 450 L/h.

3. Results and discussion

The samples selected for this study were eriodictyol 7-*O*-glucosyl-(1→2)-glucoside **1** and luteolin 7-*O*-glucosyl-(1→2)-glucoside **2** (Fig. 1). These flavonoids have to our knowledge not been examined in previous mass spectrometric studies and are thus interesting from a phytochemical and analytical point of view. In addition, compounds **1** and **2** are diglycosides consisting of two glucoside units, which are different from the most previously reported flavonoids consisting of rhamnose and glucose moieties [4,9,20–28].

3.1. Nomenclature

The major diagnostic fragmentations for flavonoid identification are those involving the cleavage of two C–C bonds of the

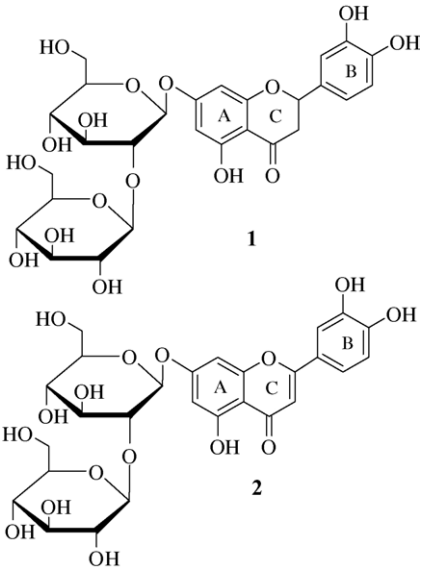
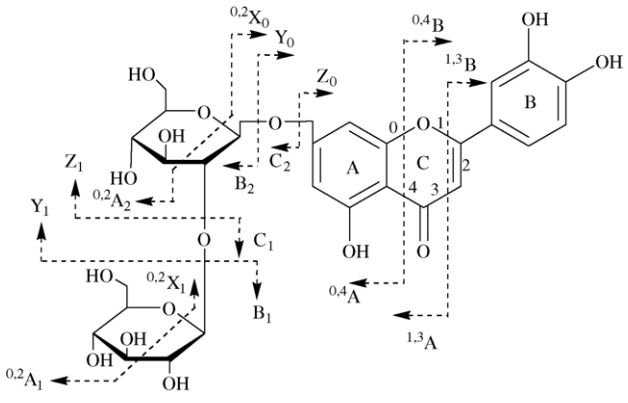


Fig. 1. Structure of the studied flavonoid glycosides.

C-ring giving two structurally informative fragment ions. These ions provide information on the number and type of substituents in A- and B-rings. In this paper, these fragment ions are designated according to the nomenclature previously proposed by Ma et al. [19]. For free aglycone, the ^{i,j}A and ^{i,j}B labels refer to the fragments containing intact A- and B-rings, respectively, in which the superscripts i and j indicate the C-ring bonds that have been broken. For flavonoid glycosides, the classical nomenclature proposed by Domon and Castello [29] for glycoconjugates was adopted to designate the fragmentations: $^{k,l}X_j$, Y_j, Z_j represents the ions still containing the aglycone, where j is the number of the interglycosidic bonds broken (counted from the aglycon) and k and l denote the cleavage within the carbohydrate rings (Scheme 1).

3.2. LC/ESI/MS

After isolation, the purity of the two studied flavonoids was determined using analytical HPLC with mass spectrometric detection (both positive and negative ion modes). When the analysis was conducted in the positive ion mode, abundant protonated molecules $[M+H]^+$ ions and protonated aglycones Y_0^+ ions at m/z 613 and 289 for **1** and m/z 611 and 287 for **2** were observed (Table 1). In addition, $[M+Na]^+$, $[M+K]^+$, and product ions allowing the partial characterization of the flavonoids were also observed. The results were consistent with their structures and their aglycone constituents, and are also consistent with available literature data. Additional weak peak signals were observed at m/z 451 and m/z 449, respectively, for compounds **1** and **2** and corresponding to the Y_1^+ in agreement with their diglycosidic structures. The presence of an abundant Y_0^+ in addition to the Y_1^+ ions are in agreement with a protonated *O*-diglucoside structure which by rearrangement reactions at the interglycosidic bonds give rise to Y_1^+ and Y_0^+ ions. In the case of *O*-*C*-diglycosides, only Y_1^+ ions are formed, whereas *C*-glycosides give only $[M+H]^+$ ions, in addition to other characteristic ion fragmentations [9,23].



Scheme 1. Ion nomenclature adopted for flavonoid glycosides fragmentation.

Table 1
Mass spectrometric data for the flavonoids **1** and **2** obtained using negative and positive LC/ESI/MS analyses

	1	**2**
Positive	613	611
$[M+H]^+$	451, 289	449, 287
Fragments		
Negative	611	609
$[M-H]^-$	475, 287	437, 285
Fragments		
Aglycone molecular mass (u)	288	286
Compound molecular mass (u)	612	610

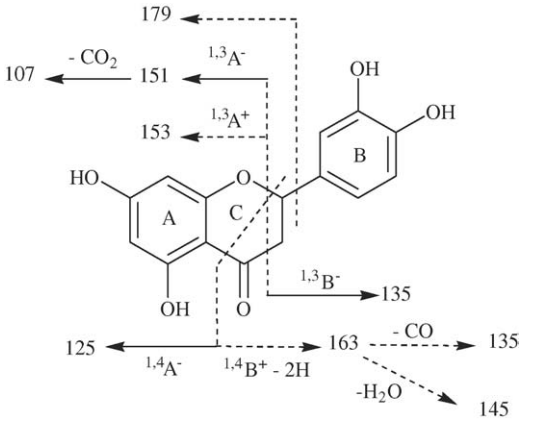
These results were also confirmed when the analyses were conducted in the negative ion mode, where the parent ions $[M-H]^-$ were observed in addition to the fragments noted in Table 1. In order to characterize the fragmentation pathways of these compounds, CID/MS/MS spectra exhibiting the protonated and deprotonated fragment ions were explored and the results are discussed below.

3.3. Positive CID MS/MS analysis

In the structure analysis of flavonoids, positive ion CID spectra are most frequently used [11,15], whereas negative ion CID spectra are often considered to be more difficult to interpret [21]. Protonation is believed to occur preferentially in the aglycone part of the molecule, in particular, at the carbonyl oxygen atom [30]. Charge delocalisation in the C-ring can lead to a protonated molecular species, which are highly stabilized by resonance. Subsequent charge-remote rearrangements take place resulting in the Y_0 and Y_1 ions, most likely involving hydrogen rearrangement from hydroxyl groups, which can sterically approach the glycosidic bonds.

3.3.1. Compound *1*

The molecular mass of compound **1** was concluded to be 612 Da on the basis of its positive ion electrospray mass spectrum, which showed parent ions ($[M+H]^+$) at m/z 613. In



Scheme 2. Main fragmentations observed in CID MS/MS spectra of the $[M+H]^+$ (dashed arrows) and $[M-H]^-$ (full arrows) ions of eriodictyol.

addition, a loss of neutral fragments corresponding to a mass of 324 was observed indicating the presence of a two hexose residues. This loss gave an ion at *m/z* 289 corresponding to an aglycone with a molecular mass of 288 Da, suggesting compound **1** as a flavanone-based compound. This conclusion was supported by the CID MS/MS spectra of the ion at *m/z* 289 (Y_0^+), which exhibited five main diagnostic fragmentations located at *m/z* 271 ($-H_2O$), 179 (–B-ring), 163 ($^{1,4}B^+$–2H), 153 ($^{1,3}A^+$), 145 ($^{1,4}B^+$–2H–H_2O) and 135 ($^{1,4}B^+$–2H–CO) (Scheme 2). Taking into account the reported data concerning the fragmentation of the flavonoid skeleton, the data indicated a 3′,4′,5,7-tetrahydroxyflavanone [31].

In order to study the fragmentations of flavonoid 1, CID spectra of the $[M+H]^+$ ion (*m/z* 613) were recorded at various collision energies. A typical CID spectrum is shown in Fig. 2. When the pseudomolecular ions at *m/z* 613 were selected as parent ion and the daughter ions were recorded at different energies, the ESI/MS/MS spectra showed, even with low relative intensity, the characteristic *m/z* 493 ($^{0,2}X_0^+$ or $^{0,2}X_1^+$) ion due to the loss of 120 Da by cleavage of the *O*-glycoside moiety. In addition, the fragmentation patterns observed for compound **1** showed several ion signals in agreement with previously reported data for flavanones [30]. Relatively abundant Y type ions are observed at *m/z* 289 (base peak Y_0^+) and 451 (Y_1^+), whereas other ions at *m/z* 595 $[M+H-H_2O]^+$, 577 $[M+H-2H_2O]^+$, 433 (Z_1^+), 373 ($^{0,2}X_0{}^{0,2}X_1^+$), 331 ($^{0,2}X_0Y_1^+$), 325 (B_2^+) and 163 (B_1^+) formed by common fragmentation routes are also present. It is worth noting that a $^{0,2}X_0{}^{0,2}X_1^+$ ion can be seen in the CID MS/MS spectrum of compound **1**. This ion can be considered as characteristic of the 1→2 interglycosidic linkage in the 7-*O*-glucoglucoside adducts, since it can not be formed in the case of other interglycosidic types. Additional peaks at *m/z* 415 (Y_1^+–2H_2O), 397 (Y_1^+–3H_2O) and 355 (Y_1^+–2H_2O–60), which are also characteristic of flavanone *O*-diglycosides [24,30], were also observed. The formation of Z_1^+ ion (*m/z* 433) and absence of the corresponding radical $Z_1^{\bullet+}$ ions are useful for establishing that the eliminated terminal carbohydrate unit is linked to another carbohydrate and not directly to the aglycone and could be of analytical value for

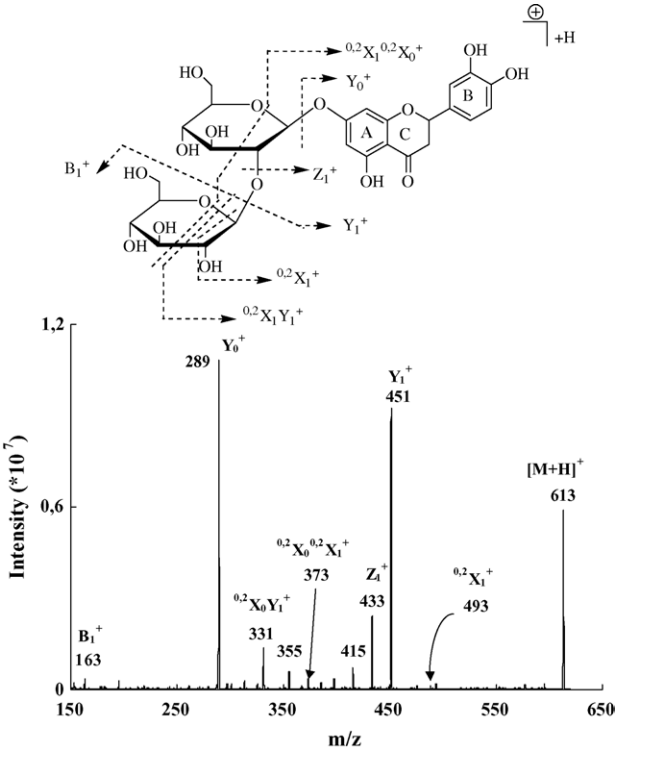


Fig. 2. CID mass spectrum of protonated eriodictyol 7-*O*-sophoroside **1** (*m/z* 613) and the proposed corresponding fragmentations.

the differentiation of *O*-diglycosyl and di-*O*-glycosyl flavonoids [9].

In the spectrum shown in Fig. 2, the most striking feature is the high intensity of the signal for the Y_1^+ ion corresponding to a neutral loss of 162 Da. In previously reported data concerning flavonoid rutinoside and neohesperoside MS/MS spectra, an internal loss of the glucose moiety leading a labeled Y^* fragment signal was observed in addition to the presence of the Y_0^+ and Y_1^+ ions [23,24]. The presence of this unusual glucose loss is easily distinguished when the two carbohydrate moieties are different as in the case of the rhamnoglycoside derivatives. This was not the case for compound **1**, which is comprised of two glucosyl moieties where the distinction between the terminal and the inner glucose was not possible. This may explain the high abundance of the observed ion located at *m/z* 451, which may include either the ion resulting from the Y_1 fragmentation $[M+H-162]^+$ corresponding to the terminal glucose loss and the Y^* fragmentation corresponding to the inner glucose loss. The fact that Y_0^+ is more abundant than Y_1^+ points to an 1→2 linkage and is in agreement with previously reported data [23,24]. This result was further confirmed through the observation of the fragmentations presented in Fig. 2, where two successive losses of 120 mass units were observed yielding the $^{0,2}X_0{}^{0,2}X_1^+$ fragment and confirming such an interglycosidic linkage.

In structural analysis of flavonoids, MS/MS techniques have been often applied to the protonated molecules, $[M+H]^+$, while few data have been reported on other cationized ions [22,32]. This fact prompted us to investigate the CID MS/MS spectra of the $[M+Na]^+$ ion of compound **1** in order to better understand their behaviour. The pseudomolecular ion at *m/z* 635 ($[M+Na]^+$)

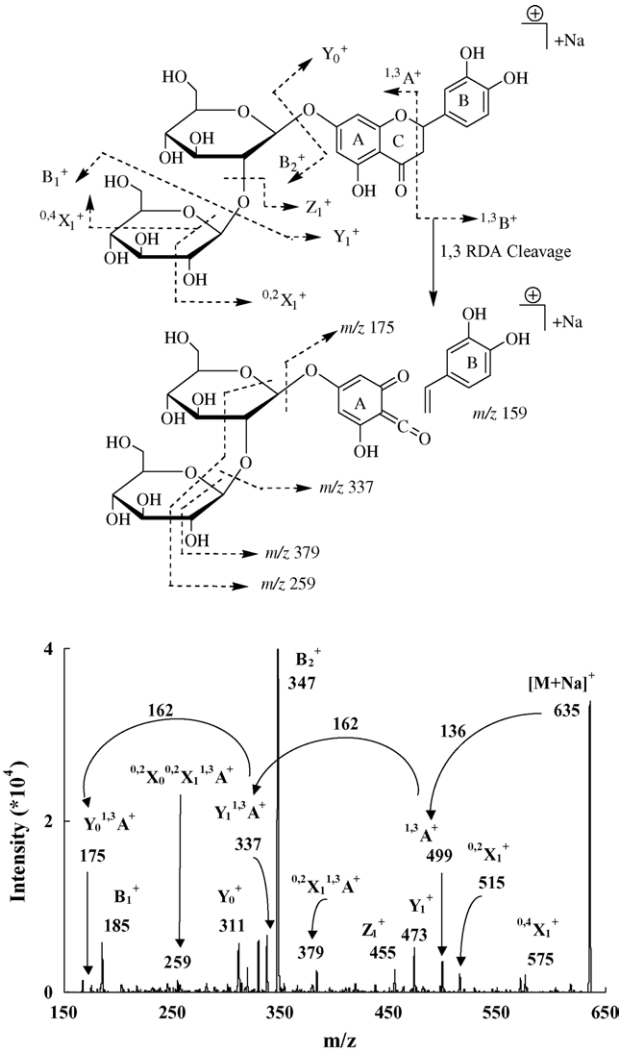


Fig. 3. CID mass spectrum of sodiated eriodictyol 7-*O*-sophoroside **1** (*m/z* 635) and the proposed corresponding fragmentations.

was thus selected as parent ion and the daughter ions were recorded at various CID collision energies. An example of a representative spectrum is shown in Fig. 3. The most striking feature of the spectrum is the high relative abundance of the ion at *m/z* 347 (B_2^+) corresponding to the loss of the aglycone moiety. This fragmentation clearly indicates that the terminal carbohydrate unit is linked to the other carbohydrate and not directly to the aglycone. This fragmentation is thus of useful analytical value for the differentiation of *O*-diglycosyl and di-*O*-glycosyl flavonoids. The Y_0^+ fragment at *m/z* 311 was observed with a relatively low abundance, as was the radical aglycone product ion observed at *m/z* 310. Another important fragment was observed at *m/z* 499 (corresponding to the $^{1,3}A^+$ fragment), while the complementary $^{1,3}B^+$ was observed at *m/z* 159 at low abundance. This showed that the fragmentation involving the C-ring could occur before those involving the diglucoside unit. This is of analytical value since it indicated that the two glycoside moieties are located on the A-ring. Further successive losses from the $^{1,3}A^+$ fragment of the terminal and inner glucose units, give ions at *m/z* 337 ($Y_1{}^{1,3}A^+$) and 175 ($Y_0{}^{1,3}A^+$), respectively.

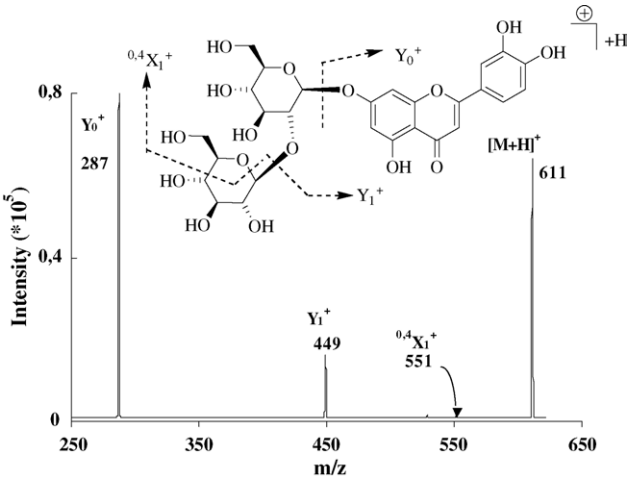
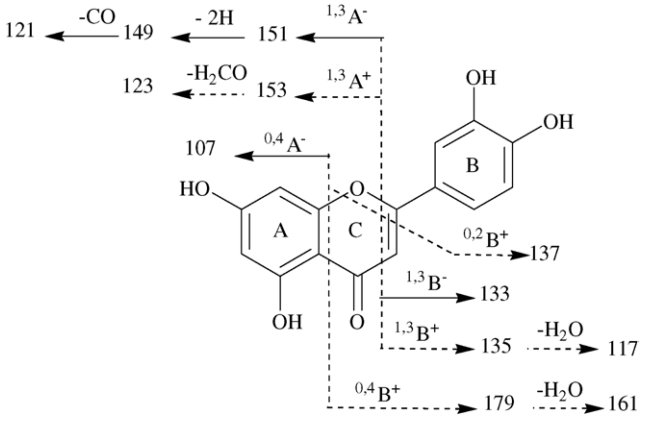


Fig. 4. CID mass spectrum of protonated luteolin 7-*O*-sophoroside **2** (*m/z* 611) and the proposed corresponding fragmentations.

Additional signals were observed at *m/z* 379 ($^{0,2}X_1{}^{1,3}A^+$) and 259 ($^{0,2}X_0{}^{0,2}X_1{}^{1,3}A^+$) and were in full agreement with the 1→2 interglycosidic linkage.

3.3.2. Compound 2

The fragmentations observed for protonated ion $[M+H]^+$ generated from compound **2** are very simple, presenting the characteristic fragmentation of flavonoid *O*-diglycosides [9,23–25] and yielding two major ion signals at *m/z* 287 and 449, respectively (Fig. 4). These signals correspond respectively to the Y_0^+ and Y_1^+ ions, which are formed by rearrangement reactions at the interglycosidic bond. Deuterium labeling experiments indicated that hydroxyl hydrogen atoms are involved in the formation of these ions [9]. The observation of a loss of a 324 Da neutral fragment in the mass spectra of compound **2** confirmed the presence of two hexose moieties. In order to characterize the aglycone part of compound **2**, the CID MS/MS spectra were recorded for the $[M+H]^+$ and Y_0^+ ions. The results presented in Scheme 3 showed that the aglycone ion observed at *m/z* 287 (Y_0^+) exhibited the following fragmentations at *m/z* 269 (Y_0^+–H_2O), 241 (Y_0^+–H_2O–CO), 179 ($^{0,4}B^+$), 161 ($^{0,4}B^+$–H_2O), 153 ($^{1,3}A^+$), 137 ($^{0,2}B^+$), 135 ($^{1,3}B^+$), 123



Scheme 3. Main fragmentations observed in CID MS/MS spectra of the $[M+H]^+$ (dashed arrows) and $[M-H]^-$ (full arrows) ions of luteolin.

($^{1,3}A^+$–H_2CO), and 117 ($^{1,3}B^+$–H_2O), which are in agreement with previously reported data for luteolin [19,33].

In the CID MS/MS spectra of the protonated $[M+H]^+$ ion, the Y_0^+ (*m/z* 287) was the base peak, while the Y_1^+ (*m/z* 449) was observed with a very low abundance (Fig. 4). This indicated the existence of a diglycoside moiety since compounds showing a high abundance for *m/z* 449 have two glucose units attached to different positions of the aglycone [34,35]. In addition, and according to previously reported analysis conducted on rhamnoglucoside derivatives, the fact that the $Y_0^+ > Y_1^+$ is more in favor with a 1→2 interglycosidic linkage [23]. In order to see if this was also the case of glucosylglucoside derivatives, the fragmentations appearing in the higher *m/z* value region were thoroughly examined. Among the observed ions, the presence of signals at *m/z* 593 $[M+H–18]^+$, 551 ($^{0,4}X_0^+$), 521 ($^{0,3}X_0^+$), 491 ($^{0,2}X_0^+$), 431 (Z_1^+) and 371 ($^{0,2}X_0{}^{0,2}X_1^+$) was observed. The latter fragmentation is of a great importance since it results from two successive losses of 120 mass units, indicating a 1→2 interglycosidic linkage.

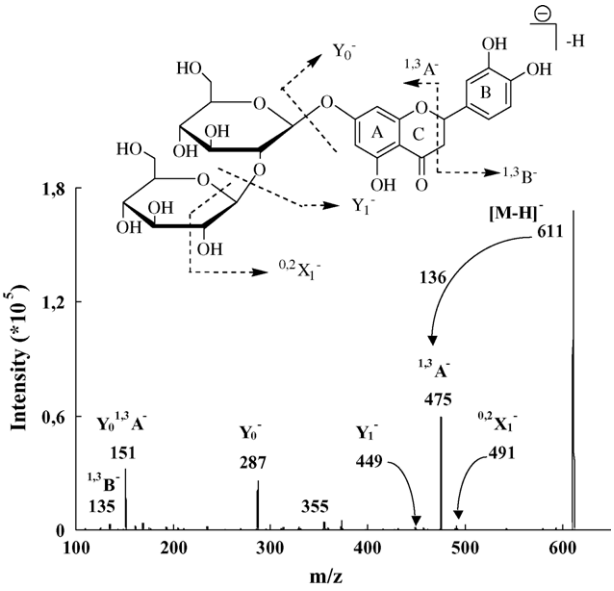


Fig. 5. CID mass spectrum of deprotonated eriodictyol 7-*O*-sophoroside **1** (*m/z* 611) and the proposed corresponding fragmentations.

3.4. Negative CID MS/MS analysis

Various positive ion mass spectrometric methods have been used in the structural analysis of flavonoids [11,15], while results obtained from experiments conducted in the negative ion mode have often been considered more difficult to interpret [21]. Studies reported on negative atmospheric pressure ionization (APCI and ESI) indicated however the improved sensitivity of such techniques in flavonoid analysis [6,7,20,36–38]. The fragmentation behavior observed was obviously different, giving additional and complementary information. This prompted us to investigate the analysis of the flavonoids of interest using negative CID MS/MS.

3.4.1. Compound *1*

In the negative mode MS/MS experiments, with the deprotonated molecular ion (*m/z* 611) generated from compound **1**, the presence of the base peak Y_0^- (*m/z* 287) was observed resulting from loss of 324 mass units. This result supports the presence of two hexose residues (Fig. 5). This elimination process gave an ion at *m/z* 287 corresponding to an aglycone with a molecular mass of 288, consistent with a flavanone based compound. The CID MS/MS spectra of the Y_0^- ion at *m/z* 287 exhibited seven main diagnostic fragmentations at *m/z* 257 (Y_0^-–H_2CO), 239 (Y_0^-–H_2CO–H_2O), 211 (Y_0^-–H_2CO–H_2O–CO), 151 ($^{1,3}A^-$), 135 ($^{1,3}B^-$), 125 ($^{1,4}A^-$) and 107 ($^{1,3}A^-$–CO_2) (Scheme 2), in agreement with a 3′,4′,5,7-tetrahydroxyflavanone [7,31,38,39]. It is worth noting that no radical anion fragments were observed for the flavanone aglycone ion generated from compound **1**. This results supports those previously reported by Hvattum and Ekeberg [26], who indicated that for flavanones and dihydrochalcone glycosides, no radical aglycones product ions were generated. This result indicates the need for a 2,3-double bond adjacent to the 4-carbonyl group in the C-ring for the formation of stable radical aglycone fragments. It may also be noted that, in contrast to these findings, that naringin, hesperidin and narirutin were reported to generate a radical aglycone product ion, indicating that a 2,3-double bond adjacent to the 4-carbonyl group is not a prerequisite to the formation of a stable radical aglycone product ion [22].

Fig. 5 shows the CID MS/MS spectrum of the $[M-H]^-$ ion (*m/z* 611) generated from compound **1**. The Y and Z type ions were observed at *m/z* 449 (Y_1^-), 431 (Z_1^-) and 287 (Y_0^-), along with other ions at *m/z* 593 $[M-H–H_2O]^-$, 491 ($^{0,2}X_0^-$ or $^{0,2}X_1^-$), 329 ($^{0,2}X_0Y_1^-$) and 311 ($^{0,2}X_0Z_1^-$). In contrast to rhamnoglucoside disaccharides where the presence of $^{0,2}X_0^-$ is characteristic of the 1→2 isomer (since it can not be formed in the case of 1→6 derivatives), this fragment could be formed in all kinds of interglycosidic linkages between two glucose moieties. Therefore, its presence could not be considered as indicative of a peculiar interglycosidic linkage.

The most striking feature of the spectra is the presence of a fragment ion at *m/z* 475 with a high relative abundance that exceeds that of Y_0^- obtained at lower CID collision energy. The complement of this ion was also detected at *m/z* 135 and corresponds to the $^{1,3}B^-$ fragment ion. This means that the ion *m/z* 475 corresponds to the $^{1,3}A^-$ fragment ion, which is an indication that the breakdown of the aglycone skeleton could occur before that of the glycosidic bonds. The importance of this ion is that it confirms that the two glycoside moieties are not located on the B-ring. The fragment ion at *m/z* 475 was subsequently selected as parent ion and its product ions were recorded at different collision energy values giving the spectrum shown in Fig. 6. The ions corresponding to the loss of the terminal glucose unit and corresponding to the Y_1^- and Z_1^- ions were observed at *m/z* 313 and 295 respectively. Further loss of the inner glucose residue yields the Y_0^- ion at *m/z* 151 corresponding to the $^{1,3}A^-$ aglycone part of the molecule, in agreement with the structure of compound **1**. Additional fragments at *m/z* 457 (–H_2O), 415 ($^{0,4}X_0^-$ or $^{0,4}X_1^-$), 373 ($^{2,5}X_0^-$ or $^{2,5}X_1^-$), 355 ($^{0,2}X_0^-$ or $^{0,2}X_1^-$), 253 ($^{0,4}X_0Y_1^-$), 235 ($^{0,2}X_1{}^{0,2}X_0^-$),

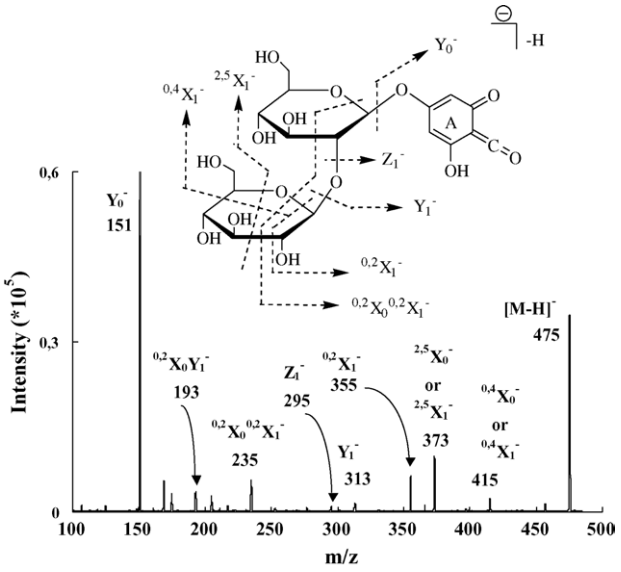


Fig. 6. CID mass spectrum of ion *m/z* 475 generated from deprotonated eriodictyol 7-*O*-sophoroside **2** (*m/z* 611) and the proposed corresponding fragmentations.

217 ($^{0,2}X_0{}^{0,2}X_1{}^-$–H_2O) and 193 ($^{0,2}X_0Y_1{}^-$). All the fragmentation pathways corresponding to theses ions are schematized in Fig. 6. The presence of the ions *m/z* 355 and 235 and corresponding to successive losses of 120 units from the 475 ion supported an 1→2 interglycosidic linkage.

3.4.2. Compound **2**

The CID MS/MS spectrum of the $[M-H]^-$ ion (*m/z* 609) generated from compound **2** exhibited the fragments typical of glycosyl derivatives (Fig. 7). Loss of the terminal sugar was observed giving a low abundance ion at *m/z* 447 ($Y_1{}^-$). Furthermore, loss of another glucosyl moiety gives an intense signal (base peak) corresponding to the aglycone moiety at *m/z* 285 ($Y_0{}^-$). The $Y_1{}^-$ ion was observed with low abundance, in agreement with a diglucoside structure of compound **2**. The CID MS/MS spectrum of the aglycone $Y_0{}^-$ ion (*m/z* 285) exhibited the following fragmentations: *m/z* 257 ($Y_0{}^-$–CO), 243 ($Y_0{}^-$–C_2H_2O), 241 ($Y_0{}^-$–CO_2), 175 ($Y_0{}^-$–C_3O_2–C_2H_2O), 151 ($^{1,3}A^-$), 149 ($^{1,3}A^-$–2H), 133 ($^{1,3}B^-$), 121 ($^{1,3}A^-$–H_2CO), and 107 ($^{0,4}A^-$) (Scheme 3), in agreement with a luteolin unit [7,38]. It is worth noting that in contrast to the behavior of eriodictyol aglycone, where no radical fragment was observed in the negative CID spectra, the glycoside 2 showed both a collision-induced homolytic and heterolytic cleavage of the *O*-glycosidic bond producing a deprotonated radical aglycone ion $[Y_0–H]^{\bullet-}$ at *m/z* 284 and an aglycone ion $Y_0{}^-$ at *m/z* 285 (Fig. 7). The results obtained in this study showed that the intensity of the scission product increases with the collision energy in agreement with previously reported data [22,26,40].

In addition to the $Y_0{}^-$ (*m/z* 285) and $Y_1{}^-$ (*m/z* 447), the presence of other fragment ions was observed at *m/z* 489 ($^{0,2}X_1{}^-$), 429 ($Z_1{}^-$), 369 ($^{0,2}X_0{}^{0,2}X_1{}^-$), 357 ($^{0,3}X_0Y_1{}^-$), 339 ($^{0,3}X_0Z_1{}^-$), 327 ($^{0,2}X_0Y_1{}^-$), 309 ($^{0,2}X_0Z_1{}^-$) and 298 ($^{0,1}X_0{}^-$). The *m/z* 369 fragment, which is formed by successive loss of two 120 mass units ($^{0,2}X_1$ and $^{0,2}X_0$ fragmentations), indicated a 1→2 dihexoside isomer, since it cannot be formed in the case of other interglycosidic linkages. The fact that the 1→2 isomer exhibits more fragment ions could be related to the hydroxyl group closest to the acetal linkage between the aglycone and the sugar part, i.e., the 2OH group of glucose. Formation of $Y_0{}^-$ appears to be more difficult compared to the rutinose analogues. This explains why the formation of other fragment ions is favored in flavonoids having interglycosidic linkages like the neohesperidoside derivatives [24].

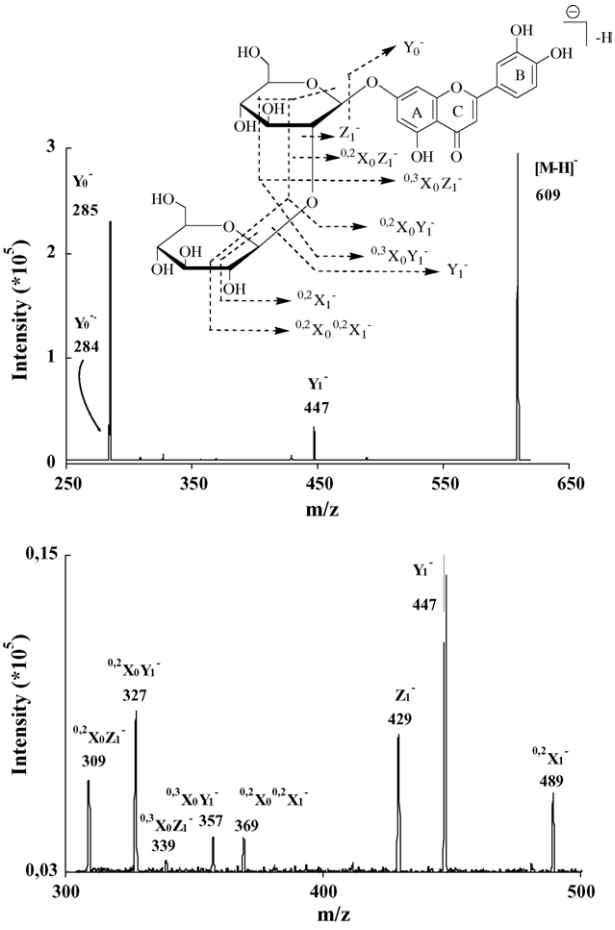


Fig. 7. CID mass spectrum of deprotonated luteolin 7-*O*-sophoroside **2** (*m/z* 609) and the proposed corresponding fragmentations.

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