Determination of Flavone, Flavonol, and Flavanone Aglycones by Negative Ion Liquid Chromatography Electrospray Ion Trap Mass Spectrometry

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Eleven naturally occurring flavonoid aglycones, belonging to the representative flavone, flavonol, and flavanone types were separated by high performance liquid chromatography and analyzed on-line with negative ion electrospray ionization tandem mass spectrometry (ESI-MS/MS). In order to resolve the MS/MS spectra obtained, each compound was reinvestigated by direct loop injections using an ion trap mass spectrometer. The MS^n spectra obtained allowed us to propose plausible schemes for their fragmentation supported by the analysis of five complementary synthetic flavonoid aglycones. The negative ion ESI-MS/MS behavior of the different aglycones investigated in this study revealed interesting differences when compared with the previously described patterns obtained using various ionization techniques in positive ion. Thus, concerning the retro Diels-Alder (RDA) fragmentation pathways, several structurally informative anions appeared highly specific of the negative ion mode. In addition, a new lactone-type structure, instead of a ketene, was proposed for a classic RDA diagnostic ion. We also observed unusual CO, CO_2, and C_3O_2 losses which appear to be characteristic of the negative ion mode. All these results and these unusual neutral losses show that the negative ion mode was a powerful complementary tool of the positive ion mode for the structural characterization of flavonoid aglycones by ESI-MS/MS. (J Am Soc Mass Spectrom 2001, 12, 707–715) © 2001 American Society for Mass Spectrometry

Flavonoids are almost universal pigments of plants. They are responsible for the coloration of flowers, fruits, and sometimes leaves. More than 4000 flavonoids, and the list is constantly expanding, have been described and occur mainly in plants as *O*-, *C*-glycosides and as free aglycones [1]. They have been found to be an important part of the human diet and are considered as active principles of many medicinal plants. Several activities have been attributed to them, i.e., antioxidants, radical scavengers [2–4], antimutagenic [5], antiinflammatory [6], anticarcinogen [7], antidepressant [8], but the antioxidative properties remain the main topic investigated in recent years.

These reasons have prompted the need for modern liquid chromatography mass spectrometry (LC-MS) or gas chromatography (GC)-MS methods to quickly characterize and identify flavonoids [9]. Several reviews widely described their structural investigation by electron ionization (EI) [10–12]. Recent methods such as fast atom bombardment (FAB), thermospray (TSP), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) were also used for their ionization [13–17]. Most publications deal with glycosides studied in the positive ion mode. The on-line detection of flavonoid glycosides by mass spectrometry is now supplanting current spectrophotometric detection and the use of tandem mass spectrometry allows us to observe the corresponding aglycone as a fragment. Ma et al. [14] and Li et al. [18] successfully investigated glycosylated derivatives by FAB-MS providing structural informations such as the nature of the sugar moiety and the order of linkage in the case of polysaccharide derivatives. Thus, it appeared impor-

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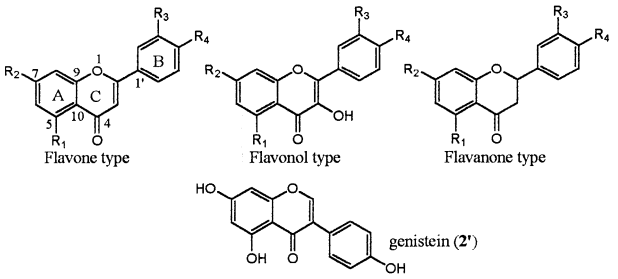


	Trivial name	[M-H]⁻	R_1	R_2	R_3	R_4
Flavone type						
1	luteolin	285	OH	OH	OH	OH
2	Apigenin	269	OH	OH	H	OH
3	genkwanin	283	H	OCH_3	H	OH
4	chrysin	253	OH	OH	H	H
5	7-OH flavone	237	H	OH	H	H
6	flavone	221	H	H	H	H
Flavonol type						
7	quercetin	301	OH	OH	OH	OH
8	fisetin	285	H	OH	OH	OH
9	kaempferol	285	OH	OH	H	OH
10	galengin	269	OH	OH	H	H
11	kaempferid	299	OH	OH	H	OCH_3
Flavanone type						
12	eriodictyol	287	OH	OH	OH	OH
13	naringenin	271	OH	OH	H	OH
14	isosakurametin	285	OH	OH	H	OCH_3
15	flavanone	223	H	H	H	H

Figure 1. Structures of the different flavonoid aglycones studied.

tant to us to investigate the fragmentation pathways of aglycones to obtain important on-line structural information. The negative ion mode was chosen because it appeared more selective and more sensitive for further LC-MS analysis of flavonoids in plants.

In the purpose of phytochemical screening of flavonoids in plants, a HPLC coupled to electrospray ion trap mass spectrometry was developed for separation and detection of 11 frequently occurring natural flavonoid aglycones (**1–4**, **7**, **9–10**, and **11–14**, Figure 1) in the negative ion mode to clarify their mass spectrometric behavior.

The LC-MS analysis of the 11 natural flavonoid aglycones was first run by mass spectrometry to obtain the ions of the molecular species. Then the negative MS/MS spectra were obtained by collision-induced dissociation (CID) from these $[M - H]^-$ ions and were analyzed to propose a plausible scheme of fragmentation for each compound studied. All the measurements have been done using the same collision energy. These fragmentations were supported by the analysis of MS^n spectra of fragments displayed by MS^2. These MS^n spectra were obtained by direct loop injection. The analysis of complementary synthetic compounds (**2′**, **5**, **6**, **8**, and **15**) displayed in Figure 1 was helpful to support plausible schemes of fragmentation.

Experimental

Chemicals

Galengin, kaempferid, eriodictyol, luteolin, isosakurametin, and genkwanin were obtained from Extrasynthese (Geney, France); quercetin, fisetin, chrysin, kaempferol, genistein, and apigenin from Sigma Chemical (St Louis, MO); flavone, 7-hydroxy flavone, flavanone, and naringenin from Aldrich Chemical (St Louis, MO).

Apparatus

The LC system consisted of a Thermo Separation Products (TSP, San Jose, CA) P4000 pump, a TSP 6000LP photodiode array detector, and a TSP AS 3000 autosampler. Separation of flavonoid aglycones was performed on a Merck (Darmstadt, Germany) C18 Lichrospher column (250 × 4.5 mm i.d., 5 μm) using a linear gradient of 0–30 min., 30%–45% of methanol (B) in formic acid 0.1% (A), 30–60 min, 45%–97% of B in A, this composition was maintained from 60 to 70 min then returned to initial conditions. The concentration of standards **1–4**, **7**, **9**, **10**, and **11–14** was adjusted to about 0.6 μg mL^{-1} in methanol/formic acid 0.1%–70/30. Flow rate was 0.7 mL min^{-1} and 20 μL of the standard solutions were injected.

Mass spectra were acquired using a LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source. The operating parameters were as follows: the spray needle voltage was set at 4.5 kV and the spray was stabilized with a nitrogen sheath gas (700 lb/in.^{2}). ES capillary voltage was −30 V, helium was used as auxiliary gas (15 lb/in.^{2}), and capillary temperature was 200 °C, collision energy 45% of 5 V for MS/MS. A syringe pump delivering 3 μL min^{-1} was used for the direct loop injections of pure standards dissolved in MeOH (about 0.3 μg μL^{-1}). The operating parameters were optimized for each standard in the various MS^n experiments.

Results and Discussion

Figure 2a shows the total ionic current of the 11 natural flavonoid aglycones (**1–4**, **7**, and **9–14**) investigated by LC-MS. The LC conditions permitted a good separation of these compounds and were optimized for further separations of crude plant extracts containing aglycones and glycosylated flavonoid derivatives in 70 min. Figure 2b, d shows the LC-MS/MS spectra obtained from the $[M - H]^-$ ions for the three aglycones luteolin, quercetin, and isosakurametin.

Tables 1, 2, and 3 display the most characteristic fragments observed for each compound obtained by MS^2 from the $[M - H]^-$ ions. In Schemes **2** to **4**, the first line of fragments is directly formed from the corresponding pseudomolecular anions. Further fragmentations of these ions were verified using MS^n experi-

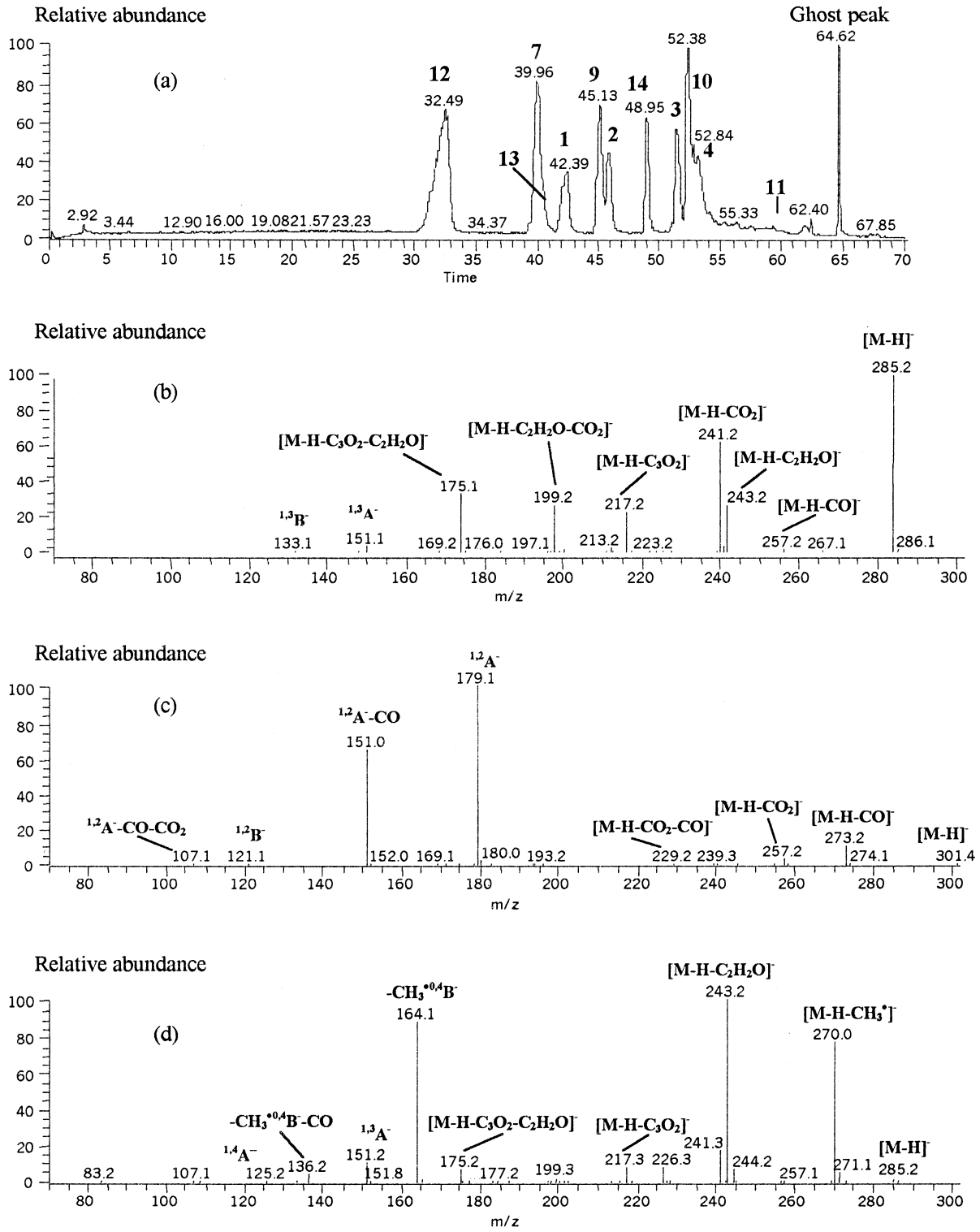


Figure 2. LC-negative ion ESI MS total ion current of flavonoid aglycones (**a**). Negative ion ESI-MS/MS spectra obtained for **1** (**b**), **7** (**c**), and **14** (**d**).

ments. Fragments, except retrocyclization ions, observed in the MS^2 spectra, were noted by the corresponding aglycone number followed by an alphabetical letter in the order of the increasing mass to charge ratio. The fragments observed by MS^n experiments (i.e., not present in the MS^2 spectra) were noted by their *m/z* value. The nomenclature adopted for the RDA cleavages was adapted from the one proposed by Ma et al. [13, 19]. Scheme **1**, adapted from [13, 19] shows the various retrocyclization fragments observed in this

Table 1. ESI-MS/MS product ions obtained from the $[M - H]^-$ ions of flavones **1–6**

	1	**2**	**2′**	**3**	**4**	**5**	**6**
$[M - H]^-$	285 (100)[a]	269 (60)	269 (100)	283 (5)	253 (100)	237 (100)	221 (100)
$[M - H - CH_3]^{\cdot -}$	—	—	—	268 (100)	—	—	—
$[M - H - H_2O]^-$	267 (1)	—	—	—	—	—	—
$[M - H - CO]^-$	257 (3)	241 (1)	241 (6)	—	225 (1)	209 (6)	193
$[M - H - C_2H_2O]^-$	243 (28)	227 (3)	227 (2)	—	—	—	—
$[M - H - CO_2]^-$	241 (65)	225 (100)	225 (23)	—	209 (75)	193 (5)	—
$[M - H - C_3O_2]^-$	217 (23)	201 (40)	201 (18)	—	—	—	—
$[M - H - CO_2 - CO]^-$	213 (3)	—	197 (4)	—	181 (2)	165 (1)	—
$[M - H - C_2H_2O - CO_2]^-$	199 (23)	183 (4)	183 (1)	—	—	—	—
$[M - H - 2CO_2]^-$	197 (1)	181 (2)	181 (16)	—	165 (2)	—	—
$[M - H - C_3O_2 - C_2H_2O]^-$	175 (35)	159 (1)	159 (1)	—	143 (1)	—	—
$^{1,3}A^-$	151 (4)	151 (10)	—	151 (1)	—	—	—
$^{1,4}B^- + 2H$	—	149 (36)	—	—	—	—	—
$^{1,3}A^- - CO_2$	—	107 (1)	—	—	—	—	—
$^{1,3}B^-$	133 (1)	117 (1)	—	—	—	—	—

[a] *m/z* (relative abundance).

study: the superscripts on the left of the A or B ring indicate the bonds that have been broken. These fragments can undergo further fragmentation as noted in Tables 1 to 3.

Degradation of Flavone Aglycones

The fragmentation behavior drawn in Scheme **2** for luteolin can be applied to the other compounds. The exceptions are mentioned in the text.

In general, all the flavones studied here (except genkwanin, **3**, which only loses a $CH_3^{\cdot}$ radical) exhibit neutral losses of CO and CO_2 that may be attributed to the C ring: the localization of this CO loss is supported by the degradation of flavone (**6**, Table 1) which also loses a CO group and by the degradation of 7-OH-flavone (**5**, Table 1). It is worth noting that the MS^2 spectrum of the flavone (**6**) was very difficult to obtain because of the lack of hydroxyl group in its structure. A maximum inject time of 8 s was necessary to observe the ionization of **6** in the negative ion mode. The contraction of the C ring due to the loss of CO (**1b**, Scheme 2) has already been described for flavonoid aglycones using EI and FAB in the positive ion mode [10, 11, 19]. We also observed using MS^3 fragmentation that **1b** loses a CO_2 group involving the A ring leading to fragment **1c** corresponding to a $[M - H - 72]^-$ anion. The structure of **1c** at *m/z* 213 may be justified by the analysis of compounds **2′** and **4** which also lose CO and then CO_2 and do not possess two hydroxyl groups on the B ring. This indicates that this last CO_2 loss can only occur in the A ring.

The contracted C-ring structure **1f** proposed, consecutive to a CO_2 loss from the pseudomolecular anion, was supported by the study in the same conditions of the 7-hydroxy flavone (**5**) which also loses CO_2. These $[M - H - CO_2]^-$ fragments (cf. **1f**) can undergo further fragmentations, notably CO loss, leading to **1g** type fragments, isomers of **1c**. The structure of **1g** and the fact that the proposed structures for the **1c** type and **1g**

Table 2. ESI-MS/MS product ions obtained from the $[M - H]^-$ ions of flavonols **7–11**. Fragment ions displayed for **11** were obtained by MS^3 from the $[M - H]^-$ and $[M - H - CH_3]^{\cdot -}$

	7	**8**	**9**	**10**	**11**[b]
$[M - H]^-$	301 (1)[a]	285 (36)	285 (100)	269 (100)	—
$[M - H - CH_3]^{\cdot -}$	—	—	—	—	284 (47)
$[M - H - CO]^-$	273 (11)	257 (22)	257 (3)	241 (3)	256 (3)
$[M - H - C_2H_2O]^-$	—	—	243 (2)	227 (11)	—
$[M - H - CO_2]^-$	257 (5)	241 (15)	241 (1)	225 (3)	240 (5)
$[M - H - 2CO]^-$	—	229 (3)	229 (3)	213 (10)	228 (10)
$[M - H - CO_2 - CO]^-$	229 (1)	213 (3)	213 (3)	197 (12)	212 (1)
$[M - H - C_2H_2O - CO_2]^-$	—	—	199 (1)	183 (1)	—
$[M - H - \text{ring B}]^-$	193 (1)	177 (1)	—	—	—
$^{1,2}A^-$	179 (100)	163 (100)	—	—	—
$^{1,2}A^- - CO$ (**7**, **8**); $^{1,3}A^-$ (**9**, **11**)	151 (67)	135 (54)	151 (1)	—	151 (100)
$^{1,3}B^-$	—	—	—	—	132 (1)
$^{1,2}B^-$	121 (1)	121 (4)	—	—	—
$^{1,2}A^- - CO - CO_2$	107 (1)	91 (2)	—	—	107 (4)

[a] *m/z* (relative abundance).
[b] MS^3 spectrum from $[M - H - CH_3]^{\cdot -}$.

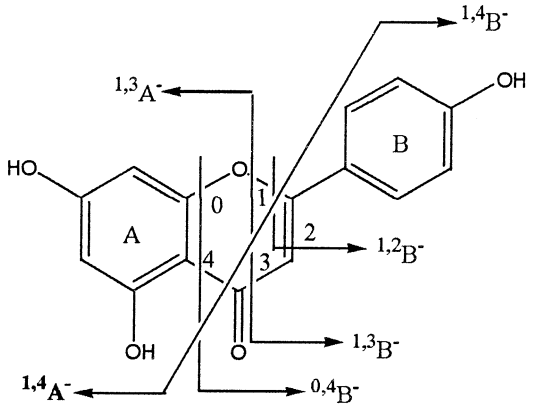
Table 3. ESI-MS/MS product ions obtained from the $[M - H]^-$ ions of flavanones **12–15**

	12	13	14	15
$[M - H]^-$	287 (0)[a]	271 (0)	285 (2)	223 (70)
$[M - H - CH_3]^{\cdot -}$	—		270 (75)	
$[M - H - CO]^-$	—	—	257 (1)	195 (100)
$[M - H - C_2H_2O]^-$	—	—	243 (100)	—
$[M - H - CO_2]^-$	—	227 (1)	241 (18)	—
$[M - H - C_2H_2O - CH_3]^{\cdot -}$			228 (1)	
$[M - H - CO_2 - CH_3]^{\cdot -}$			226 (8)	
$[M - H - C_3O_2]^-$	—	—	217 (8)	—
$[M - H - C_2H_2O - CO_2]^-$	—	—	199 (2)	—
$[M - H - \text{ring B}]^-$	—	177 (18)	—	—
$[M - H - C_3O_2 - C_2H_2O]^-$	—	—	175 (6)	—
$^{1,3}A^-$	151 (100)	151 (100)	151 (12)	—
$- CH_3{}^{0,4}B^{\cdot -}$	—	—	164 (88)	—
$[- CH_3{}^{0,4}B - CO]^{\cdot -}$	—	—	136 (3)	—
$^{1,3}B^-$	135 (3)	119 (3)	—	—
$^{1,4}A^-$	125 (1)	—	125 (1)	—
$^{1,3}A^- - CO_2$	107 (1)	107 (3)	107 (1)	—

[a] *m/z* (relative abundance).

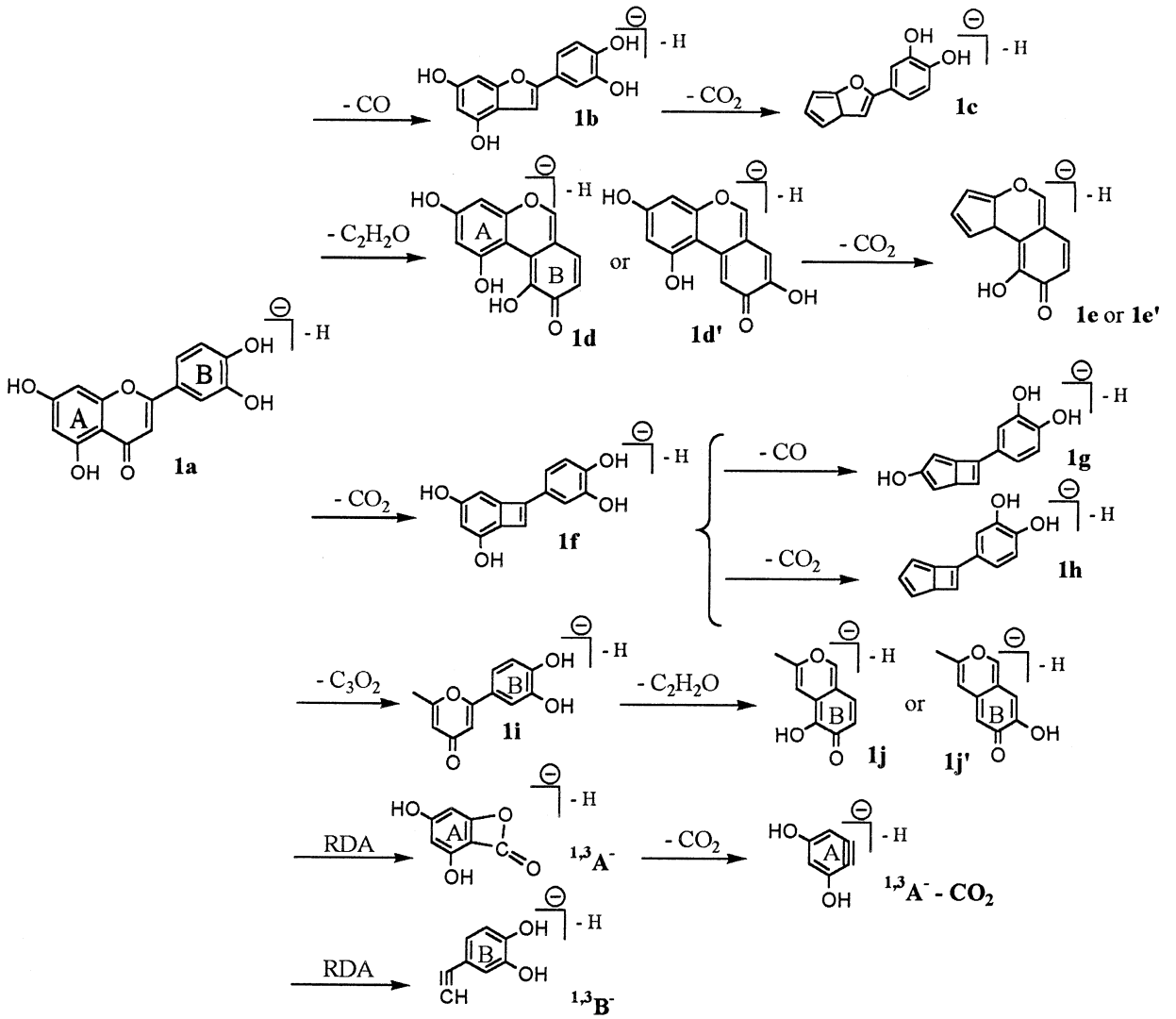
type fragments are different is supported by the ion at *m/z* 165 ($[M - H - 72]^-$) observed for the 7-hydroxy flavone (**5**) which is formed from the $[M - H - CO_2]^-$ ion and not from the $[M - H - CO]^-$ ion. This reinforces the hypothesis that these CO and CO_2 neutral losses first concern the C ring and then the A ring and supports the fragment **1h** type consecutive to two CO_2 losses (−88 u) from the pseudomolecular ion. Furthermore, this $[M - H - 88]^-$ anion is also observed for compound **4** devoid of any hydroxyl group on the B ring.

Another small neutral loss, sometimes prominent, concerns the C_2H_2O (−42 u) cleavages. It is very hard to localize this fragmentation from the $[M - H]^-$ ion **1a** because it may occur on different sites of the molecule. We propose that this fragmentation occurs mainly on the C ring followed by a new cyclization implying the B ring and leading to the two plausible fragments **1d** or **1d′**. The possibility that the A ring should be implicated for this neutral loss was eliminated by the study in the same conditions of the 3′,4′-dihydroxyflavone (data not shown) which revealed this C_2H_2O loss from the corresponding $[M - H]^-$ ion and which does not contain any hydroxyl group on the A ring. The fact that this loss is not observed for compounds **4**, **5**, and **6** (Table 1) should be due to a fragmentation intermediate implying the hydroxyl on position 4′ which is absent in **4**, **5**, and **6**. Genkwanin **3** gave only the $[M - H - CH_3]^{\cdot -}$ radical ion without other fragmentation. This type of cleavage leading to further cyclization was already observed and a mechanism proposed by Ma et al. in positive ion FAB [13]. It is worth mentioning that this C_2H_2O loss is also observed for the isoflavone **2′** and should yield to a different fragment than **1d** involving the A or B ring. The consecutive losses of CO_2 from **1d** or **1d′** give rise to the corresponding contracted A-ring ions **1e** or **1e′**. The latter cleavage concerns the A ring because it is also observed for apigenin (**2**) which possesses only one hydroxyl on the B ring.



Scheme 1. Nomenclature adopted for the different retrocyclization cleavages observed in this study (adapted from [13, 18]).

In addition, the fact that this C_2H_2O cleavage did not involve the B ring (that should be possible and may occur in isoflavone aglycones) may be explained by the fragmentation consecutive to the C_3O_2 loss (−68 u). Indeed, examining the product ions obtained for the different flavones studied and the position of the hydroxyl groups in the different structures, this C_3O_2 loss implies unambiguously the β-dihydroxy configuration displayed by the A ring. The resulting **1i** fragment proposed includes a methyl group and undergoes further C_2H_2O loss leading to two plausible fragments (**1j** and **1j′**). In a similar way as proposed for **1d** and **1d′**, **1j** and **1j′** involve the carbons 3 and 4 for luteolin (**1**) and apigenin (**2**) and probably carbons 4 and 10 for genistein (**2′**) (where the B ring is connected on carbon 3). This allows the formation of two bicyclic fragments (**1j** or **1j′**) depending on the free rotation of the bond between carbons 2 and 1′. Moreover, the presence of the fragment at *m/z* 143 for **4** (Table 1), consecutive to the loss of



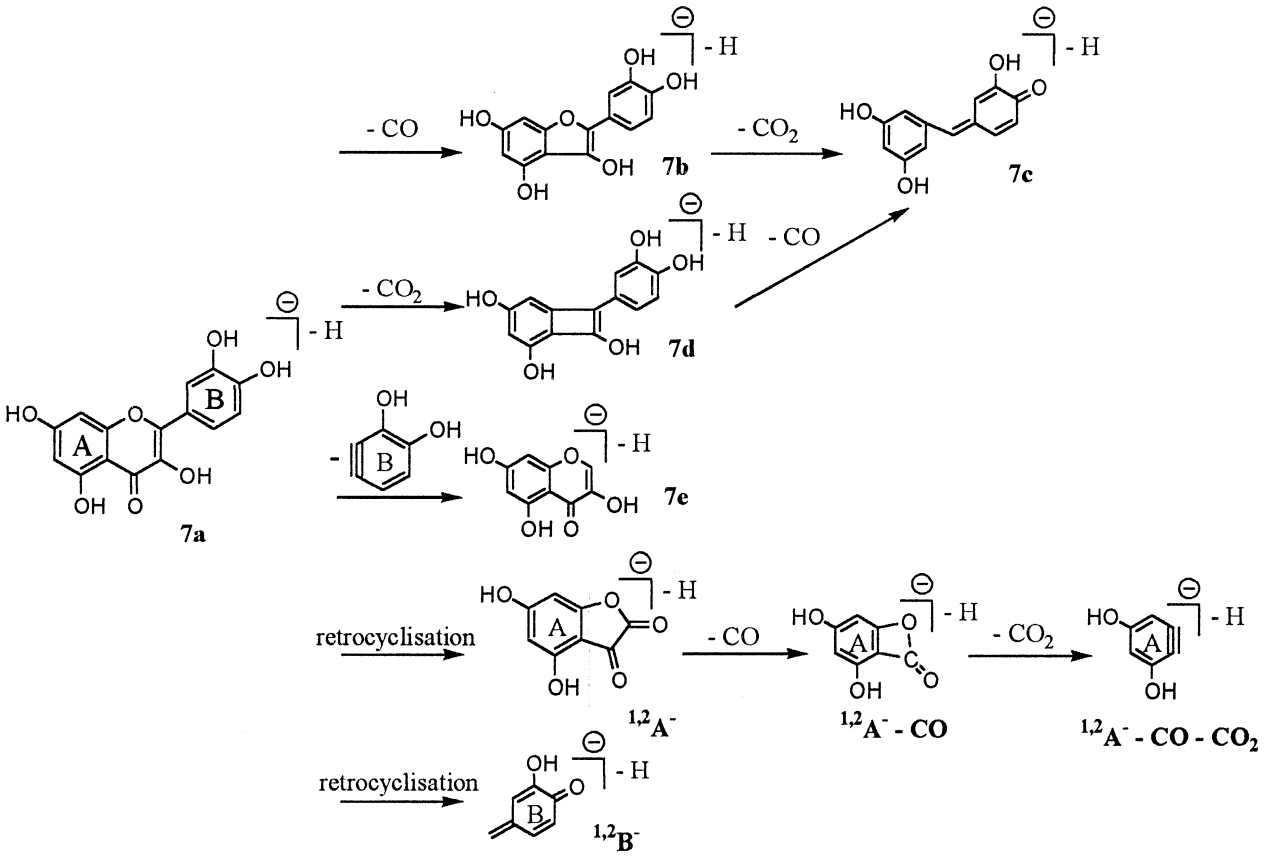
Scheme 2. Proposed fragmentation from the pseudomolecular anion flavone type luteolin (**1**).

C_3O_2 then C_2H_2O, supports a C-ring localized cleavage for the C_2H_2O loss from the $[M - H]^-$ ion.

Concerning the RDA fragmentations, we noted that the $^{1,3}\mathbf{A}^-$ ions systematically undergo further CO_2 loss leading to an ion at *m/z* 107 noted $^{1,3}\mathbf{A}^- - \mathbf{CO_2}$. This ion appears in the MS^2 spectra when the $^{1,3}\mathbf{A}^-$ fragment is prominent (compound **2**, Table 1), and in the MS^3 spectra for compounds **1** and **3**. The structure usually proposed for these RDA fragments is a ketene [11, 13, 19] which we also observed in positive ion ESI (data not shown) at *m/z* 153 (noted $^{1,3}\mathbf{A}^+$ in positive ion FAB by Ma et al. who discussed this fragmentation in detail [13, 19]). Furthermore, MS^n experiments on this $^{1,3}\mathbf{A}^+$ fragment did not show any consecutive CO_2 loss, but a CO loss. This suggests that the structure of the $^{1,3}\mathbf{A}$ RDA fragments seems different in negative and positive ion modes. That is why we propose a lactone form for $^{1,3}\mathbf{A}^-$ that more easily undergoes further CO_2 loss (Scheme **2**) than the ketene proposed in the positive ion mode.

Flavone aglycones also displayed common features but we also noted interesting differences between the two isomers apigenin (**2**) and genistein (**2′**). The latter is fragmented as apigenin except for the RDA fragmentation that has not been observed in negative ion ESI-MS/MS and thus permits us to distinguish these two isomers. On the contrary, apigenin (**2**) exhibits various RDA fragments, particularly the prominent ion at *m/z* 149 (**2**, Table 1) attributed to a $^{1,4}\mathbf{B}^- + \mathbf{2H}$ fragment, on the basis of the very reduced possibilities of structures. Unfortunately, MS^3 experiments on this fragment ion were fruitless.

The MS/MS spectrum of genkwanin (**3**) exhibits only a $[M - H - CH_3]^{\cdot -}$ radical anion as base peak (Table 1) without any other fragmentation except a small (1%) $^{1,3}\mathbf{A}^-$ ion at *m/z* 151. No fragmentation was observed using MS^3 experiments on this $[M - H - CH_3]^{\cdot -}$ ion. This means that this radical anion is probably rearranged in a 7-keto-diconjugated-3,4-enol derivative avoiding any other fragmentation for this 7-methoxy-4′-hydroxyflavone aglycone and supports the hypothe-



Scheme 3. Proposed fragmentation from the pseudomolecular anion flavonol type quercetin (**7**).

sis of the existence of an intermediate ion inducing the fragmentations described for the other flavones.

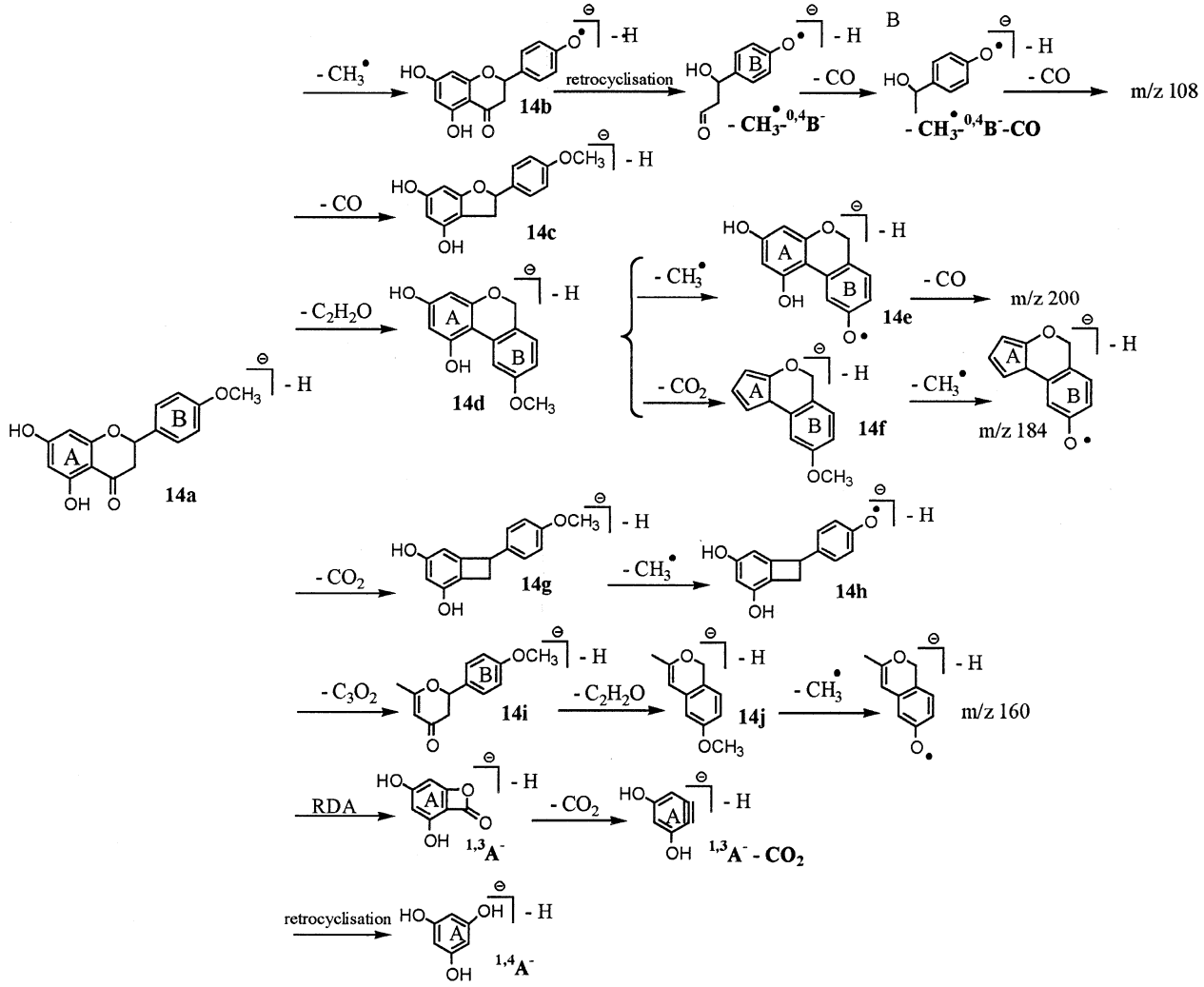
Finally, it is worth noting that the qualitative and quantitative fragmentation in negative ESI-MS/MS is related to the degree of hydroxylation on the B ring. In general, this observation may also be extended to the other types of flavonoid aglycones.

Fragmentation of Flavonol Aglycones

The fragments displayed in Table 2 and obtained from the various flavonol aglycone pseudomolecular anions show the same types of small neutral losses as those described for the flavone aglycones but also interesting differences. The fragments proposed (Scheme **3**) for quercetin (**7**) consecutive to CO and CO_2 losses (**7b** and **7d**) are similar to the corresponding luteolin fragments. Further losses of CO_2 and CO from **7b** and **7d**, respectively, give rise to the resonance-stabilized ion **7c**. These pathways are justified by examining the fragmentation of fisetin (**8**) that has only one hydroxyl group, on position 5, on the A ring and galengin (**10**) which has no substitution on the B ring. This reinforces the previous discussed hypothesis that these successive CO and CO_2 losses involve first the C ring. No further fragmentation was observed from **7c**. With regard to the C_2H_2O loss, it occurs only for kaempferol (**9**) and galengin (**10**) involving unambiguously the A ring because of flavonol aglycones are hydroxylated on position 3 on the C ring and compounds **9** and **10** are unsubstituted on the B ring.

The fragmentation of the 4′-methoxylated flavonol **11**, displayed in Table 2, was obtained on-line using the MS^3 spectrum from the $[M - H - CH_3]^{\cdot -}$ radical anion. The product ions produced join the fragmentation of its 4′-hydroxylated homolog **9** except for the $^{1,3}\mathbf{A}^-$ ion, which is the base peak for **11** (Table 2).

The most interesting fragments concern the base peaks at *m/z* 179 and 163 for **7** and **8**, respectively. Indeed, exhaustive MS^n experiments allowed us to propose a specific RDA pathway leading to the previously mentioned $^{1,3}\mathbf{A}^-$ and $^{1,3}\mathbf{A}^- - \mathbf{CO}_2$ ions (*m/z* 151 and 107 for the 5,7-dihydroxyflavones). For flavonols, the proposed scheme shows that this new retrocyclization pathway concerns bonds 1 and 2 leading to $^{1,2}\mathbf{A}^-$ and $^{1,2}\mathbf{B}^-$ fragments at *m/z* 179 and 121 for **7**. This $^{1,2}\mathbf{A}^-$ diagnostic ion undergoes further loss of CO giving rise to a $^{1,2}\mathbf{A}^- - \mathbf{CO}$ ion at *m/z* 151 for **7** and *m/z* 135 for **8** which exhibit similar structures than the $^{1,3}\mathbf{A}^-$ ion but obtained from a different pathway. Another further loss of CO_2 leads to $^{1,2}\mathbf{A}^- - \mathbf{CO} - \mathbf{CO}_2$ ion at *m/z* 107 having the same structure as the $^{1,3}\mathbf{A}^- - \mathbf{CO}_2$ fragment previously explained for the flavone **1** (Scheme **2**). These results are supported by the fragmentation of



Scheme 4. Proposed fragmentation from the pseudomolecular anion flavanone type isosakurametin (**14**).

fisetin (**8**) that exhibits the same $\mathbf{A}^-$ type fragments with 16 u less and the same $\mathbf{B}^-$ type ions with an equal mass to charge ratio. It is worth mentioning that this specific pathway, involving the 1,2 bonds, was only observed for quercetin and fisetin and highly increases the specificity of 3′,4′-dihydroxyflavonol derivatives in negative ion ESI-MS/MS.

Fragmentation of Flavanone Aglycones

The various product ions provided from the four pseudomolecular ions of the flavanone aglycones studied here are displayed in Table 3. The fragmentation of the flavanone **15** yielded a $[M - H - CO]^-$ base peak without any other fragmentation. This reinforces the proposed contracted C rings consecutive to this type of small neutral loss. The MS^2 spectra of eriodictyol (**12**) and naringenin (**13**) yielded the $^{1,3}\mathbf{A}^-$ ions as base peaks with few other RDA fragments detailed previously for the other flavonoid aglycones.

The most complex fragmentation for this type of aglycones concerned the 4′-methoxylated one (**14**). Scheme **4** proposes two main pathways for the fragmentation of isosakurametin (**14**). The first one deals with the radical anion **14b** formed after the $CH_3^{\cdot}$ loss from the pseudomolecular anion **14a**. With the ion trap, we show that this **14b** radical anion at *m/z* 270 leads to the prominent radical anion at *m/z* 164. A retrocyclization fragmentation involving the 0 and 4 bonds is proposed for this fragment noted $-\mathbf{CH_3}^{0,4}\mathbf{B}^{\cdot -}$. This radical anion further loses two successive CO as shown in Scheme **4**, supporting the proposed structure. The second main pathway concerns almost all the other fragments which, if compared with the fragmentation of luteolin (**1**, Table 1), revealed the same neutral losses of CO, C_2H_2O, CO_2, and C_3O_2 obtained from the corresponding $[M - H]^-$ ion. This result is very important because it allows us to support the proposed fragments for **1** consecutive to these small neutral losses. Indeed, these similar fragmentations may be obtained from a similar intermediate for **1** and **14**. This means that sites implicated in these fragmentations should be the same. MS^n experiments operated on isosakurametin (Scheme **4**) support our previously discussed hypothesis show-

ing that these neutral losses from the pseudomolecular ions are localized on rings A and C. All these fragmentations, including the retrocyclization cleavages, were described above, except for the formation of anion at *m/z* 125, observed for **12** and **14** and attributed to a $^{1,4}\mathbf{A}^-$ ion, corresponding to the natural product phloroglucinol. This fragmentation has already been observed on flavan-3-ol derivatives [20]. It is interesting to note that this fragmentation, which was considered by Miketova et al. as diagnostic for the presence of two hydroxyl groups on the A ring of flavan-3-ols is not observed here for most of the flavonoids studied.

Conclusion

This study concerning various flavonoid aglycones investigated using ES ion trap mass spectrometry in the negative ion mode revealed new fragmentation pathways. Interesting diagnostic ions allows us to increase the structural specificity in the detection of these flavonoid aglycones.

The structural informative fragments described here may be very interesting from a phytochemical screening point of view, particularly in high throughput screenings using selected ion monitoring or selected reaction monitoring. Furthermore, a few tests using a triple quadrupole allowed us to observe the same fragments as those obtained with the ion trap.

In crude plant phytochemical analysis, negative ion mode should be more selective and more sensitive than the positive ion mode. Some problems reside in the low intensity of some characteristic fragments described here. Furthermore, some of the diagnostic RDA ions observed in the positive ion mode that are helpful for the structural determination of the A- and B-ring substitution patterns are lacking in the negative ion mode. For these reasons, the negative ion mode appeared to be a complementary method to the positive ion mode for the structural study of flavonoid aglycones by mass spectrometry.

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References

1. Bruneton, J. *Pharmacognosie-Phytochimie-Plantes médicinales, 3rd ed.*; Tec & Doc: Paris, 1999; p 310.
2. Leenen, R.; Roodenburg, A. J. C.; Tijburg, L. B. M.; Wiseman, S. A. *Eur. J. Clin. Nutr.* **2000,** *54*, 87–92.
3. Dugas, A. J. J.; Castaneda-Acosta, J.; Bonin, G. C.; Price, K. L.; Fischer, N. H.; Winston, G. W. *J. Natl. Prod.* **2000,** *63*, 327–331.
4. Ng, T. B.; Liu, F.; Wang, Z. T. *Life Sci.* **2000,** *66*, 709–723.
5. Miyazawa, M.; Okuno, Y.; Nakamura, S. I.; Kosaka, H. *J. Agric. Food Chem.* **2000,** *48*, 642–647.
6. Crespo, M. E.; Galvez, J.; Cruz, T.; Ocete, M. A.; Zarzuelo, A. *Planta Med.* **1999,** *65*, 651–653.
7. Weber, G.; Shen, F.; Yang, H.; Prajda, N.; Li, W. *Anticancer Res.* **1999,** *19*, 3703–3709.
8. Butterweck, V.; Jurgenliemk, G.; Nahrstedt, A.; Winterhoff, H. *Planta Med.* **2000,** *66*, 3–6.
9. Merken, H. M.; Beecher, G. R. *J. Agric. Food Chem.* **2000,** *48*, 577–599.
10. Mabry, T. J.; Markham, K. R. *In the Flavonoids*, Harborne, J. B.; Mabry, T. J.; Mabry, H., Ed.; Chapman and Hall: London, 1975; p 78.
11. Drewes, S. E. In *Chroman and related compounds*; Verlag Chemie: Wheinheim, 1974; p 41.
12. Porter, Q. N.; Baldas, J. In *Mass Spectrometry of Heterocyclic Compounds*; Weissberger, A.; Taylor, A. C., Ed.; Wiley: New York, 1971; p 89.
13. Ma, Y. L.; Li, Q. M.; Van den Heuvel, H.; Clayes, M. *Rapid Commun. Mass Spectrom.* **1997,** *11*, 1357–1364.
14. Ma, Y. L.; Vedernikova, I.; Van den Heuvel, H.; Clayes, M. *J. Am. Soc. Mass Spectrom.* **2000,** *11*, 136–144.
15. Lin, Y. Y.; Ng, K. J.; Yang, S. *J. Chromatogr.* **1993,** *629*, 389–393.
16. Nielsen, S. E.; Freese, R.; Cornett, C.; Dragsted, L. O. *Anal. Chem.* **2000,** *72*, 1503–1509.
17. Nindi, M. M.; Kgarebe, B. V.; Wolfender, J. L.; Abegas, B. M. *Phytochem. Anal.* **1999,** *10*, 69–75.
18. Li, D. T.; Sheen, J. F.; Her, G. R. *J. Am. Soc. Mass Spectrom.* **2000,** *11*, 292–300.
19. Ma, Y. L.; Van den Heuvel, H.; Clayes, M. *Rapid Commun. Mass Spectrom.* **1999,** *13*, 1932–1942.
20. Miketova, P.; Schram, K. H.; Whitney, J.; Li, M.; Huang, R.; Kerns, E.; Valvic, S.; Timmermann, B.; Rourick, R.; Klohr, S. *J. Mass Spectrom.* **2000,** *35*, 860–869.