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A tandem mass spectrometric study of selected characteristic flavonoids

Richard J. Hughes, Timothy R. Croley[1], Chris D. Metcalfe, Raymond E. March*

Water Quality Centre, Trent University, Peterborough, Ontario K9J 7B8, Canada

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Abstract

A study of 14 flavonoids, monohydroxy- to pentahydroxy-substituted, has been carried out using electrospray ionization coupled with high performance liquid chromatography and tandem mass spectrometry. The flavonoids are of increasing interest due to their biological activity and, in particular, those isoflavones that are phytoestrogens. This study is presented in two parts. First, the product ion mass spectra of $[M-H]^-$ of the 14 flavonoids and of $[M+H]^+$ of naringenin and galangin are discussed broadly in terms of five common fragmentation processes. Second, the identification of genistein in wood pulp, in untreated waste water, and in treated (or final) effluent from a wood pulp mill is described. (Int J Mass Spectrom 210/211 (2001) 371–385) © 2001 Elsevier Science B.V.

Keywords: Flavonoids; Flavones; Isoflavones; Tandem mass spectrometry

1. Introduction

The study of the ubiquitous class of phytochemicals known as the flavonoids has been confined largely heretofore to their distribution in the plant kingdom, elucidation of their structures, and the pathways by which they are synthesized. The advent of fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) combined with tandem mass spectrometry (MS/MS) has permitted ready study of the flavonoids, their ion chemistry, and the determination of flavonoids in low concentrations in aqueous systems.

FAB/MS/MS has been employed for structure determination of steroid and flavonoid glycosides [1], differentiation of 6-C- and 8-C-glycosidic flavonoids [2,3] and of O-diglycosyl, O-C-diglycosyl, and di-C-glycosyl flavonoids [4], and the investigation of prenylated flavonoids [5,6]. Collisional activation of $[M+H]^+$ or $[M-H]^-$ ions of steroid and flavonoid glycosides [1] led to sequential losses of glycoside moieties in a manner which permitted establishment of the sequence of glycosides. Differentiation of 6-C- and 8-C-glycosidic flavonoids was achieved using collision-activated dissociation (CAD) with mass-analyzed ion kinetic energy spectra (MIKES) [2] and CAD with linked scanning at constant *B*/*E* [3]. High energy product ion spectra permitted differentiation of isomeric di-6,8-C-glycosides [4]. A review of the

* Corresponding author. E-mail: rmarch@trentu.ca

[1]Present address: University of Arkansas for Medical Sciences, Department of Pharmacology, Slot 611, 4301 W. Markham Street, Little Rock, Arkansas 72205

Dedicated to Professor N.M.M. Nibbering for his many contributions to mass spectrometry.

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application of FAB/MS/MS to the study of flavonoid glycosides has appeared recently [7]. Prenylated flavonoids have been investigated using B^2/E^2 and B/E linked scans [5,6]. Thermospray LC/MS/MS has been used for the characterization of flavonoids [8] and the rapid screening of fermentation broths for flavones [9]. Using ion spray LC/MS/MS, parent ion scans of the two protonated aglycons, quercitin, and kaempferol, indicated the presence of more than 12 different flavonol glycosides among nine hop varieties [10]. Ion spray LC/MS/MS has been used also for the characterization of flavonoids in extracts from Passiflora incarnata [11,12]. APCI/MS/MS has been employed for the quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer [13], for a study of flavonone absorption following naringin, hesperidin, and citrus administration [14] and for the identification of 26 aglycons from the leaf surfaces of Chrysothamnus [15]. ESI/MS/MS has been employed for analysis of 6′-O-malonylated β-D-glucosides in plants [16], and for the investigation of apigenin anionic clusters in the gas phase [17] and of Na^+-bound clusters of quercetin in the gas phase [18]. The combination of MS/MS with these ionization techniques for polar compounds has proven to be a valuable technique that has great sensitivity, specificity, mass range, and mass resolution.

Flavonoid is a collective noun given to several classes of structurally similar, naturally occurring compounds. The major classes are flavones, isoflavones, flavans, anthocyanins, proanthcyanidins, flavanones, chalcones, and aurones; the basic structures of flavanones, flavones, and isoflavones are shown in Fig. 1. The flavonoids were reviewed extensively in 1994 [19]. The isoflavonoids are a large and distinctive subclass of the flavonoids. The isoflavonoids enjoy only a limited distribution in the plant kingdom, and are almost entirely restricted to the subfamily Papilionoideae of the leguminosae.

Plants synthesize flavonoids, along with other secondary metabolites such as terpenoids, alkaloids, lignans, tannins, and coumarins, for protection against pathogens and herbivores. Flavonoids are ubiquitous in the environment; they are found primarily in petals, the foliage of trees and bushes, and are widely

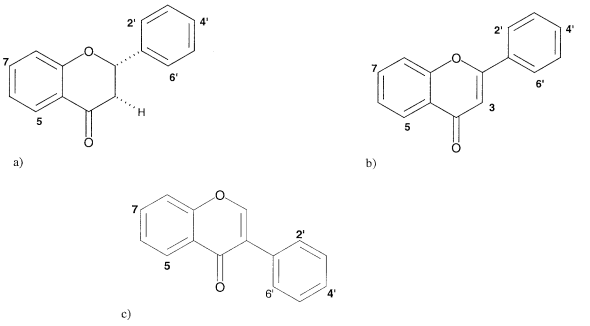

Fig. 1. Flavonoid structures: (a) flavanone; (b) flavone; (c) isoflavone.

distributed in the edible parts of plants. Flavonoids may be of ecotoxicological importance since they are present in the heartwood of tree species used for wood pulp [20,21] and are to be found in a variety of fruits, vegetables and Graminae (e.g. soy) that are important components of the diets of humans and animals [22,23]. Flavonoids are of environmental significance because several flavonoid aglycones are known to be biologically active [24] whereas some isoflavones are also phytoestrogens, that is, they mimic the sex hormone estrogen. Although phytochemicals in the heartwood and sapwood of trees make the wood disease-resistant [20], flavonoids can affect reproduction in mammals by acting upon the pituitary-gonadal axis, either as competitors for steroid receptor sites [25] or by inhibiting aromatase [26].

Pulp mill effluents are complex mixtures of chemicals and their composition depends on the species of wood, the pulping process, bleaching technology, and wastewater treatment [27]. Thus, isolation and identification of the toxic or bioactive chemicals in these effluents is a challenge. Effluents and wood pulp from a bleached kraft mill have been studied to determine whether flavonoid compounds are present [28]. LC-MS and LC-MS/MS techniques have been employed for the identification and quantification of flavonoid compounds in samples of wood pulp after oxygen delignification, as well as mill effluents collected prior to treatment and after treatment. These investigations are described below.

Flavonoids are also present in herbal medicines [29] and preventative therapeutics [30]. The existence

Table 1.
Flavonoids, in order of increasing molecular weight, examined in this study

Flavonoid type	Common name	M.W.	Hydroxylation position
Flavones	Monohydroxy	238	4′-; 3-; 6-; 7-
Isoflavone	Daidzein	254	7,4′
Flavone	···	254	6,4′-
Flavone	Chrysin	254	5,7-
Flavone	Apigenin	270	5,7,4′-
Flavone	Galangin	270	3,5,7-
Isoflavone	Genistein	270	5,7,4′
Flavanone	Naringenin	272	5,7,4′
Flavone	Kaempferol	286	3,5,7,4′-
Flavan	Catechin	290	3,5,7,3′,4′-
Flavone	Quercetin	302	3,5,7,3′,4′-

of these compounds as monomers, dimers and trimers has been linked to increased antioxidant activity [31]. The potential health effects of flavonoids demand that methods be developed for their determination and quantification in food products, plant extracts, and serum. Mass spectrometric methods coupled to liquid chromatography show great promise for the analysis and quantification of these compounds in biological samples [32–34], food products [35], plant extracts [36–38], and for fundamental studies [39–45].

Fourteen flavonoids have been selected for study by HPLC-ESI/MS/MS and the results of this study are reported here. The selected flavonoids are listed, in order of increasing molecular weight, in Table 1. The results of this study are presented below in two parts. First, the product ion mass spectra of $[M-H]^-$ of the 14 flavonoids and of $[M+H]^+$ of naringenin and galangin are discussed broadly in terms of five common fragmentation processes. Second, the identification of genistein in wood pulp, in untreated waste water, and in treated (or final) effluent from a wood pulp mill is described.

2. Experimental

Flavonoid standards were purchased from Sigma-Aldrich Inc., Toronto, ON, and from Apin Chemicals Ltd., Oxfordshire, England. They were dissolved in HPLC grade methanol (Caledon Laboratories Ltd., Georgetown, ON) to produce stock solutions of approximately 1 mg/mL; these stock solutions were diluted with methanol as required to provide 1 μg/mL flavonoid standards.

Chromatographic separations were performed initially on a Hewlett Packard Model 1100 LC-MSD, employing a 3 μm Phenomenex Luna C18 column (150×4.6 mm). The binary mobile phase consisted of solvents A (0.5% acetic acid in HPLC grade water) and B (HPLC grade methanol). The column was flushed with 100% A for at least 10 min before each run; the concentration of B was increased linearly to 70% from time $t=0$–3 min, and held until $t=15$ min, then decreased back to zero in preparation for the next run. Detection was achieved by single ion monitoring (SIM) of the parent ion at *m/z* $[M-1]^-$. An isocratic mobile phase consisting of 65% B (methanol)/35% A (20% formic acid) was employed on the same column, at a flow rate of 0.3 mL/min, for introduction of the standards and the pulp mill effluent samples into the Micromass Quattro LC-MS/MS. The capillary voltage was 2.8 kV; cone voltage 35–55 V; source block 150°C; nebuliser gas 300°C. For MS/MS experiments, UHP argon was used as the collision gas at 10 psi inlet pressure and the collision voltage adjusted for optimum performance.

3. Results and discussion

The overall findings were, first, the fragmentation patterns of CID mass spectra of a series of mono- and

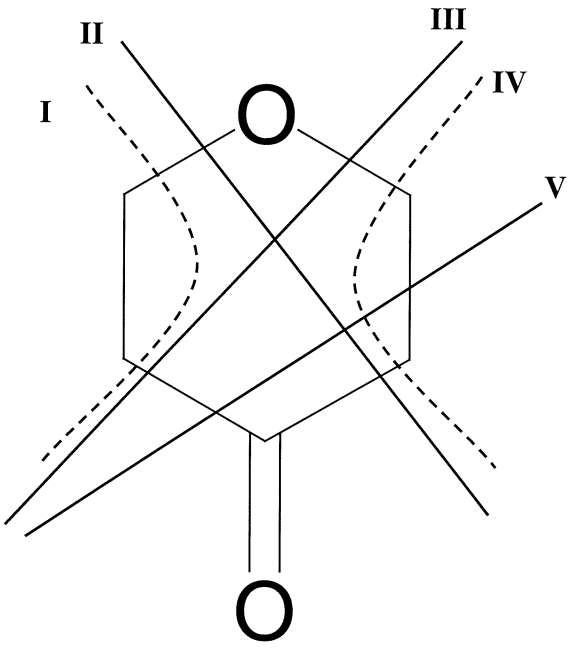

Fig. 2. Schematic representation of five common fragmentation processes observed in the CID of flavonoids.

poly-hydroxy flavonoids could be rationalized according to the position and degree of hydroxylation, thus allowing for their differentiation [46,47]; second, detection of the isoflavone genistein in microgram/litre concentrations confirmed the presence of this isoflavone in pulp mill effluents [31].

3.1. Tandem mass spectrometry of flavonoids

Tandem mass spectrometry entails the selection or isolation of ions falling within a narrow band (1–2 Da) of mass/charge ratios, dissociation of the selected or isolated ions by collision(s), and mass analysis of the fragment ions or product ions formed to yield a product ion mass spectrum. The major product ions observed in the tandem mass spectrometric experiments described below can be ascribed to five fragmentation processes of the $[M-H]^-$ ion or of the protonated molecule. The five fragmentation processes, I–V, are shown in Fig. 2.

3.1.1. Four monohydroxyflavones (4′-; 3-; 6-; and 7-)

The product ion mass spectra of four monohydroxyflavones, obtained at a laboratory collision energy of 20 eV, are shown in Fig. 3. The product ion mass spectrum of $[M-H]^-$ at *m/z* 237 of 4′-hydroxyflavone, shown in Fig. 3(a), is the simplest such mass spectrum of the four shown in Fig. 3. There is but a single product ion, *m/z* 117, that has been formed in a retro-Diels Alder reaction, that is, by fragmentation process IV; thus, the charge is retained on the B ring of the flavone at the site of hydroxylation. The structure proposed for *m/z* 117 is $HC{\equiv}C{-}(C_6H_4){-}O^-$. The product ion mass spectrum of $[M-H]^-$ at *m/z* 237 of 3-hydroxyflavone [Fig. 3(b)] shows only two peaks of low signal intensity at *m/z* 193 and 208. It is proposed that these product ions are due to the respective losses of CO_2 and HCO. The mechanism for the loss of CO_2 is not known; however, the loss of CHO may be from the 3-position of the $[M-H]^-$ ion.

Each of the two product ion mass spectra following, Figs. 3(c) and 3(d) shows the same three product ions at *m/z* 181, 193, and 208. Although it is proposed that these product ions are due to the respective losses of 2(CO), CO_2, and HCO, the mechanisms are not known. Differentiation of these four monohydroxyflavones is accomplished readily by MS/MS.

3.1.2. Three di-hydroxy isomers, chrysin, 6,4′-dihydroxyflavone, and daidzein

The flavone chrysin is di-hydroxylated on the A ring whereas the flavone 6,4′-dihydroxyflavone (which lacks a trivial name) and the isoflavone daidzein are mono-hydroxylated on each of the A and B rings. The $[M-H]^-$ ion (*m/z* 253) of chrysin exhibits many fragmentation pathways as shown in the product ion mass spectrum of Fig. 4; some 12 product ion species are observed with *m/z* 143 as the base peak. The exhibition of many fragmentation pathways is shown by a number of flavonoids examined in this study. The mechanism of *m/z* 143 is somewhat obscure. Because the empirical formula of *m/z* 143 is probably $C_9H_3O_2^-$, the ion most certainly contains the B ring, but the condition for three hydrogen atoms can be satisfied only upon opening of the B ring. The *m/z* 145 ion, possibly with the empirical formula $C_9H_5O_2^-$, may be $C_6H_5{-}C(-O^-){=}C{=}C{=}O$. A retro-Diels Alder reaction (fragmentation IV) may be invoked again to account for the formation of *m/z* 151 plus the neutral

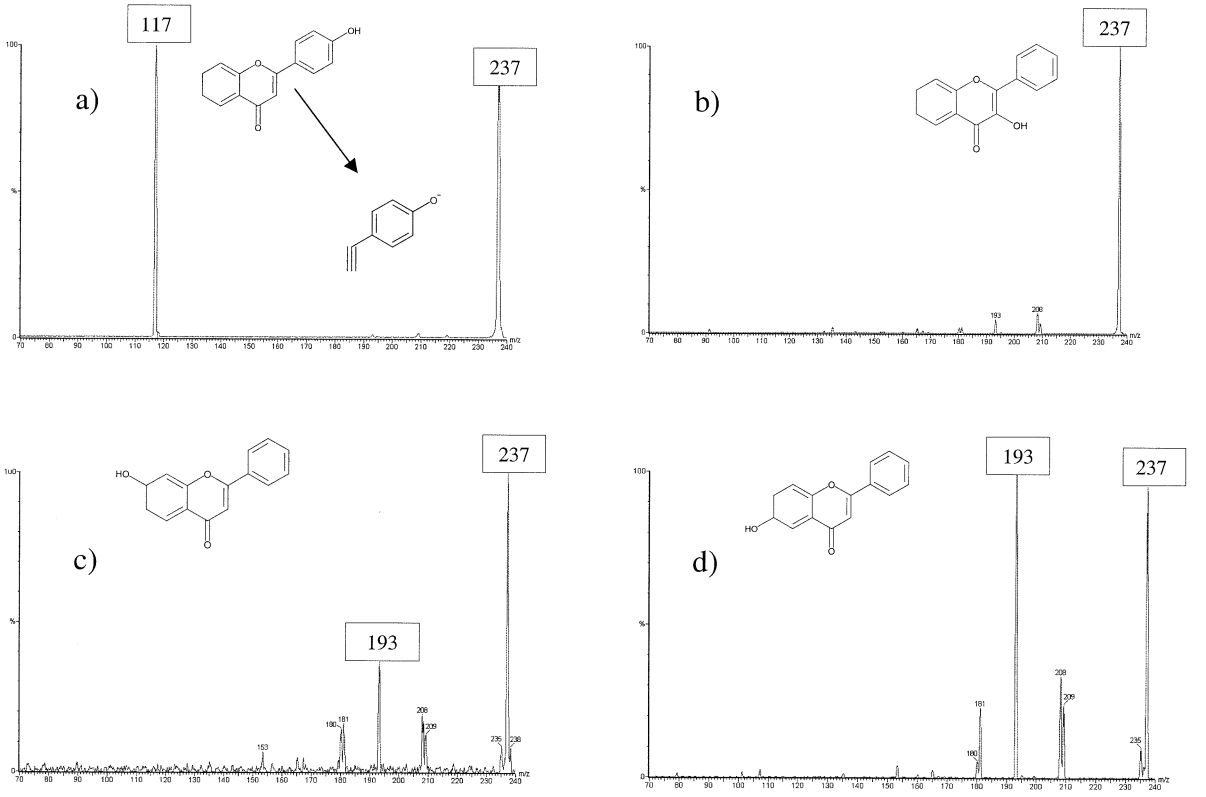

Fig. 3. Product ion mass spectra of $[M-H]^-$ of each of four monohydroxyflavones, obtained at a laboratory collision energy of 20 eV: (a) product ion mass spectrum of 4′-monohydroxyflavone; (b) 3-monohydroxyflavone; (c) 6-monohydroxyflavone; and (d) 7-monohydroxyflavone.

$HC{\equiv}C{-}(C_6H_5)$ moiety. The ion species which is complementary to *m/z* 151, that is, *m/z* 101, $[C{\equiv}C{-}(C_6H_5)]^-$, is observed though of low signal intensity. The ions of *m/z* 211 and 209 may be due to losses of $HC{\equiv}C{-}OH$ and $H_2C{=}CHOH$, respectively, from the $[M-H]^-$ ion. Subsequent losses of CO and O from each of *m/z* 211 and 209 could lead to formation of *m/z* 183 and 181, then to *m/z* 167 and 165. From the ratios of ion signal intensities, these ions appear to be related in a simple manner.

The product ion mass spectra of the $[M-H]^-$ ion of 6,4′,-dihydroxyflavone are shown in Fig. 5(a) and (b). These product ion mass spectra, obtained at laboratory collision energies of 35 and 90 eV, respectively, are dominated by *m/z* 117 that arises from the B ring. The product ion is proposed as $HC{\equiv}C{-}(C_6H_4){-}O^-$ (that is, the same structure as was proposed for *m/z* 117 from 4′-hydroxyflavone above) formed in a retro-Diels Alder reaction (fragmentation IV). The product ions *m/z* 107, 134, and 160, each of relatively low signal intensity and observed only with the lower collision energy, may be due to the ions $CH_3{-}(C_6H_4){-}O^-$, $HO{-}(C_6H_4){-}C_2HO^-$ formed by fragmentation II, and $HO{-}(C_6H_4){-}C_4H_3O^-$, respectively, derived from the B ring.

The product ion mass spectra of the $[M-H]^-$ ion of the isoflavone daidzein are shown in Figs. 5(c) and (d); these product ion mass spectra were obtained at laboratory collision energies of 30 and 43 eV, respectively. At a collision energy of 35 eV, the daidzein $[M-H]^-$ ion shows little fragmentation. At 43 eV, some 12 product ions were observed though of low signal intensity; indeed, the sum of the product ion signal intensities barely exceeded the remaining signal intensity of the parent $[M-H]^-$ ion. The most notable product ions are *m/z* 91, 117, 133, and 208.

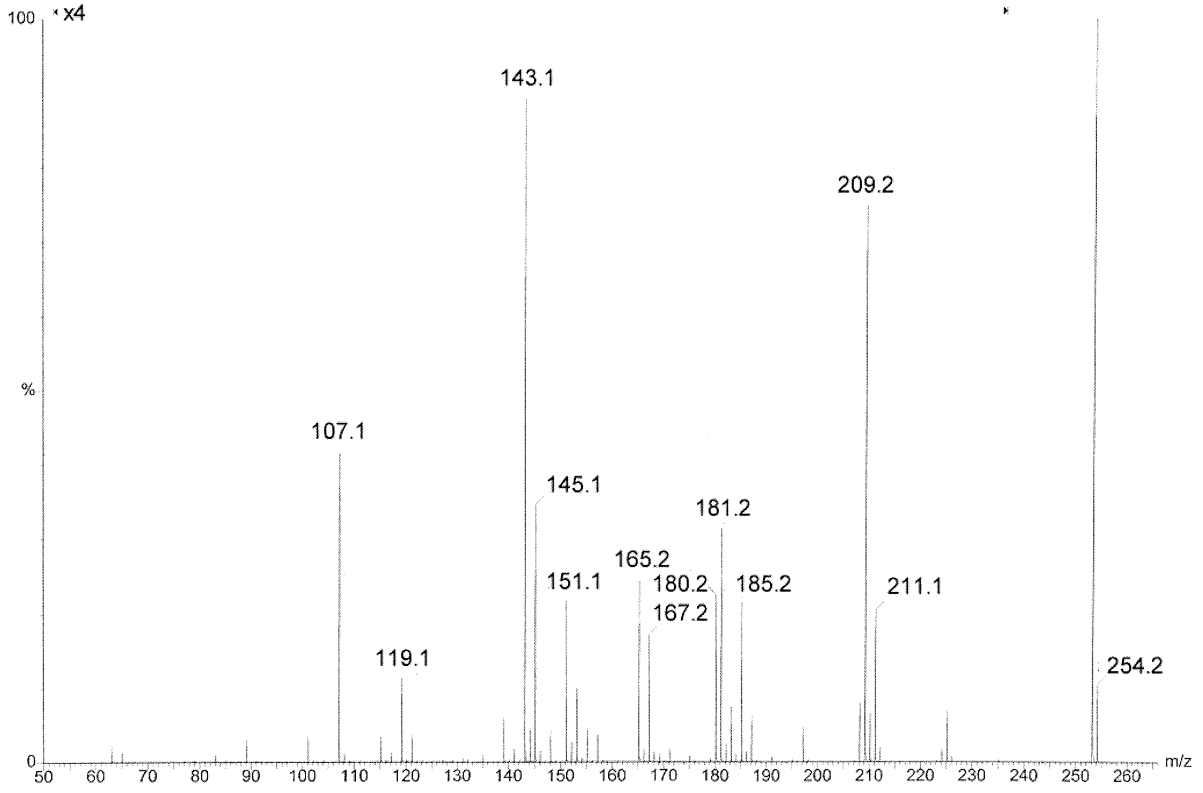

Fig. 4. Product ion mass spectrum of $[M-H]^-$ of chrysin.

The *m/z* 91 ion is probably $C_7H_7^-$. The *m/z* 117 ion is due to a fragmentation IV process as described above for 4′-hydroxyflavone and 6,4′-dihydroxyflavone. The base peak, *m/z* 133, is the product of a fragmentation process II where the charged entity contains the B ring. The structure of the *m/z* 133 ion is proposed as $HO–C{\equiv}C–(C_6H_4)–O^-$. The *m/z* 208 ion formed by loss of a moiety of 45 Da, possibly C_2H_5O, is of unknown structure.

3.1.3. Four tri-hydroxy flavonoids; naringenin, apigenin, galangin, and genistein

The flavanone naringenin, the flavone apigenin, and the isoflavone genistein have 5,7,4′- substitution whereas the flavone galangin has 3,5,7-substitution. A comparison of the product ion mass spectra of $[M-H]^-$ and $[M+H]^+$ of naringenin, shown in Fig. 6(a) and (b), respectively, permits examination of the CID behavior of these pseudomolecular ions of naringenin.

The base peak in the product ion mass spectrum from $[M-H]^-$ of naringenin is *m/z* 151 whereas the signal intensity of the complementary ion of *m/z* 119 is some 83% of that of the base peak. Both product ions arise from fragmentation IV. The product ion of *m/z* 151 contains the substituted A ring. The product ion mass spectrum of $[M+H]^+$ of naringenin [Fig. 6(b)] shows evidence also for fragmentation process IV in that *m/z* 153, the base peak, was observed. This protonated moiety arose from the A ring of naringenin as did the negatively charged ion of *m/z* 151. The signal intensity of the complementary ion of *m/z* 119 is some 14% of that of the base peak. The observation of *m/z* 179 from $[M+H]^+$ of naringenin shows that scission of the B ring has occurred. The *m/z* 147 ion from $[M+H]^+$ of naringenin may arise by fragmentation III and where the B ring bears the proton.

A comparison of the product ion mass spectra of $[M-H]^-$ of naringenin, shown in Fig. 6(a) and of

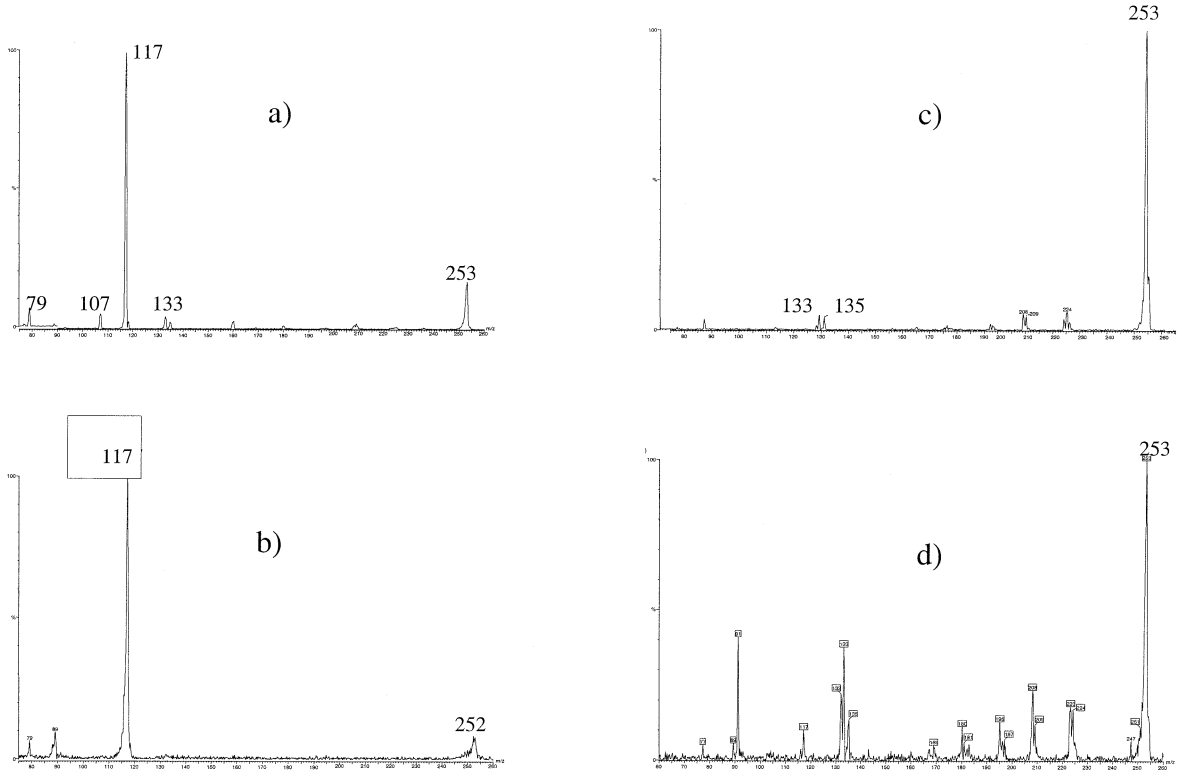

Fig. 5. Product ion mass spectra of $[M-H]^-$ of each of 6,4′-dihydroxyflavone and daidzein: (a) 6,4′-dihydroxyflavone at a collision energy of 35 eV; (b) 6,4′-dihydroxyflavone at a collision energy of 90 eV; (c) daidzein at a collision energy of 30 eV; and (d) daidzein at a collision energy of 43 eV.

$[M-H]^-$ of apigenin [Fig. 7(a)] permits examination of the effect of hydrogenation of apigenin at the 2,3 position; naringenin and apigenin have 5,7,4′ substitution in common. The product ion of *m/z* 151, that contains the substituted A ring, was observed from each mass-selected ion species, together with the complementary ions, *m/z* 119 for naringenin and *m/z* 117 for apigenin, as may be expected. The *m/z* 119 ion is possibly the hydrogenated analogue of *m/z* 117. The $[M-H]^-$ ions of both naringenin and apigenin yielded a product ion of *m/z* 107; this ion is due possibly to CH_3–(C_6H_4)–O^- derived from the B ring, as was proposed for 6,4′-dihydroxyflavone.

The product ion mass spectra of the $[M-H]^-$ ions from the flavones apigenin and galangin and the isoflavone genistein are compared here because they are isomers. Intuitively, one might expect a degree of similarity particularly among the $[M-H]^-$ product ion mass spectra of the similarly substituted flavone and isoflavone, that is, apigenin [Fig. 7(a)] and genistein [Fig. 7(b)]. As discussed previously, the $[M-H]^-$ ion from apigenin suffers fragmentation IV to produce *m/z* 151 which contains the A ring; however, the $[M-H]^-$ ion from genistein suffers fragmentation process II to produce *m/z* 133, as a base peak, where the charged entity contains the B ring. The structure of the *m/z* 133 ion is proposed as HO–C≡C–(C_6H_4)–O^-. For apigenin, the *m/z* 117 ion is the base peak whereas the signal intensity of the complementary ion of *m/z* 151 was some 70% of that of the base peak. The similarity of the product ion mass spectra of $[M-H]^-$ of naringenin and apigenin is due to the common fragmentation process IV. The only common feature of the $[M-H]^-$ product ion mass spectra from 6,4′,-dihydroxyflavone [Fig. 5(a)], naringenin [Fig. 6(a)], apigenin [Fig. 7(a)], and

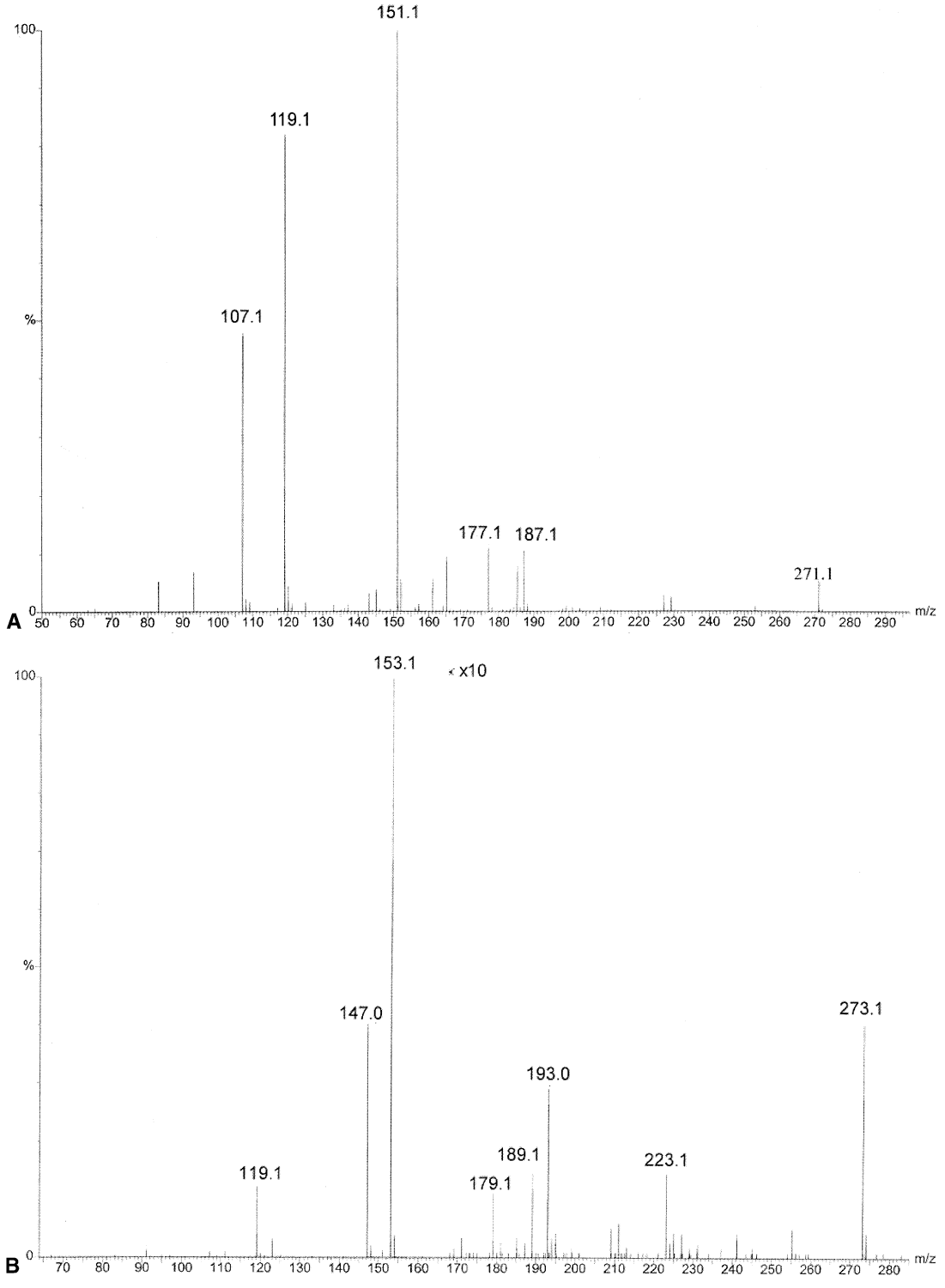

Fig. 6. Product ion mass spectrum of (a) $[M-H]^-$ of naringenin; (b) $[M+H]^+$ of naringenin.

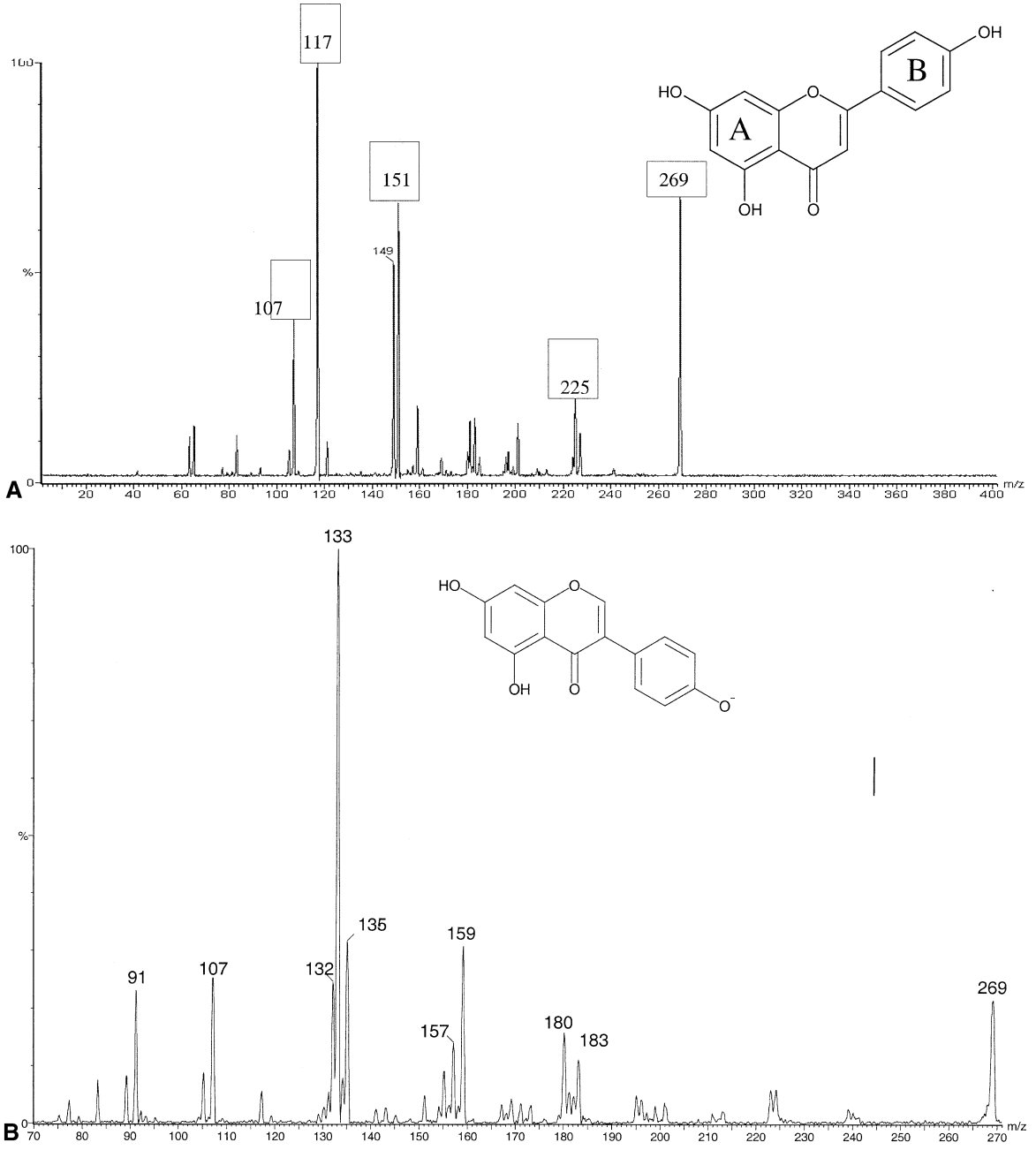

Fig. 7. Product ion mass spectrum of $[M-H]^-$ of (a) apigenin; (b) genistein.

genistein [Fig. 7(b)] was *m/z* 107; this ion species may be due to $HO–(C_6H_2)–O^-$ which contains the ring and is derived from *m/z* 151 by loss of CO_2. One question remains; if the *m/z* 151 product ions from naringenin and apigenin are identical, what is the origin of *m/z* 149 observed only in the product ion mass spectrum of apigenin? It is proposed that *m/z* 149 contains the B ring that accounts for 92 Da; the remaining 57 Da

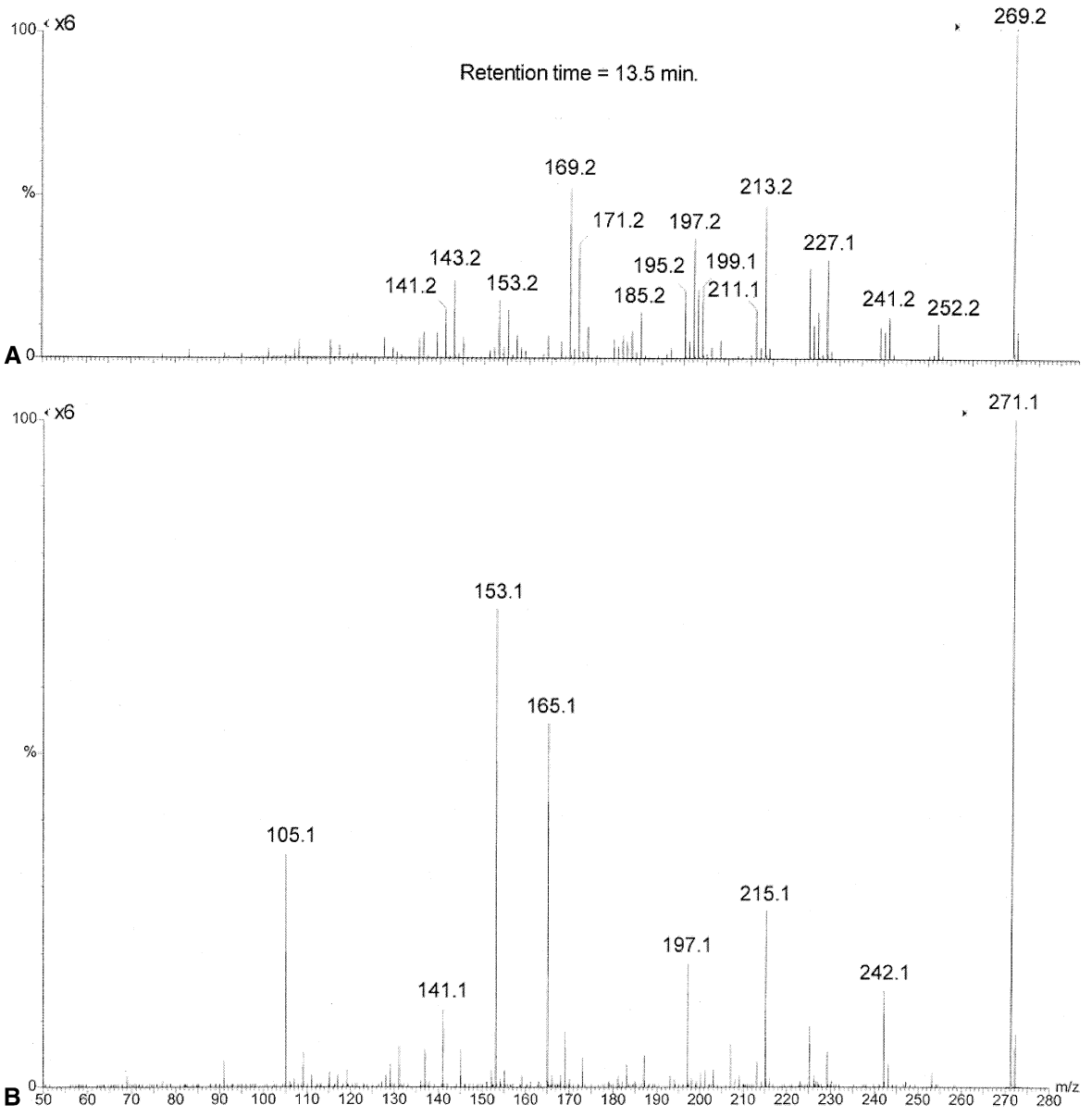

Fig. 8. Product ion mass spectrum of (a) $[M-H]^-$ of galangin; (b) $[M+H]^+$ of galangin.

may be due to C_3H_5O or C_2HO_2. C_2HO_2 may be favored because the C_2O_2 can be lost. If an ion can rearrange to eject a C_2O_2 moiety, as does the $[M-H]^-$ ion of galangin, then $HC_2O_2C_6H_5O^-$ or $HO_2C_2C_6H_5O^-$ structures are plausible.

The $[M-H]^-$ ion of galangin upon CID behaves similarly to that of chrysin in that it exhibits many fragmentation pathways; more than 20 product ions were observed of signal intensity ≥10% of the signal intensity of the base peak as shown in Fig. 8(a). Fragmentation processes I–IV would be expected to lead to product ions of *m/z* 107, 135, 123, and 151, yet none of these ions is observed. The product ion of *m/z* 213 may have been produced by fragmentation pro-

cess V with the loss of the moiety C_2O_2 of 56 Da. Because the charge is spread over the many fragment ions observed, the behavior of the $[M-H]^-$ species of galangin is incompatible with an efficient tandem mass spectrometric protocol. It is proposed that the lack of B ring substitution eliminates the low energy fragmentation processes I–IV. Only when the collision energy in the laboratory frame is increased to >30 eV, are the numerous dissociation pathways of relatively high activation energy accessed leading to many product ion species.

The positively charged product ion mass spectrum was determined by CID of the $[M+H]^+$ species of galangin, and is shown in Fig. 8(b). The base peak is due to *m/z* 153 formed by fragmentation process IV where the A ring is retained in the charged product. The product ion of *m/z* 215 may have been produced by fragmentation process V with the loss of the moiety C_2O_2 of 56 Da; this process is analogous to the formation of the product ion of *m/z* 213 from the $[M-H]^-$ ion of galangin. It is proposed that the incidence of three ions species spaced equally with a separation of 12 Th, that is, *m/z* 141, 153, and 165, may be indicative of a single carbon–carbon bond in the 2,3 position of the $[M+H]^+$ ion.

3.1.4. Tetrahydroxy flavonoid

The $[M-H]^-$ ion of kaempferol (3,5,7,4′-tetrahydroxyflavone) showed virtually no dissociation until a collision energy of about 25 eV was reached; at that point, the parent ion yielded some 60 ionic fragments, only a few of which are common to the other flavonoids. The major fragment occurs at *m/z* 93, and is probably the negatively charged phenoxy ion, $C_6H_5O^-$. With so many product ion species in the product ion mass spectrum, one would expect all of the fragmentation processes to be accessed and, indeed, they are. For fragmentation I, *m/z* 107 could contain the A or B ring or could be two isobaric species. For fragmentation II, *m/z* 135 was observed and should contain the A ring. For fragmentation III, only the ion containing the B ring, *m/z* 161, was observed. For fragmentation IV, *m/z* 151 was observed and may contain either the A or B ring. For fragmentation V, *m/z* 229 was observed and, of course, contains both A and B rings.

3.1.5. Pentahydroxy flavonoids

A ready comparison can be made between two pentahydroxy flavonoids with 3,5,7,3′,4′-substitution, quercetin (3,5,7,3′,4′-pentahydroxy flavone) on one hand and catechin (3,5,7,3′,4′-pentahydroxy flavan) on the other. The product ion mass spectrum of $[M-H]^-$ of catechin, shown in Fig. 9, consists of some 32 product ion species with ion signal intensity that is ≥10% of the ion signal intensity of the base peak, *m/z* 109. The base peak ion is possibly $HO–(C_6H_4)–O^-$ and is probably due to scission of the dihydroxylated B ring. Product ions formed from fragmentation processes I–V would yield ions of *m/z* 107, 121, 123, 137, and 245; all but *m/z* 107 were observed.

The product ion mass spectrum of Quercetin ($[M-H]^-$) consists essentially of four peaks and is shown in Fig. 10. The base peak, *m/z* 151, is formed by fragmentation process IV. The ion of *m/z* 107, $CH_{3-}(C_6H_4)–O^-$, occurs by fragmentation process I. The ions of *m/z* 121 and 179 are complementary and may be formed by cleavage of the C2 to C3 bond in the parent ion. The ion of *m/z* 121 contains the B ring whereas that of *m/z* 179 contains the A ring. No product ions were observed from fragmentation processes I, III, and V.

3.2. The identification and measurement of genistein in pulp mill effluent

In August 1998, samples of jackpine and spruce wood pulp and pulp mill effluent were collected from the E.B. Eddy pulp mill located in Espanola, ON, Canada. The pulp samples were obtained from the pulping line immediately after delignification by treatment with an oxygen reactor, whereas pulp mill effluent samples were collected prior to both biological treatment in the secondary wastewater treatment facility and to discharge into the receiving water of the Spanish River. The details concerning sample treatment are given elsewhere [32]. Extracts were analyzed initially by LC-MS (Hewlett Packard Series

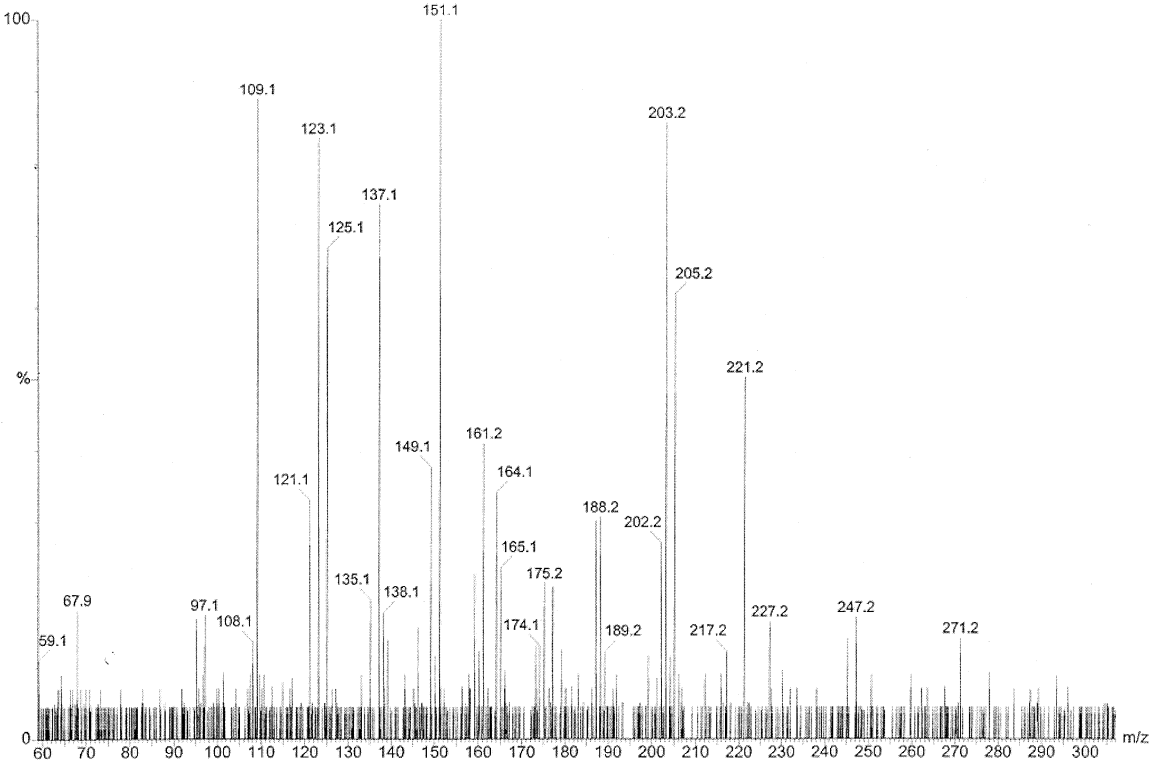

Fig. 9. Product ion mass spectrum of $[M-H]^-$ of catechin.

1100 HP/MSD) and a specific flavonoid was identified by HPLC-ESI/MS/MS using a Micromass QUATTRO LC instrument.

A mixture containing seven flavonoid compounds was chosen initially for investigation; the flavonoids were chrysin, genistein, apigenin, galangin, kaempferol, catechin, and quercetin. All of these flavonoids have been reported in the heartwood and bark of trees that are processed commonly in the pulp and paper industry. For example, kaempferol and quercetin are the most abundant aglycone flavonoids in Douglas fir and Larix species, whereas catechin is the monomer flavonoid that comprises the condensed tannins in chestnut wood and in Eucalyptus species. Some 9.4 g of flavonoids were extracted by Fang et al. [48] from 1.1 kg of the heartwood of Pinus morrisonicola, native to Taiwan; they found relatively large quantities of chrysin (7.2 g), apigenin (14.5 mg) and galangin (17.4 mg). The color change of wood chips during storage at pulp mills is attributed to the aerial oxidation of flavanones to flavonoids [49].

LC-MS analysis of extracts from wood pulp and treated and untreated mill effluent showed several peaks that corresponded to the $[M-H]^-$ ions of the seven flavonoids chosen originally for investigation. However, only one peak was observed at a retention time that corresponded to a flavonoid in the mixture of standards; that peak was due to the $[M-H]^-$ (*m/z* 269) of genistein (5,7,4′-trihydroxyisoflavone). Further analysis by LC-MS/MS yielded the product ion mass spectra of *m/z* 269 obtained from each of the treated effluent, the untreated effluent, and a genistein standard, as are shown in Fig. 11(a)–(c), respectively.

In Fig. 11(c), the base peak of the product ion mass spectrum is *m/z* 133 which has been shown above to be characteristic of isoflavones. Additional ion signals of lower signal intensity are seen at *m/z* 61, 63, 91, 107, 135, 157, 159, and 180. Although the signal/

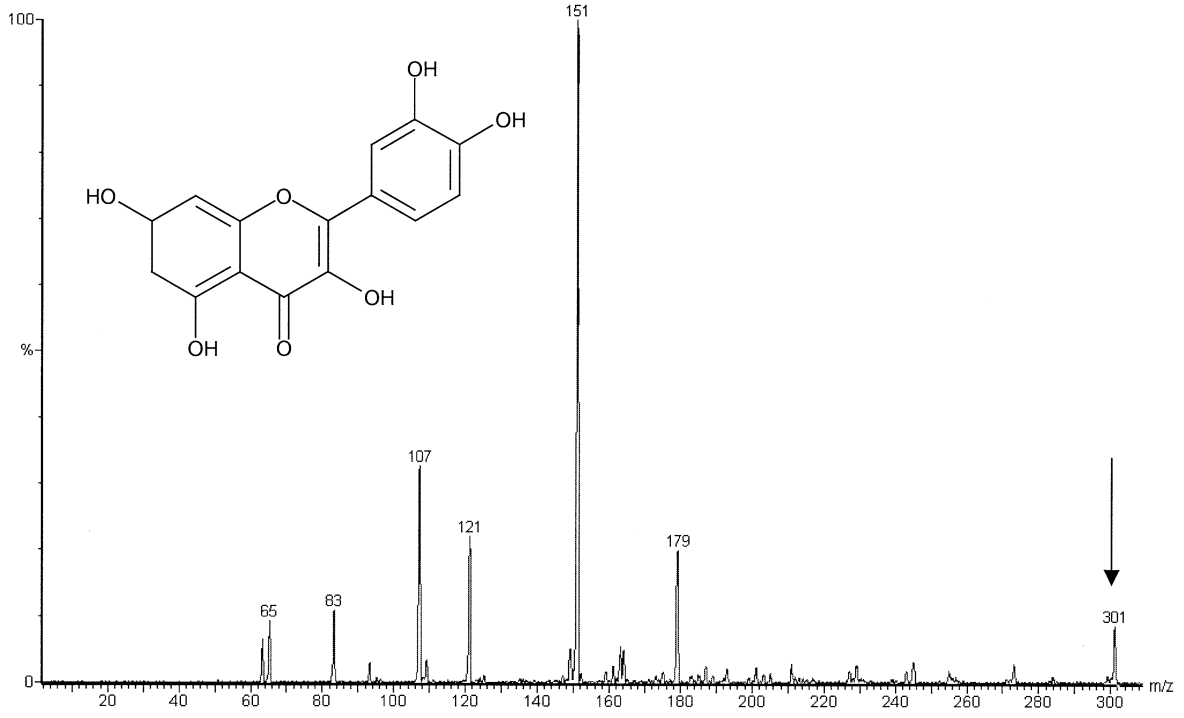

Fig. 10. Product ion mass spectrum of $[M-H]^-$ of quercetin.

noise ratio in Fig. 11(a) and (b) is rather low, the high degree of similarity between these product ion mass spectra and that of the genistein standard together with the retention time confirm the identification of genistein in the effluent samples. Quantification by LC-MS analysis of genistein in the subfractions of the extracts revealed that this isoflavone was present in wood pulp at a concentration of 30 μg/kg, and at concentrations of 13.1 and 10.5 μg/L in untreated and treated effluent, respectively.

These experiments showed that the isoflavone genistein persisted through the "OCdEHD" bleaching process (i.e. elemental oxygen, molecular chlorine with chlorine dioxide, caustic extraction, hypochlorite and chlorine dioxide) [50] and through the wastewater treatment process. This latter treatment consisted of a settling basin (primary treatment) and a 7 day aerated stabilization basin (secondary treatment). Because the pulp mill sampled in this study has treatment facilities, it is probable that genistein and possibly other flavonoids are present in the receiving waters of other pulp mills. Because genistein was the only flavonoid identified of the seven flavonoids selected for study, either the other six flavonoids are absent from the tree species used in the Espanola pulp mill or they failed to survive the pulping, bleaching, and effluent treatment processes.

4. Conclusions

The product ion mass spectra $[M-H]^-$ of the 14 flavonoids and of $[M+H]^+$ of naringenin and galangin have been discussed in terms of five common fragmentation processes. Fragmentation process I, in which the A ring becomes a fragment, was observed only in those cases where the $[M-H]^-$ exhibited many fragmentation pathways. Fragmentation process II was observed only for isoflavones. Apart from the multifragmentation of $[M-H]^-$ from quercitin and kaempferol, fragmentation process III was observed only for apigenin. The incidence of fragmentation

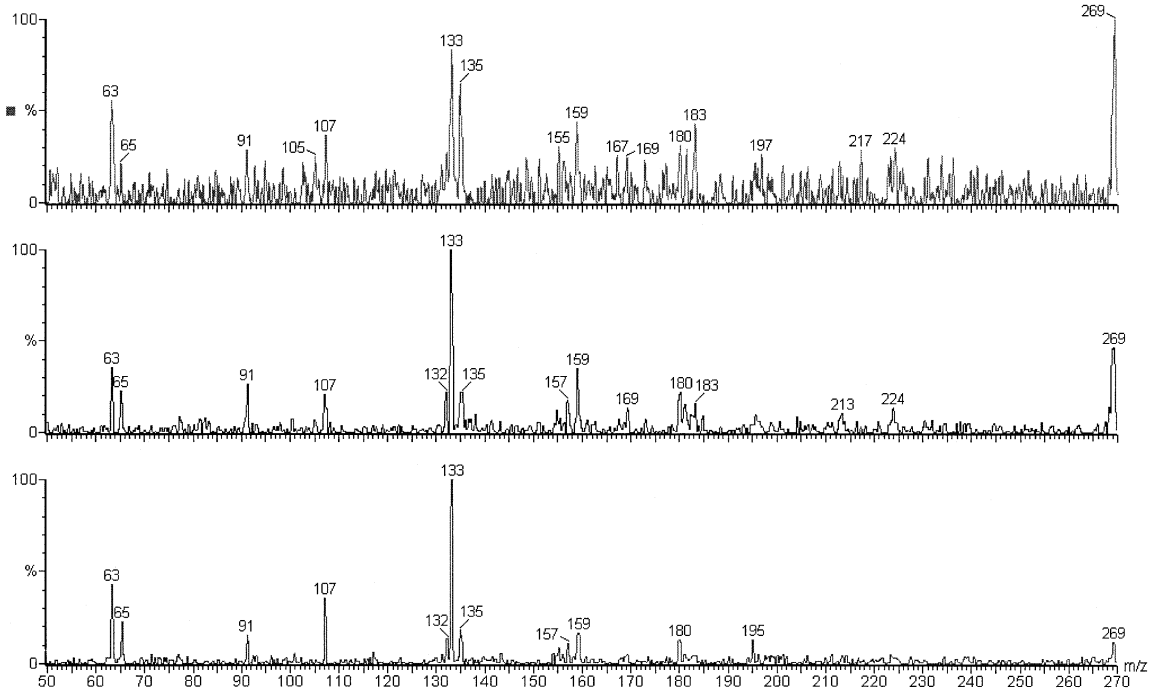

Fig. 11. Product negative ion mass spectra of *m/z* 269 from (a) a treated effluent sample; (b) an untreated effluent sample; and (c) a genistein standard.

process IV during CID, wherein the B ring in its entirety becomes a fragment, is widespread among the flavonoids examined. Fragmentation process IV is observed for almost all of the flavones, with the exception of galangin and 6,4′-dihydroxyflavone, for the single flavan and flavanone examined, for one of two isoflavones, and for the B ring monohydroxy substituted flavones. In some cases, both the A and B ring moieties are observed as fragment ions. Apart from the multiple fragmentation paths of $[M-H]^-$ from quercitin and kaempferol, fragmentation process V was observed only for galangin.

It is proposed that the low energy fragmentation processes I–IV can be observed when the B ring is substituted. When the B ring is not substituted, a collision energy in the laboratory frame of >30 eV is required for fragmentation, at which point numerous dissociation pathways of relatively high activation energy are accessed leading to many product ion species.

Fragmentation of $[M-H]^-$ into many moieties does not augur well for tandem mass spectrometric identification because the product ion charge is spread over many species. However, in the two cases examined here, fragmentation of $[M-H]^+$ appears to offer an alternative method although, generally, the intensity of $[M-H]^+$ ions is about one order of magnitude less than that for $[M-H]^-$ ions.

The identification and measurement of genistein in low concentration in wood pulp, untreated waste water, and treated (or final) effluent from a wood pulp mill has been described.

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