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Electrospray tandem mass spectrometry analysis of methylenedioxy chalcones, flavanones and flavones

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RATIONALE: Several methylenedioxy chalcones, flavanones and flavones substituted with mono-, di- and trimethoxy groups have been used in the treatment of proliferative conditions like cancer and inflammatory diseases. The application of these flavonoids in biology requires an analytical method to ensure a detailed knowledge of their structures after drug metabolism.
METHODS: Electrospray ionization mass (ESI-MS) and tandem mass (ESI-MS/MS) spectra were acquired using a Q-TOF 2 instrument. Fragmentation patterns and their pathways were analyzed by CID-MS^{2-3} spectra acquired in a LXQ linear ion trap mass spectrometer using standard isolation and excitation procedures (activation *q* value of 0.25, activation time of 30 ms). ESI-MS and ESI-MS^n conditions: spray voltage 5 kV, nitrogen 8.00 sheath gas flow rate (arb), heated capillary temperature 275°C, capillary voltage 10.99 V; tube lens voltage 75.01 V.
RESULTS: The ESI-MS/MS spectra of chalcones were nearly identical to their corresponding isomeric flavanones with $^{0,\alpha}A^+/^{1,3}A^+$ and $^{0,1'}B^+/^{1,4}B^+$ cleavages. Other common losses are of $\cdot CH_3$, H_2O, HCHO and C_2H_2O. The characteristic loss of C_2H_2O and absence of a $^{0,\alpha}B^+/^{1,3}B^+$ product ion allows to distinguish between the 2- or 4-methoxy-substituted chalcones and flavanones. Common losses of $\cdot CH_3$, $\cdot CH_3$ and $\cdot H$, and $C_2H_2O_2$ characteristic for the presence of methylenedioxy groups were observed in flavones.
CONCLUSIONS: The substitution pattern on the B-ring leads to distinct base peak formation in the flavones. In addition, differentiation of isomers with methoxy substituents in *ortho* and *para* positions of the B-ring was achieved using MS/MS in chalcones and flavanones. This method will be helpful for identification of these compounds in biological mixtures.

Flavonoids and their derivatives which form a large class of food constituents are naturally present in fruits, vegetables as well in beverages, such as red wine and tea. They form an integral part of the human diet as many of them alter metabolic processes and have a positive impact on health.[1] Scientific interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds.[2] As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity in both *in vivo* and *in vitro* systems.[3] Apart from antioxidant activities, flavonoids also have scavenging effects on activated carcinogens and mutagens, act on proteins that control cell cycle progression and altered gene expression.[4]

In this regard newer synthetic flavonoid moieties based on naturally occurring ones having specific groups have been synthesized and tested for biological activity. For example, chalcones having the methylenedioxy group at 3,4 positions have been tested for treatment of proliferative condition like cancer and inflammatory conditions.[5] The 3,4-methylenedioxy-3′,4′,5′-trimethoxychalcone **1** is a potent anticancer prodrug activated by the tumor-selective catalytic activity of the cytochrome P450 enzyme CYP1B1 to give the 3,4-dihydroxy group which is a potent broad spectrum tyrosine kinase inhibitor.[6] Synthetic flavones like 7,8,3′,4′-tetrahydroxyflavone **2** and their analogues are known to inhibit telomerase, and have been intensively explored as anticancer agents.[7] The corresponding tetramethoxychalcone **3** is also known to inhibit the generation of an inflammatory mediator (Fig. 1).[8]

Several methoxy-substituted flavonoids like tangeretin, a 5,6,7,8,4′-pentamethoxyflavone, found in the peel of most citrus fruits, can act as a prodrug, forming its primary metabolite 4′-hydroxy-5,6,7,8-tetramethoxyflavone, and both inhibit cell cycle progression in primary hepatocytes.[9] Tangeretin also protects against the development of experimentally induced Parkinson's disease in rats.[10] In addition, nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), along with tangeretin detected and isolated from orange juice, was found to inhibit P-glycoprotein drug efflux transporter, but not the cytochrome P450 (CYP) isozyme CYP3A4 and can be potential agents for reversing multidrug resistance or for recovering the bioavailability of certain drugs.[11]

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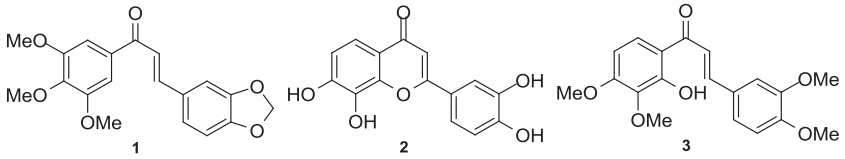


Figure 1. Structures of chalcones and flavones with biological activity.

Because all these biochemical activities like antioxidant, cardiovascular, anticancer, and other age-related disease protective properties are dependent on the individual flavonoid structure, each new compound needs to be studied systematically to assess its structure before and after drug metabolism. Mass spectroscopy is routinely used for the structural elucidation of flavonoid metabolites in *in vivo* studies. The main conjugations expected for *in vivo* metabolites are *O*-methylation, *O*-glucuronidation and *O*-sulfation and they can be easily identified by the mass they add to the original molecule.[12] Mass spectrometric techniques are indispensible tools for the identification and structural studies of flavonoid glycosides using electrospray ionization (ESI) and liquid chromatography/mass spectrometry (LC/MS) and have been previously reviewed.[13]

Considering the importance of methylenedioxy- and methoxy-substituted flavonoids, and in continuation of our previous study,[14] it was desirable to synthesize new flavonoids with such substitution patterns for biological evaluation. In this paper we describe the detailed mass spectral characterization of synthetic chalcones, flavanones and flavones derived from 2′-hydroxy-3′,4′-methylenedioxyacetophenone (**4**) and mono (2- and 4-), di (3,4-) and tri (3,4,5-) methoxybenzaldehyde using electrospray ionization tandem mass spectrometry (ESI-MS/MS).

EXPERIMENTAL

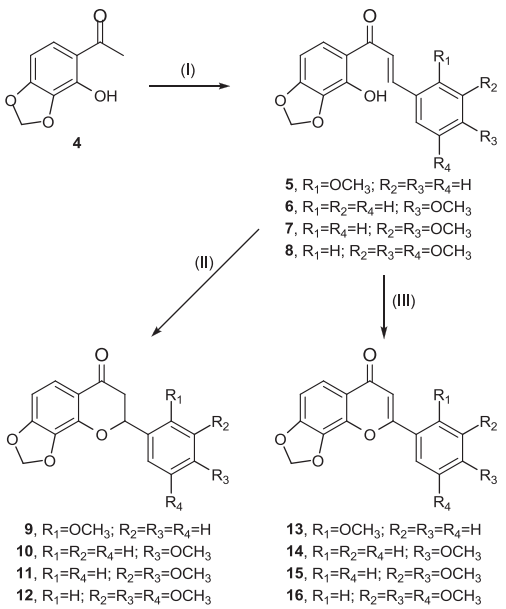
Mass spectrometry

Positive ion ESI mass spectra and tandem mass spectra were acquired using a Q-TOF 2 instrument (Micromass, Manchester, UK). The samples for ESI analyses were prepared by diluting 1 μL of the flavonoid solutions in chloroform (~10^{-5} M) in 200 μL of methanol. Nitrogen was used as the nebulizer gas and argon as the collision gas. Samples were introduced into the mass spectrometer at a flow rate of 10 μL min^{-1}, the needle voltage was set at 3000 V and the ion source temperature was 80°C. The cone voltage was 35 V. Collision-induced decomposition tandem mass spectra were acquired by selecting the desired ion with the quadrupole section of the mass spectrometer and using a collision energy of 30–50 eV. The product ions were analyzed with the time-of-flight (TOF) analyzer. In MS and MS/MS experiments, the TOF resolution was set to ~9000. In MS/MS experiments, the Q1 resolution was set to ~0.7 Da. Data acquisitions were carried out with a Micromass Mass Lynx data system. The lock mass in each product ion spectrum was the calculated monoisotopic mass/charge of the precursor ion and the empirical formula, observed and calculated mass/charge ratio, double-bond equivalent and mass error were determined for the fragment ions observed in the MS/MS spectra of the studied compounds.

MS^3 spectra were acquired in a LXQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) with an ESI source. Samples were introduced into the mass spectrometer at a flow rate of 8 μL min^{-1}. ESI conditions used to obtain ESI-MS and ESI-MS^n spectra were: spray voltage 5 kV, nitrogen 8.00 sheath gas flow rate (arb), heated capillary temperature 275°C, capillary voltage 10.99 V, and tube lens voltage 75.01 V. CID-$MS^{2–3}$ experiments were performed on mass-selected precursor ions using standard isolation and excitation procedures (activation *q* value of 0.25, activation time of 30 ms). The collision energy used was between 20–24 (arbitrary units). Data acquisition was carried out with an Xcalibur data system (ThermoFinnigan, San Jose, CA, USA).

Synthesis

The syntheses of chalcone, flavanone and flavones derivatives were carried out using the reported procedure (Scheme 1).[14] The experimental details of the flavonoids used in this study are described in the Supporting Information.



Scheme 1. Synthesis of methylenedioxy-substituted mono-, di- and trimethoxychalcones, flavanones and flavones; (I) Benzaldehyde derivative, alcoholic KOH; (II) TFA, reflux, 2 h; (III) Cat. I_2, DMSO, reflux, 45 min.

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RESULTS AND DISCUSSION

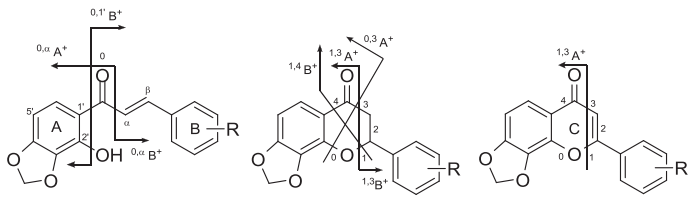
The ESI-MS spectra of the chalcones, flavanones and flavones **5–16** showed the $[M+H]^+$ ions and a minor $[M+Na]^+$ ion. The fragmentations of the $[M+H]^+$ ions were induced by collision with a gas under tandem mass spectrometry conditions (ESI-MS/MS). The MS/MS spectra of the chalcones were similar to the isomeric flavanones with similar substituents. The cyclization of 2′-hydroxychalcones to the corresponding isomeric flavanones has been observed in the gas phase and justifies their similar behavior.[15] Hence, the discussion of chalcones and flavanones (**5–12**) will be presented together based on the similar fragmentation patterns observed while those of flavones (**13–16**) will be discussed separately.

The ion nomenclature for the product ions for chalcones, flavanones and flavones has been adapted as proposed in literature (Scheme 2). The $^{i,j}A$ and $^{i,j}B$ labels are used to designate product ions containing intact A- and B-rings, and the subscripts i and j refer to the C-ring bonds that have been broken.[16] The fragmentations or proposed structures in chalcones, flavanones and flavones were confirmed by MS^3 experiments. The product ions observed in the MS^n experiments and not observed in the MS^2 spectra were noted by their *m/z* values and are presented in Supplementary Tables S1 and S2 (see Supporting Information).

Fragmentation of chalcones and flavanones 5–12

The main product ions observed in the ESI-MS/MS spectra of the $[M+H]^+$ ion of chalcones and flavanones (**5–12**), the most probable identification and the corresponding relative abundance (RA) are summarized in Table 1. The fragmentation pathways of the studied chalcones and flavanones show several common features but also show interesting differences. The common fragmentation observed for chalcones or flavanones involves $^{0,\alpha}A^+/^{1,3}A^+$ cleavage giving a product ion at *m/z* 165 for all compounds **5–12** and $^{0,1'}B^+/^{1,4}B^+$ product ions at *m/z* 161 for **5**, **6**, **9**, and **10**; *m/z* 191 for **7**, and **11**; and *m/z* 221 for **8** and **12**. These pairs of product ions ($^{0,\alpha}A^+/^{1,3}A^+$ and $^{0,1'}B^+/^{1,4}B^+$) indicate that the methylenedioxy substituent is attached to the A-ring and is constant for all compounds whereas the B-ring of each compound carries either the mono-, di- or trimethoxy substituents.

The $^{0,1'}B^+/^{1,4}B^+$ product ions are the base peak in the spectra of compounds **6–8** and **10–12**, while, in the case of compounds **5** and **9**, this ion was observed in 12% and 16% relative abundance (RA) (Table 1). It is worth mentioning here that differences in the position of substituents on the B-ring of chalcones (or flavanones), or the *ortho*-position in the cases of **5** and **9**, and *para*-position in the case of **6** and **10**, lead to similar fragmentation but with different RAs, thus allowing the differentiation of these pairs of isomers. The presence of the



Scheme 2. Ion nomenclature used for the fragmentation of flavonoids.[18]

Table 1. Identification of the $[M+H]^+$ ions and summary of the main product ions observed in the ESI-MS^2 spectra for the chalcones **5–8** and flavanones **9–12** with the indication of their relative abundances (%RA)[a]

Compound	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Formula	$C_{17}H_{15}O_5$	$C_{17}H_{15}O_5$	$C_{18}H_{17}O_6$	$C_{19}H_{19}O_7$	$C_{17}H_{15}O_5$	$C_{17}H_{15}O_5$	$C_{18}H_{17}O_6$	$C_{19}H_{19}O_7$
$[M+H]^+$ *m/z*	299.0	299.0	329.1	359.1	299.0	299.0	329.0	359.1
$[P-CH_3]^{+\bullet}$	-	284 (3)	314 (2)	344 (<1)	-	284 (3)	314 (1)	344 (<1)
$[P-H_2O]^+$	-	281 (4)	311 (2)	341 (2)	-	281 (4)	311 (2)	341 (2)
$[P-HCHO]^+$	-	269 (2)	299 (1)	-	-	269 (2)	299 (1)	-
$[P-HCHO-H_2O]^+$	-	251 (3)	281 (<1)	-	-	251 (3)	281 (<1)	-
$[P-C_2H_2O]^+$	257.0 (100)	257 (<1)	287 (1)	317 (9)	257.0 (100)	257 (<1)	287 (<1)	317 (8)
$[P-CO-H_2O]^+$	-	253 (1)	-	-	-	253 (1)	-	-
$[P-C_2H_2O_2]^+$	-	241 (2)	271 (<1)	-	-	241 (2)	271 (<1)	-
$[P-CO-HCHO-H_2O]^+$	-	223 (1)	253 (<1)	-	-	223 (1)	253 (<1)	-
$[^{0,1'}B^+/^{1,4}B^+\text{-}\cdot CH_3]^{+\bullet}$	-	-	-	206 (2)	-	-	-	206 (2)
$^{0,\alpha}A^+/^{1,3}A^+$	165 (38)	165 (36)	165 (20)	165 (13)	165 (86)	165 (39)	165 (18)	165 (15)
$^{0,1'}B^+/^{1,4}B^+$	161 (12)	161 (100)	191 (100)	221 (100)	161 (16)	161 (100)	191 (100)	221 (100)
$[P-B]^+$	191 (3)	191 (2)	-	-	191 (3)	191 (3)	-	-
$[^{0,1'}B^+/^{1,4}B^+]$-HCHO	-	-	-	191 (28)	-	-	-	191 (34)
$[^{0,1'}B^+/^{1,4}B^+\text{-}\cdot OCH_3]^{+\bullet}$	-	-	-	190 (4)	-	-	-	190 (4)
$^{0,\alpha}B^+/^{1,3}B^+$	-	133 (5)	163 (3)	193 (4)	-	133 (5)	163 (3)	193 (4)
$[P-C_2H_2O-B]^+$	151 (76)	-	-	151 (3)	151 (88)	-	-	151 (4)

[a]P refers to the precursor ion, i.e. the ion undergoing MS^2 $[M+H]^+$ (*m/z*) and B indicates the B-ring of chalcone/flavanone.

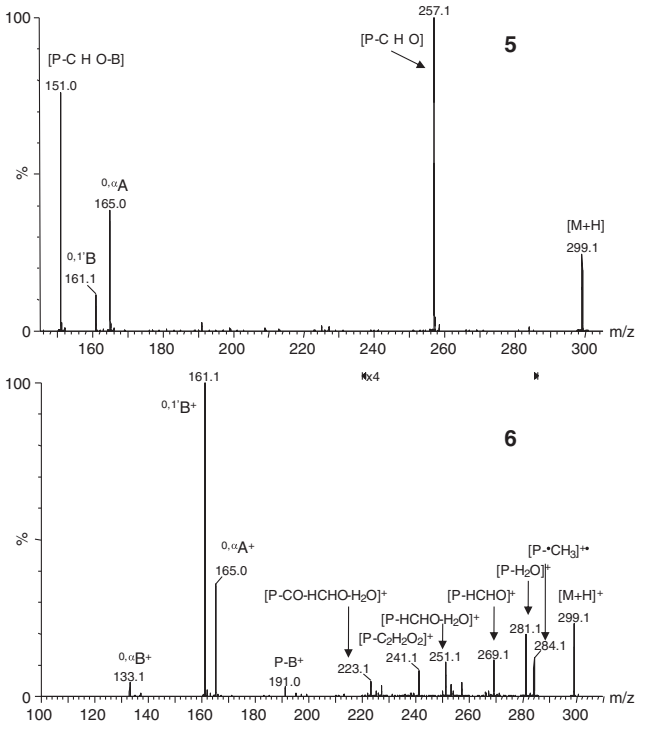


Figure 2. ESI-MS^2 spectra of the $[M+H]^+$ ions at *m/z* 299 of positional isomeric chalcones **5** and **6**.

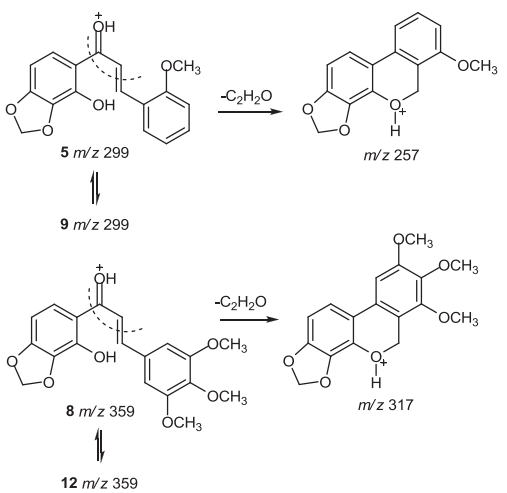
electron-donating methoxy substituent at the *ortho*-position in chalcone **5** (or flavanone **9**) changes the fragmentation pattern compared to the *para*-position in **6** (or **10**) and serves as a diagnostic feature for differentiating between the positional isomers [see Fig. 2 for chalcones **5** and **6**; see Supplementary Figs. S3 and S4 (Supporting Information) for flavanones **9** and **10**]. Previous works have shown that substituted chalcones (*o*- and *p*-methoxy-substituted) show different fragmentation patterns for isomeric chalcones depending on the position of substituents.[17] Similarly, specific product ions were formed/observed in the case of *o*-nitro substituents in the B-ring of chalcones and were absent in the *m*- or *p*-nitro-substituted chalcones.[18]

The $^{0,\alpha}B^+/^{1,3}B^+$ product ions were also observed for the studied compounds and were detected at *m/z* 133 for **6** and **10**, *m/z* 163 for **7** and **11**, and *m/z* 193 for **8** and **12**, while they were absent in case of **5** and **9**. [see Fig. 2 for **5** and **6**; Supplementary Figs. S1, S2, S3, S4, S5 and S6 (Supporting Information) for **7**, **8**, **9**, **10** and **11**.] These $^{0,\alpha}B^+/^{1,3}B^+$ product ions can also assist as diagnostic product ions to distinguish between the positional isomers **5** versus **6** and **9** versus **10** (see Table 1).

MS^3 experiments for the ions at *m/z* 161, 191 and 221 ($^{0,1'}B^+/^{1,4}B^+$), in the case of compounds **6**, **10**; 7, **11**; and **8**, **12**, gave the product ions at *m/z* 133, 163 and 193 ($^{0,\alpha}B^+/^{1,3}B^+$), respectively, by loss of CO (–28 Da). The MS^3 experiment on the product ion observed at *m/z* 221 for compound **8** and **12** gave the product ions observed in its MS^2 spectrum at *m/z* 190 (-•OCH_3) and 191 (-HCHO), confirming the fragmentation pathway for these ions along with the product ions at *m/z* 206 (-•CH_3) and 189 (CH_3OH) (see Supplementary Table S1, Supporting Information).

Apart from the $^{i,j}A^+$ and $^{i,j}B^+$ product ions chalcones and flavanones also show losses of small molecules and/or radicals from the $[M+H]^+$ ion. The most common losses observed are 15 Da (•CH_3), 18 Da (H_2O) which are typical of chalcones and flavanones[17] along with losses of neutral species with 30 Da (HCHO), 42 Da (C_2H_2O), 58 Da ($C_2H_2O_2$); combined losses of 46 Da (CO+H_2O), 48 Da (HCHO+H_2O) and 76 Da (CO+HCHO+H_2O). Losses of 15 Da, 18 Da, 30 Da and 48 Da were observed in the case of chalcones **6**, **7**, **8**; flavanones **10**, **11**, **12** and were curiously found to be absent in chalcone **5** and flavanone **9** wherein the methoxy group is in the *ortho*-position in the B-ring. Generally methoxy-substituted chalcones showed product ions derived by loss of 15 Da and 18 Da and combined losses of 46 Da (CO+H_2O), as observed in the present cases.[17] Loss of 30 Da was proposed[17] as loss of CO followed by 2H in case of non-methoxylated chalcones, but can also be considered as loss of HCHO[19] from the methoxy substituents by addition of H to the ring. Similarly, combined loss of 46 Da (CO+H_2O) was only seen for chalcone **6** and its isomeric flavanone **10**. Loss of 76 Da (CO+HCHO+H_2O) was observed for chalcones **6**, **7** and flavanones **10** and **11**. Loss of 58 Da ($C_2H_2O_2$) is characteristic for the presence of a methylenedioxy group[19] and was observed with low RA for chalcones **6**, **7** and flavanones **10** and **11** (see Table 1; Fig. 2 for chalcone **6**; see Supplementary Figs. S1, S4 and S5, Supporting Information, for **7**, **10** and **11**).

The loss of 42 Da (C_2H_2O) is known to be characteristic for substituted chalcones and flavanones[17,20] and it was observed as the base peak at *m/z* 257 for compounds **5** and **9**; at *m/z* 257 in compounds **6**, **10**; and at *m/z* 287 in **7**, **11** with low RA (see Table 1; Scheme 3). This loss in compound **5** and **9** (100%RA) can be rationalized as arising due to the proximity of the 2-methoxy substituent on the B-ring as shown in 2-methoxy- or hydroxychalcones.[20] This cleavage of 42 Da (C_2H_2O) is also proposed for flavones and flavonols[16,21] and can also account for the formation of these ions in the chalcones **5**, **8** and flavanones **9**, **12**. In compounds **8** and **12** the loss of 42 Da produced a product ion at *m/z* 317 with 9% and 8% RA, respectively. This fragmentation serves as a characteristic for distinguishing between the *ortho*- and *para*-methoxychalcones (**5** and **6**) and -flavanones (**9** and **10**) and also between the 3,4-dimethoxy- and 3,4,5-trimethoxychalcones (7 and **8**) and -flavanones (**11** and **12**).



Scheme 3. Proposed fragmentation pathway observed in the MS^2 spectra of chalcones **5**, **8** and flavanones **9**, **12**.

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Table 2. Identification of the $[M+H]^+$ ions and summary of the main product ions observed in the ESI-MS^2 spectra for the flavones **13–16** with the indication of their relative abundances (%RA)[a]

Compound	13	14	15	16
Formula	$C_{17}H_{13}O_5$	$C_{17}H_{13}O_5$	$C_{18}H_{15}O_6$	$C_{19}H_{17}O_7$
$[M+H]^+$ *m/z*	297.0 (40)	297.0 (24)	327.0 (23)	357.0 (25)
$[P\text{-}CH_3]^{+\cdot}$	282 (66)	282 (26)	312 (11)	342 (2)
$[P\text{-}{\cdot}CH_3\text{-}{\cdot}H]^+$	281 (18)	-	311 (100)	341 (25)
$[P\text{-}HCHO]^+$	-	-	-	327 (44)
$[P\text{-}CH_3OH]^+$	265 (19)	-	-	-
$[P\text{-}{\cdot}CH_3\text{-}{\cdot}H\text{-}H_2O]^+$	-	-	-	323 (14)
$[P\text{-}CO_2]^+$	-	-	283 (28)	313 (11)
$[P\text{-}{\cdot}CH_3\text{-}HCHO]^{+\cdot}$	-	-	282 (5)	312 (5)
$[P\text{-}CO\text{-}H_2O]^+$	-	-	-	311 (10)
$[P\text{-}{\cdot}CH_3\text{-}CO]^{+\cdot}$	254 (13)	254 (100)	-	-
$[P\text{-}C_2H_2O_2]^+$	239 (5)	239 (14)	269 (2)	299 (6)
$[P\text{-}{\cdot}CH_3\text{-}H_2O\text{-}CO]^{+\cdot}$	-	-	266 (13)	296 (100)
$[P\text{-}HCHO\text{-}CH_3OH]^+$	-	-	-	295 (5)
$[P\text{-}{\cdot}CH_3\text{-}H_2O\text{-}2CO]^{+\cdot}$	-	-	-	268 (23)
$[P\text{-}{\cdot}CH_3\text{-}H_2O\text{-}CO\text{-}{\cdot}HCO]^+$	-	-	-	267 (28)
$[P\text{-}2HCHO\text{-}{\cdot}OCH_3]^{+\cdot}$	-	-	-	266 (5)
$[P\text{-}{\cdot}CH_3\text{-}2CO]^{+\cdot}$	-	226 (12)	-	-
$[P\text{-}C_2H_2O_2\text{-}{\cdot}CH_3]^{+\cdot}$	-	224 (3)	254 (2)	-
$[P\text{-}C_2H_2O_2\text{-}CO]^+$	-	211 (11)	241 (<1)	-
$[P\text{-}C_2H_2O_2\text{-}CO\text{-}{\cdot}CH_3]^{+\cdot}$	-	196 (4)	-	-
$[P\text{-}{\cdot}CH_3\text{-}CO\text{-}C_3O_2]^{+\cdot}$	-	186 (6)	-	-
$[P\text{-}C_2H_2O_2\text{-}2CO]^+$	-	183 (6)	213 (<1)	-
$^{1,3}A^+$	165 (6)	165 (12)	165 (<1)	165 (2)
$^{1,3}A^{+\cdot}$	164 (100)	-	-	-

[a]P refers to the precursor ion, i.e. the ion undergoing MS^2 $[M+H]^+$ (*m/z*).

Fragmentation of flavones 13–16

The main product ions $[M+H]^+$ observed in the ESI-MS/MS spectra of flavones (**13–16**) and the corresponding relative abundance (RA) are summarized in Table 2. The common fragmentation pathways for all these compounds will be discussed followed by the interesting differences which help in differentiating between the two monomethoxy (**13** and **14**; see Fig. 3) and also the dimethoxy- and trimethoxyflavones (**15** and **16**; see Supplementary Figs. S7 and S8, Supporting Information). The characteristic retro-Diels-Alder (RDA) fragmentation for flavones or the $^{1,3}A^+$ product ion at *m/z* 165 was observed for all flavones **13**, **14**, **15** and **16**. In case of compound **13** the same RDA product ion losses the ·H radical to give a radical ion as base peak at *m/z* 164 (Fig. 3). Here the ketene type intermediate is proposed since, under MS^3 experiment, loss of 28 Da was observed (see Supplementary Table S2 for MS^3 product ions in Supporting Information).[16] This product ion serves as a diagnostic for distinguishing the *o*-methoxychalcone **13** from its *p*-methoxy isomer and the di- and trimethoxy derivatives in which this ion is absent.

The most common loss of 15 Da (·CH_3) was observed for all flavones **13**, **14**, **15** and **16**. This loss is easily detected and proves the presence of the methoxy groups in flavonoids.[16] Loss of 16 Da seen for flavones **13**, **15** and **16** is considered as a combined loss of ·CH_3 and ·H. This fact was proved by MS^3 experiments which showed that ion at *m/z* 282 in **13**, *m/z* 312 in **15** and *m/z* 342 in **16** (all due to loss of 15 Da or ·CH_3) leads to the product ions at *m/z* 281, 311 and 341, respectively. This product ion (–16 Da) corresponds to the base peak of the spectrum of flavone **15** (see Supplementary Fig. S7, Supporting Information). It is worth mentioning that, although the loss of 16 Da is observed for all flavones in this series except for flavone **14**, it was specifically observed as base peak for the 3,4-dimethoxyflavone **15**, indicating that it can serve as diagnostic ion to distinguish it from the other derivatives in this series. Similar sequential loss of 16 Da was confirmed by MS^n experiments for methoxychalcones.[17]

Loss of 58 Da ($C_2H_2O_2$) characteristic for the presence of a methylenedioxy group[19] leads to the formation of the product ion at *m/z* 239 for compound **13**, **14**; *m/z* 269 for **15**; and at *m/z* 299 for **16**(see Supplementary Fig. S8 Suporting Information). Comparatively this product ion was observed with higher % RA in the case of flavone **14** (see Table 2).

The loss of small molecules and/or radicals from the $[M+H]^+$ ion was also observed. Loss of 44 Da (CO_2) was seen in flavones **15** and **16** to give product ions at *m/z* 283 and 313, and this loss has been shown to occur by the contraction of the C-ring of flavonoids using ESI-MS^2 in the negative mode.[21] Loss of 46 Da ($CO+H_2O$) gave a product ion at *m/z* 311 in flavone **16**. Specific loss of 32 Da (CH_3OH) which proves the presence of a methoxy group was observed only in the case of flavone **13** to give a product ion at *m/z* 265. Combined loss of 43 Da ($CO+{\cdot}CH_3$) giving an ion at *m/z* 254 was seen for flavone **13** and forms the base peak for **14**; thus, this product ion is able to differentiate between the *p*-methoxyflavone **14** from its *o*-methoxy isomer **13** which has a base peak arising from the RDA reaction and loss of ·H as

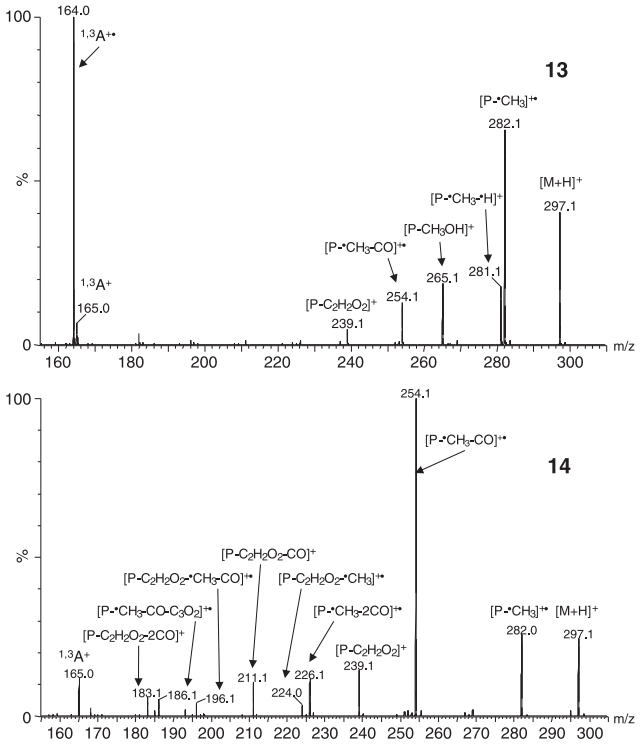
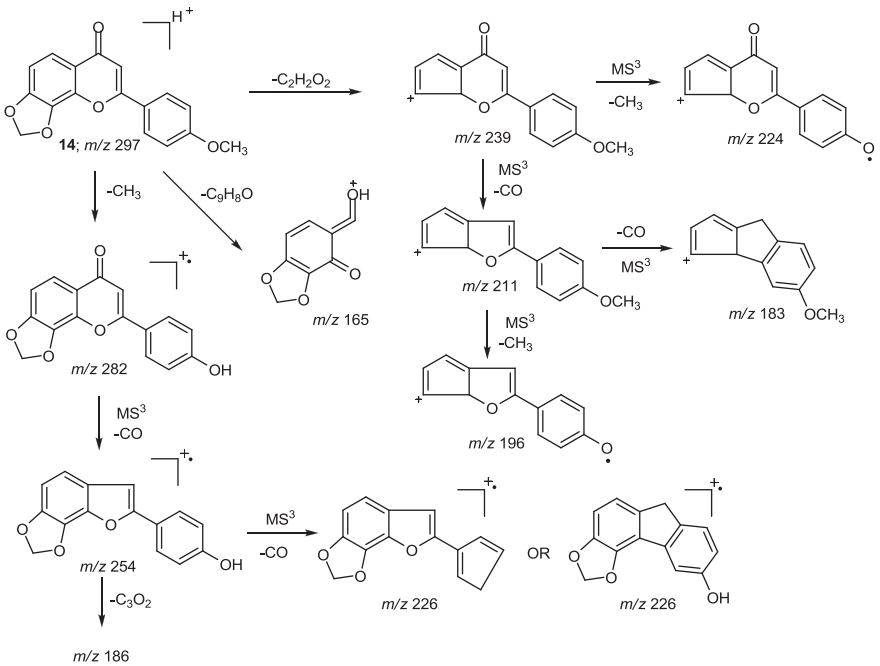


Figure 3. ESI-MS^2 of the [M+H]$^+$ ions at *m/z* 297 of positional isomer flavones **13** and **14**.

discussed earlier (see Fig. 3). An MS^3 experiment on the ion at *m/z* 282 (-•CH_3) in **13** and **14** gave rise to *m/z* 254 due to combined loss of •CH_3 followed by CO. This type of combined loss has been specifically observed in flavones having the methoxy group at the *p*-position on the B-ring using FAB CID MS/MS.[16] A further MS^3 experiment on *m/z* 254 in the case of **14** gave rise to *m/z* 226 (100% RA) which was also seen in the MS^2 spectrum in 12% RA and was deduced to be due to further loss of CO. A product ion at *m/z* 186 observed in the MS^2 spectrum was also seen as second most abundant ion in MS^3 and as the base peak in the MS^4 on the ion of *m/z* 254 (see Supplementary Table S2, Supporting Information). This loss was deduced to have arisen from combined loss of 43 Da (CO+•CH_3) followed by 68 Da (C_3O_2) which has been observed in flavonoids and has been ascribed to arise from the A-ring containing a 1,3-dihydroxy group.[21] In the present case this loss of 68 Da (C_3O_2) can be proposed as arising from the methylenedioxy group on the A-ring. Some of the above proposed fragmentation mechanisms in the MS^2 and MS^3 of flavone **14** are represented in Scheme 4.

Loss of 61 Da (•CH_3+H_2O+CO) gives ions at *m/z* 266 for **15** and forms the base peak at *m/z* 296 for flavone **16** (see Supplementary Fig. S8, Supporting Information). The stepwise losses of these product ions were derived by MS^3 experiments on the product ion at *m/z* 312 in **15** and *m/z* 342 in **16** which explained their formation and confirmed the pathway by comparing their %RA in the MS^2 as well as MS^3 spectra (see Table 2 and Supplementary Table S2, Supporting Information). This stepwise loss is in complete agreement with the fragmentation of flavones having higher or lower degree of methoxylation which starts with loss of the methyl radical, followed by H_2O and a carbonyl group.[22] Combined loss of •CH_3 and HCHO (45 Da) gives ions at *m/z* 282 for **15** and *m/z* 312 for **16**. Characteristic loss of a formaldehyde group was observed only in the case of flavone **16** to give an ion at *m/z* 327; although unusual, it has been observed for methoxy- and methylenedioxy-substituted lignans.[19]



Scheme 4. Proposed fragmentations pathways observed in the MS^2 and MS^3 of flavone **14**.

Product ions at *m/z* 266 and 267 observed only in flavone **16** can be explained by combined loss of 91 Da (2 HCHO and $^{\bullet}OCH_3$) and 90 Da (combined loss of $^{\bullet}CH_3$+H_2O+CO +HCO$^{\bullet}$). The fragmentation pathway for 90 Da was confirmed as that proposed by carrying out MS^3 experiments. It was observed that the product ion at *m/z* 267 was derived from product ions at *m/z* 296 (loss of $^{\bullet}CH_3$+H_2O+CO) indicating the stepwise formation. Proposed fragmentations in the MS^2 and MS^3 of flavone **16** are represented in Supplementary Scheme S1 (see Supporting Information).

The most probable elemental compositions of the product ions were obtained with a high degree of confidence (see Supplementary Table S3, Supporting Information). The errors between the observed masses and calculated values are around –35.7 to 17.6 mDa (–176.5 to 69.2 ppm), indicating very good mass accuracy and confirming the compositions of the observed product ions.

CONCLUSIONS

The electrospray ionization tandem mass spectrometry of methylenedioxy-substituted mono-, di- and trimethoxychalcones, flavanones and flavones show several interesting features. The ESI tandem mass spectra of all the studied 2′-hydroxychalcones were identical to the corresponding isomeric flavanones due to cyclization in the gas phase. The most common fragmentation of chalcones or flavanones involved $^{0,\alpha}A^+/^{1,3}A^+$ and $^{0,1'}B^+/^{1,4}B^+$ cleavages giving common ions for all studied compounds. The common losses observed in chalcones and flavanones are 15 Da ($^{\bullet}CH_3$), 18 Da (H_2O), 30 Da (HCHO) and 42 Da (C_2H_2O). The characteristic loss of 42 Da (C_2H_2O) serves as a diagnostic product ion between the *o*-methoxy- or *p*-methoxychalcones (**5** and **6**) and -flavanones (**9** and **10**) and also between the 3,4-dimethoxy- and 3,4,5-trimethoxychalcones (7 and **8**) and -flavanones (**11** and **12**).

All the studied flavones have shown different fragmentations leading to distinct base peaks dependent on the substitution pattern on the B-ring. The common losses observed in flavones are 15 Da ($^{\bullet}CH_3$), 16 Da ($^{\bullet}CH_3$ and $^{\bullet}H$) and 58 Da ($C_2H_2O_2$) which are characteristic for the presence of methylenedioxy groups.[19] In the flavones it was possible to distinguish the two mono-methoxy-substituted (2- and 4-methoxy) compounds by the distinct base peak due to RDA cleavage followed by loss of a $^{\bullet}H$ radical in the former while sequential loss of $^{\bullet}CH_3$ followed by CO in the latter. The di- and trimethoxy flavones also showed loss of 16 Da and combined loss of methoxy and formaldehyde groups, respectively. This study enables to distinguish between the two different mono-methoxychalcones, flavanones and di- or trimethoxy derivatives. It is clear that the flavones in this study fragment differently depending on the substitution pattern on the B-ring and this will be helpful for identification in biological mixtures.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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