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The formation and fragmentation of flavonoid radical anions☆

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Dedicated to Professor Michael Gross, on the occasion of his 70th birthday and in recognition of his important contributions to organic, organometallic and biological mass spectrometry and his service to the mass spectrometry community.

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ABSTRACT

Negative electrospray ionization of iron(III) salen complex of flavonoids, M, was used in conjunction with collision-induced dissociation (CID) to examine the formation and subsequent fragmentation reactions of their radical anions $[M-2H]^{\bullet -}$. Sixteen different flavonoids were investigated from three different sub-groups (flavanone, flavone and flavanol). All formed the desired iron salen complex, $[Fe^{III}(salen)(M-2H)]^-$, and all but one of these complexes produced the radical anion upon CID. The CID fragmentation reactions of these radical anions, $[M-2H]^{\bullet -}$, were compared to their even electron counterparts $[M-H]^-$. Generally the former provided more structural information, with novel cross-ring cleavages of sugar(s) often being observed. Isomeric flavonoids can often be distinguished based on the differences in the fragmentation pathways of their radical anions.

1. Introduction

Since the first mass spectrometry based study on peptides using electron ionization (EI) appeared over 50 years ago [1], the diverse nature of biomolecules has offered interesting challenges and opportunities for the mass spectrometry community. Over the intervening period, two major breakthroughs have occurred: (i) the invention of a series of new ionization methods; (ii) the development of tandem mass spectrometry techniques. Professor Mike Gross has been at the forefront of applying these new technologies to address fundamental and applied problems for different classes of biomolecules. Some of the highlights of his pioneering work include: (i) development and application of the powerful combination of fast atom bombardment (FAB) and tandem mass spectrometry on multisector instruments [2] for the analysis of a range of biomolecules including cyclopeptides [3], lipids [4] and nucleic acids [5]; (ii) the discovery [6] and coining of the term "charge remote fragmentation" [7,8]; (iii) some of the first studies on the gas phase chemistry of metal–peptide interactions [9].

Although radical cleavage reactions of biomolecules have been known from early EI/MS studies [10] and from high energy CID of FAB generated $[M+H]^+$ [11], these have largely remained a curiosity due to challenges with volatility or ready access to appropriate instrumentation. Thus a major contemporary research theme in bioanalytical mass spectrometry has been the development of new methods that utilize radical cleavage reactions to gain novel structural information. While most efforts have been devoted to methods with potential applications in the analysis of peptides and proteins [12,13], reports have also appeared on other classes of biomolecules, including oligonucleotides [14–21], oligosaccharides [22–24] and lipids [25,26]. The new types of radical fragmentation methods developed fall into four broad areas:

(i) Ion–electron interactions, which can be further classified according to the nature of the ion (e.g., multiply charged versus singly charged, cation versus anion), the energy of the electron and the nature of the radical chemistry (for reviews see [27,28]).

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(ii) Ion–ion interactions, which often proceed via electron transfer to form radical ions (for reviews see [29,30]).
(iii) UV–vis photodissociation of native (for Refs. see [31–33]) and derivatized biomolecules (for a review see [13]).
(iv) Collision-induced dissociation (CID) on derivatized biomolecules. These can include CID of covalently modified biomolecules which install a weak bond that is susceptible to homolysis [34–39] and CID on ternary metal complexes that involve one electron redox reactions in which the metal centre is reduced and the biomolecule is oxidized [40–45]. The latter of these two methods has been used to form radical ions of peptides [40–45] and nucleobases [19,20], and here we explore whether it can be extended to the formation of $[M-2H]^{\bullet-}$ of flavonoids.

Flavonoids are an important class of biomolecules formed as secondary plant metabolites. They perform a wide range of functions in plants, including: comprising the coloured pigments of flowers; acting as enzyme inhibitors; defending against UV radiation and insects; and acting as metal chelating agents to prevent damage to plants [46]. The potential benefits of flavonoids to human health have been extensively examined, and recent studies have shown anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity [47–49]. Flavonoids are best known for their ability to act as antioxidants. Because of these important biological properties, flavonoids have been the subject of numerous mass spectrometry based studies (for reviews see [50–52]) including CID on metal complexes [53–55]. Recently Davis and Broadbelt showed that the $[M-H]^-$ of flavonol 3-O-glycosides undergo both homolytic and heterolytic saccharide cleavage and that the resultant odd electron $[Y_0-H]^{\bullet-}$ flavone product ions fragmented differently to their even electron $[Y_0]^-$ counterparts (for flavonoid fragment ion nomenclature, see Scheme 2 and corresponding text below) [56]. Inspired by this work, here we report that: (i) CID on ternary metal complexes of the flavonoids shown in Scheme 1 with Fe^{III}(salen), **17**, form radical ions for all flavonoids except Luteolin-7,3′-di-O-glucoside, **14**, and (ii) the subsequent CID behaviour of these $[M-2H]^{\bullet-}$ are different to their even electron $[M-H]^-$ counterparts.

2. Experimental

Homoorientin **2**, Hesperidin **15**, Luteolin-7,3′-di-O-glucoside **14**, Luteolin-7-O-glucoside **5**, Orientin **3**, Peltatoside **13**, Syringetin-3-O-galactoside **9**, Syringetin-3-O-glucoside **10** and Vitexin-2-O-rhamnoside **11** were all purchased from INDOFINE Chemical Company, Hillsborough, NJ, USA and were used as received. Naringin **12** and Quercitrin **4** hydrate were purchased from Sigma–Aldrich (Steinheim, Germany), Quercetin-3-beta-D-glucoside **6** and Quercetin-3-galactoside **7** from Fluka (Steinheim, Germany), and Rutin **16**, Myricetin **1** and Spiraeoside **8** were a gift of Prof. Owen Woodman of RMIT University and were used as received. The iron salen salt was available from a previous study [42].

All experiments were carried out on a Finnigan-LTQ-FT (Thermo, Bremen, Germany) mass spectrometer equipped with electrospray ionization (ESI) source [57,58] described in detail elsewhere [59,60]. Samples were typically prepared by combining a 0.137 mM methanolic Fe(III)salen solution with less than 0.1 mM of a methanolic solution of the flavonoid. Once prepared, the samples were immediately introduced to the mass spectrometer via the ESI source, using a flowrate of 5.0 μL/min. Typical ESI conditions used were: spray voltage, 2.7–4.5 kV; capillary temperature, 250 °C; nitrogen sheath pressure, 10–30 (arbitrary units). The capillary voltage and the tube lens offset were tuned to maximize the desired peak. The injection time was set using the automatic gain control function. The LTQ-FT mass spectrometer consists of: (i) linear ion trap; (ii) ion transfer optics; and (iii) FT-ICR mass analyzer. For the tandem mass spectrometry experiments, the desired ions produced via ESI were mass selected, trapped in the linear ion trap and subjected to CID at a He bath gas pressure of ca. 5×10^{-3} Torr at the room temperature. CID was carried out by mass selecting the desired ions with a 1.5–6 m/z units window and subjecting them to the following typical conditions: normalized collision energy between 16% and 40%, activation (Q) 0.25–0.35, and activation time of 30 ms. The wider isolation widths were used to select the $[Fe^{III}(salen)(M-2H)]^-$ complexes. In the case of the radical anions $[M-2H]^{\bullet-}$, the selected window for the MS^3 experiment was always less than 1.8 Da, to ensure that there was no contribution from the even electron $[M-H]^-$ to the CID spectrum of $[M-2H]^{\bullet-}$. The even electron anions $[M-H]^-$ were directly isolated from the electrospray in a MS/MS experiment, and thus are not contaminated by the ^{13}C isotopomer associated with the radical anions $[M-2H]^{\bullet-}$ as would be the case in a MS^3 experiment in which the $[M-H]^-$ were formed via CID on the $[Fe^{III}(salen)(M-2H)]^-$ complex. For high-resolution mass analysis, the ions were transferred via the ion optics transfer region ($\sim 2 \times 10^{-7}$ Torr) into an FT-ICR cell at a pressure below 1.5×10^{-9} Torr.

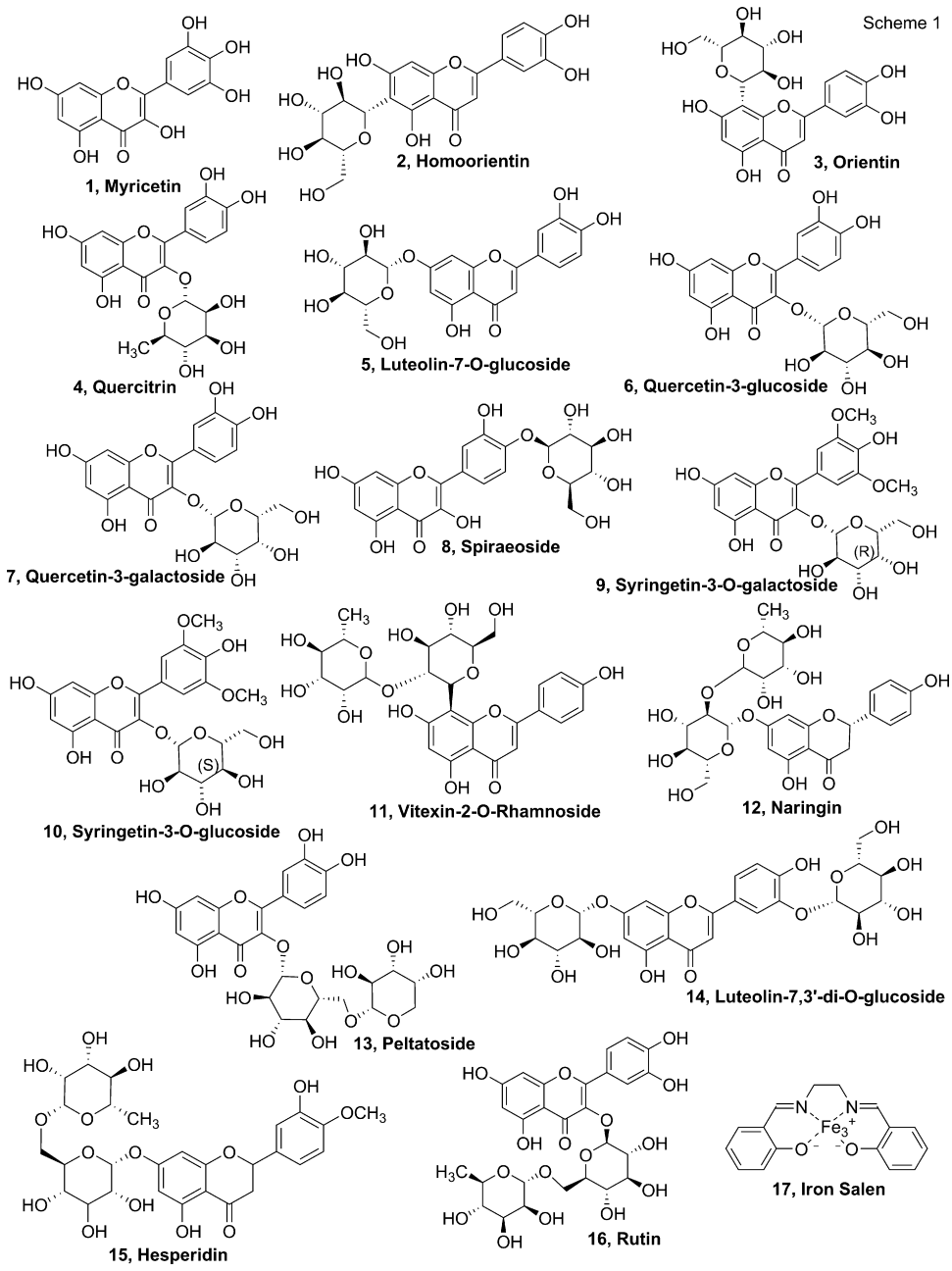
3. Results and discussion

Negative ion ESI of mixtures of the $[Fe^{III}(salen)]Cl$ salt and the flavonoid, M, result in a range of anions, including the desired $[Fe^{III}(salen)(M-2H)]^-$ complex for all flavonoids examined. Each of these complexes was subjected to CID with the aim of forming radical anions, $[M-2H]^{\bullet-}$, of the flavonoid through electron transfer to the metal. The fragmentation reactions of each of these flavonoid radical anions were examined in a series of MS^3 experiments and were compared to the CID spectra of their even electron counterparts, $[M-H]^-$. The Supplementary material section contains all of the CID spectra. Before describing the fragmentation reactions of the $[Fe^{III}(salen)(M-2H)]^-$ complexes (Section 3.1) and comparing the CID spectra of the $[M-2H]^{\bullet-}$ and $[M-H]^-$ of the flavonoids (Section 3.2), we briefly review the fragment ion nomenclature for flavonoids.

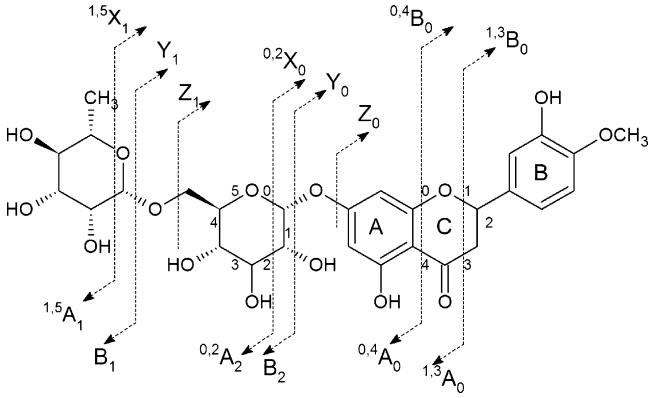
The nomenclature for labelling fragment ions of flavonoids has been derived from nomenclature for carbohydrates with further modification [50,61,62]. An example is illustrated in Scheme 2 on Hesperidin **15**. The most useful fragmentations in terms of flavonoid aglycone identification are those that require cleavage of one C–C and one C–O bond of the C-ring, resulting in ${}^{i,j}A_0^{+/-}$ and ${}^{i,j}B_0^{+/-}$ ions (Scheme 2), A and B indicating intact ring. The superscripts i and j indicate which of the C-ring bonds have been broken. For conjugated aglycones, an additional subscript 0 to the right of the letter is used to avoid confusion with A_n^+ and/or B_n^+ ($n \geq 1$) labels that have been used to designate carbohydrate fragments containing a terminal sugar unit (non-reducing end), which also use the superscripts i and j to indicate the bonds that have been broken. The X_m, Y_m and Z_m represent ions still containing the aglycones (or the reducing sugar unit). Finally, in a number of cases the radical anion and even electron anion fragment to give related X_m or Y_m fragments that only differ by one or two H atoms and these are differentiated in the nomenclature. For example, the $[M-2H]^{\bullet-}$ of hesperidin gives a $[Y_0-2H]^-$ at m/z 229 (Supplementary material spectrum 16B), while the $[M-H]^-$ gives a $[Y_0^-]^-$ at m/z 301 (Supplementary material spectrum 16B).

3.1. Fragmentation reactions of the $[Fe(salen)(M-2H)]^-$ complexes

Previous studies on the fragmentation reactions of ternary metal complexes of biomolecules have demonstrated that other reactions compete with the desired redox reaction for radical ion formation



Scheme 1. Flavonoids investigated in the present study **1**–**16**, and iron salen complex **17**.



Scheme 2. Ion nomenclature used for the fragmentation of di-substituted flavonoid glycosides, illustrated for Hesperidin **15**.

[19,20,41]. We have found this to be true for the CID spectra for the $[Fe^{III}(salen)(M-2H)]^-$ complexes of flavonoids, which fragment via four pathways:

$$[Fe^{III}(salen)(M-2H)]^- \rightarrow [M-2H]^{\bullet -} + [Fe^{II}(salen)] \quad (1)$$

$$[Fe^{III}(salen)(M-2H)]^- \rightarrow [M-H]^- + [Fe^{III}(salen-H)] \quad (2)$$

$$[Fe^{III}(salen)(M-2H)]^- \rightarrow [Fe^{III}(M-4H)]^- + (salen + 2H) \quad (3)$$

$$[Fe^{III}(salen)(M-2H)]^- \rightarrow [Fe^{III}(salen)(M-2H-X)]^- + X \quad (4)$$

where X is a fragment of the flavonoid.

The relative abundances of the product ions produced from each of the flavonoid containing complexes are summarized in Table 1 and Fig. 1 provides four representative spectra (all spectra are provided in Supplementary material). Formation of the radical anion (Eq. (1)) occurs for all of the complexes with the exception of the Luteolin-7,3′-di-O-glucoside (**14**), furthermore it is the pre-

Table 1
Summary of CID fragmentation reactions of the iron(III) salen flavonoid complexes, $[Fe(salen)M\text{-}2H]^-$.

Flavonoid name and number	Type	Molecular formula	Molecular weight	Electron transfer (Eq. (1))	Proton transfer (Eq. (2))	Flavonoid fragmentation (Eq. (3))	Salen loss (Eq. (4))	Spectra in Appendix
Myricetin, **1**	Flavanol	$C_{15}H_{10}O_8$	318	100%	–	–	–	Appendix 2A
Homoorientin, **2**	Flavone	$C_{21}H_{20}O_{11}$	448	100%	40%	–	–	Appendix 3A
Orientin, **3**	Flavone	$C_{21}H_{20}O_{11}$	448	100%	10%	–	–	Appendix 6A
Quercitrin, **4**	Flavanol	$C_{21}H_{20}O_{11}$	448	100%	25%	–	–	Appendix 5A
Luteolin-7-O-glucoside, **5**	Flavone	$C_{21}H_{20}O_{11}$	448	100%	25%	–	–	Appendix 4A
Quercetin-3-B-glucoside, **6**	Flavanol	$C_{21}H_{20}O_{12}$	464	100%	45%	–	–	Appendix 8A
Quercetin-3-galactoside, **7**	Flavanol	$C_{21}H_{20}O_{12}$	464	100%	50%	–	–	Appendix 7A
Spiraeoside, **8**	Flavanol	$C_{21}H_{20}O_{12}$	464	100%	40%	622, 15%	–	Appendix 9A
Syringetin-3-O-galactoside, **9**	Flavanol	$C_{23}H_{24}O_{13}$	508	100%	35%	–	–	Appendix 11A
Syringetin-3-O-glucoside, **10**	Flavanol	$C_{23}H_{24}O_{13}$	508	100%	45%	–	–	Appendix 10A
Vitexin-2-O-Rhamnoside, **11**	Flavone	$C_{27}H_{30}O_{14}$	578	100%	85%	–	–	Appendix 12A
Naringin, **12**	Flavanone	$C_{27}H_{32}O_{14}$	580	100%	55%	–	–	Appendix 13A
Peltatoside, **13**	Flavanol	$C_{26}H_{28}O_{16}$	596	100%	23%	–	648, 35%	Appendix 14A
Luteolin-7,3′-di-O-glucoside, **14**	Flavone	$C_{27}H_{30}O_{16}$	610	–	23%	768, 30%	662, 100%	Appendix 15A
Hesperidin, **15**	Flavanone	$C_{28}H_{34}O_{15}$	610	34%	35%	610, 30%	622, 47%	Appendix 16A
Rutin, **16**	Flavanol	$C_{27}H_{30}O_{16}$	610	100%	40%	–	662, 35%	Appendix 17A

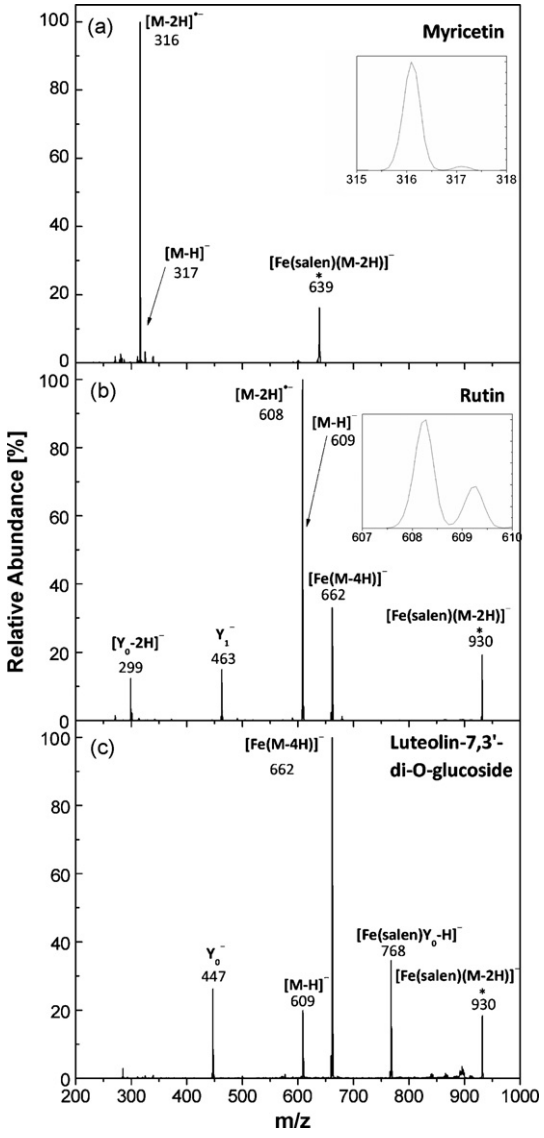


Fig. 1. CID mass spectra of the ternary metal complexes $[Fe^{III}(salen)(M-2H)]^-$ of the following flavonoids: (a) Myricetin **1**; (b) Rutin **16**; (c) Luteolin-7,3′-di-O-glucoside **14**.

dominant fragmentation pathway for all the other complexes with the exception of Hesperidin (**15**). The fact that $[M-2H]^{\bullet -}$ are readily formed for most of the flavonoids studied is likely due to the combination of two factors: (i) all flavonoids studied have a least two phenolic groups and these are the most likely sites for these proton losses to generate the $[Fe^{III}(salen)(M-2H)]^-$ precursor ions; (ii) the flavonoid radical anion products are stabilized by resonance. Formation of the even electron anion, $[M-H]^-$, via proton transfer (Eq. (2)), is the second most common pathway, occurring for all complexes except Myricetin (**1**), the only flavonoid not to have an appended glycoside examined here. Fragmentation of the bound flavonoid occurs for Spiraeoside (**8**), Luteolin-7,3′-di-O-glucoside (**14**) and Hesperidin (**15**) largely through cleavage of weak O-glycosidic bonds to provide product ions corresponding to $[Fe^{III}(salen)(Y_0-H)]^-$. Salen loss (Eq. (4)) only occurs for the more complex flavonoids containing a linkage to a disaccharide or in the case of Luteolin-7,3′-di-O-glucoside, two glycosidic linkages. In addition to the product ions produced by the four pathways indicated above, fragments of the flavonoid are also observed. For example, Fig. 1b shows the CID spectrum of $[Fe^{III}(salen)(Rutin-2H)]^-$, in this case the $[Y_0-2H]^-$ and Y_1^- ions are present. We suggest that these products probably form upon further fragmentation of the $[M-2H]^{\bullet -}$ ion as they are present in the MS^3 CID spectrum of this radical ion but not in the CID spectrum of the even electron anion (see Supplementary material). Elimination of neutral salen (Eq. (4)) requires the transfer of two additional protons from the already doubly deprotonated flavonoid. That this pathway does not occur for smaller flavonoids probably reflects a lack of proton donor sites, furthermore, one might envisage that a larger flavonoid may more effectively solvate the ferric ion providing a driving force for the elimination of the salen.

It is interesting to compare the likely coordination mode of the current complexes with those studied previously by Brodbelt, such as $[Co^{II}(bipy)(M-H)]^+$ [51,53–55]. Brodbelt has suggested that coordination of the flavonoid most likely occurs via the deprotonated hydroxyl of the A ring and the ketone of the C ring. In contrast to bipy, the salen ligand utilized here is tetradentate and preferentially coordinates the metal ion in a planar arrangement, with two vacant coordination sites *trans* to each other. As a consequence coordination of the flavonoid as a bidentate ligand concurrently with coordination of the salen as a tetradentate ligand may be difficult to accomplish. It may be the case that in an $[Fe^{III}(salen)(M-2H)]^-$ complex only one of the ligands can effectively coordinate as a poly-dentate ligand at a time. This would explain why the complexes containing the simpler flavonoids readily dissociate via pathways involving elimination of

Table 2
Comparison of the CID fragmentation reactions of the $[M-H]^-$ and $[M-2H]^{\bullet -}$ of flavonoids.

Flavonoid name and structure number	Number of sugars attached	Ion	*m/z*	B_0	$A_{0\text{ or }1\text{ or }2}$	$Y_{0\text{ or }1}$	$Z_{0\text{ or }1}$	$X_{0\text{ or }1}$	Spectra in Appendix
Myricetin, **1**	0	$[M-H]^-$	317	192, 8% 193, 6%	151,22% 179, 63%	N/A	N/A	N/A	Appendix 2B,C *Note*: Loss of H_2O and CH_2O from parent radical anion
		$[M-2H]^{\bullet -}$	316	163, 3% 164,21%	151, 15% 152, 3% 179, 92%	N/A	N/A	N/A	
Homoorientin, **2**	1	$[M-H]^-$	447	0%	0%	0%	N/A	327, 20% 357, 12%	Appendix 3B,C *Note*: Loss of H_2O from parent radical anion
		$[M-2H]^{\bullet -}$	446	0%	0%	284, 14% 285, 6%	N/A	297, 7% 298, 84% 313, 100% 326, 94% 356, 56% 385, 11%	
Orientin, **3**	1	$[M-H]^-$	447	0%	0%	0%	N/A	327, 72% 357, 32%	Appendix 4B,C *Note*: Loss of H_2O and CH_4 from parent anion.
		$[M-2H]^{\bullet -}$	446	0%	0%	0%	N/A	297, 48% 298, 30% 313, 100% 326, 43% 356, 13%	
Quercitrin, **4**	1	$[M-H]^-$	447	0%	0%	300, 3% 301, 18%	0%	0%	Appendix 5B,C
		$[M-2H]^{\bullet -}$	446	0%	0%	299, 100% 300, 70% 301, 8%	0%	0%	
Luteolin-7-O-glucoside, **5**	1	$[M-H]^-$ $[M-2H]^{\bullet -}$	447 446	0% 0%	0% 0%	285, 100% 283, 62% 284, 73% 285, 35%	0% 0%	327, 2% 297, 12% 298, 5% 313, 100% 327, 10% 328, 5%	Appendix 6B,C
Quercetin-3-B-glucoside, **6**	1	$[M-H]^-$	463	0%	0%	300, 17% 301, 100%	0%	343, 2%	Appendix 7B,C
		$[M-2H]^{\bullet -}$	462	0%	0%	299, 68% 300, 42%	0%	314, 15% 343, 2% 344, 4%	
Quercetin-3-galactoside, **7**	1	$[M-H]^-$	463	0%	0%	300, 26% 301, 100%	0%	343, 3%	Appendix 8B,C
		$[M-2H]^{\bullet -}$	462	0%	0%	299, 57% 300, 78% 301, 15%	0%	314, 100% 342, 2% 343, 13% 344, 11%	
Spiraeoside, **8**	1	$[M-H]^-$ $[M-2H]^{\bullet -}$	463 462	0% 0%	0% 0%	301, 100% 299, 23% 300, 100% 301, 15%	0% 0%	0% 313,5% 314,8% 342, 14% 343, 7% 371, 10% 372, 5%	Appendix 9B,C
Syringetin-3-O-galactoside, **9**	1	$[M-H]^-$	507	0%	0%	343, 5% 344, 92% 345, 100%	0%	387, 17%	Appendix 10B,C
		$[M-2H]^{\bullet -}$	506	0%	0%	343, 100%	0%	0%	
Syringetin-3-O-glucoside, **10**	1	$[M-H]^-$	507	0%	0%	343, 4% 344, 100% 345, 86%	0%	387,21%	Appendix 11B,C
		$[M-2H]^{\bullet -}$	506	0%	0%	343, 100%	0%	0%	
Vitexin-2-O-Rhamnoside, **11**	2	$[M-H]^-$ $[M-2H]^{\bullet -}$	577 576	0% 0%	0% 0%	413, 100% 429, 15% 431, 100% 412,26% 413, 70% 414,42%	0% 0%	293, 15% 293, 42% 339, 80% 475, 50%	Appendix 12B,C

Table 2 (*Continued*)

Flavonoid name and structure number	Number of sugars attached	Ion	*m/z*	B_0	$A_{0\text{ or }1\text{ or }2}$	$Y_{0\text{ or }1}$	$Z_{0\text{ or }1}$	$X_{0\text{ or }1}$	Spectra in Appendix
Naringin, **12**	2	$[M-H]^-$ $[M-2H]^{\bullet-}$	579 578	0% 0%	459, 100% 459, 14% 472, 10%	271, 35% 269, 100% 270, 35% 271, 64%	0% 415, 12%	313, 18% 0%	Appendix 13B,C *Note*: Loss of ring C_6H_5O from parent anion
Peltatoside, **13**	2	$[M-H]^-$ $[M-2H]^{\bullet-}$	595 594	0% 0%	0% 0%	300, 22% 301, 100% 299, 100% 300, 14% 462, 5% 463, 38%	0% 0%	343, 6% 314,8% 342, 8% 372, 7%	Appendix 14B,C
Luteolin-7,3′-di-O-glucoside, **14**	2	$[M-H]^-$ $[M-2H]^{\bullet-}$	609 –	0% –	0% –	447, 100% –	0% –	0% –	Appendix 15C
Hesperidin, **15**	2	$[M-H]^-$ $[M-2H]^{\bullet-}$	609 608	0% 0%	0% 0%	301, 100% 299, 82% 300,51% 301,48% 463, 40%	0% 284, 20%	0% 0%	Appendix 16B,C
Rutin, **16**	2	$[M-H]^-$ $[M-2H]^{\bullet-}$	609 608	0% 0%	0% 0%	300, 10% 301,41% 299, 36% 300, 4% 462, 4% 463, 43%	0% 0%	0% 0%	Appendix 17B,C

the flavonoids (Eqs. (1) and (2)) rather than fragmentation of the coordinated flavonoids (Eq. (4)). In contrast larger and thus more complex flavonoids are able to effectively compete with salen as metal ion chelators, thereby promoting salen loss (Eq. (3)) or fragmentation of the bound flavonoid (Eq. (4)). Finally, it is curious that Luteolin-7,3′-di-O-glucoside (**14**) is the only flavonoid that did not form a radical anion. A possible explanation is that this flavonoid is the only one with two monosaccharides on two different rings (the A and B rings), which may affect the bonding to the Fe^{III}(salen) moiety.

3.2. Comparing the CID spectra of radical anions, $[M-2H]^{\bullet-}$, and even electron anions, $[M-H]^-$, of the flavonoids

We have undertaken CID of both the $[M-H]^-$ and $[M-2H]^{\bullet-}$ ion of the flavonoids. The former were produced via direct ESI/MS of the flavonoids, while the latter were produced via oxidative dissociation (Eq. (1)) from the corresponding ESI generated $[Fe^{III}(salen)(M-2H)]^-$ complex. Table 2 summarizes the relative abundance of the major products observed in these CID experiments, and all spectra are provided in Supplementary material. Fig. 2 highlights the differences in the CID spectra of the $[M-H]^-$ and $[M-2H]^{\bullet-}$ ions of four isomeric flavonoids Quercitrin (**4**), Luteolin-7-O-glucoside (**5**), Orientin (**3**) and Homoorientin (**2**).

CID of the $[M-H]^-$ anion of the O-linked glycosides leads almost exclusively to cleavage of the glycosidic bond to give the corresponding Y_0^- ion. Syringetin-3-O-glucoside (**10**), Syringetin-3-O-glucoside (**9**), Luteolin-7-O-glucoside (**5**) and Peltatoside (**13**) also produce minor product ions which seem to correspond to the $[^{0,2}X-H]^-$ ion. In contrast to the other O-glycoside, Naringin (**12**) produces a considerably more complex spectrum which includes cleavage of the naringenin core. The C-linked glucosides Orientin (**3**) and Homoorientin (**2**) fragment via cross-ring cleavages of the glucose giving $[^{0,2}X-H]^-$ and $[^{1,4}X-H]^-$ ions. Additionally, Homoorientin provides a product ion at $m/z = 301$. Vitexin-2″-O-Rhamnoside primarily fragments via formation of the $[Y_1-H_2O]^-$ ion.

Dissociation of the ternary $[Fe^{III}(salen)(M-2H)]^-$ complex via Eq. (1) has allowed the further isolation and CID of the corresponding flavonoid radical anion, $[M-2H]^{\bullet-}$, for all the flavonoids with the exception of Luteolin-7,3′-di-O-glucoside (**14**). The relative abundances of the product ions from both the even electron $[M-H]^-$ and $[M-2H]^{\bullet-}$ are summarized in Table 2. The greater complexity of the radical anion spectra may provide a means of distinguishing between isomeric flavonoids which contain the same core and thus are not readily distinguishable by the Y_0 ion produced upon CID of their $[M-H]^-$. Quercetin-3-galactoside (**7**) and Quercetin-4′-glucoside (Spiraeoside, **8**) are isomeric with Quercetin-3-B-glucoside (**6**). Although their radical anion spectra are qualitatively similar, CID produces the same types of product ions, there are quantitative differences between the three. For example the $^{0,1}X_0$ ion is the major product ion for the galactoside whereas the $[Y_0-2H]^-$ is the major product for both the glucosides. Similarly the product ion at $m/z = 371$ in the Spiraeoside is significantly greater than in the other two spectra.

It is interesting to note that minor changes in the flavonoids core may substantially alter the fragmentation of the radical anion. For example, syringetin-3-O-glucoside (**10**) and Syringetin-3-O-galactoside (**9**) differ from the Quercetin-3-glycosides (**6** and **7**) only in the substitution on the B ring of the core, but the radical anion spectra provide only the $[Y_0-2H]^-$ ion in contrast to the relatively rich spectra from the Quercetin-3-glycoside radical anions.

The difference between the CID spectra of the $[M-H]^-$ and $[M-2H]^{\bullet-}$ ions is highlighted in Fig. 2, which shows the CID spectra of the four isomeric flavonoids Quercitrin (**4**), Luteolin-7-O-glucoside (**5**), Orientin (**3**) and Homoorientin (**2**). Orientin (**3**) and Homoorientin (**2**) are isomeric C-linked Luteolin glucosides with substitution at the 8 and 6 positions, respectively. The CID spectrum of their $[M-H]^-$ is similar, with both providing $[^{0,2}X-H]^-$ and $[^{1,4}X-H]^-$ ions. An additional ion at $m/z = 301$ is observed in the homoorientin spectrum, which provides a means of differentiating both isomers. Several product ions corresponding to cross-ring cleavages are observed in the radical anion spectrum and unique product ions at $m/z = 398$ and $m/z = 284$ for Orientin and Homoorientin, respectively provide a basis for distinction between the two. Quercitrin (**4**) and Luteolin-7-O-glucoside (**5**) are both isomeric with Orientin and Homoorientin but are O-linked glycosides that differ in their core structures. The radical anion spectrum of Quercitrin (**4**) provides almost exclusively Y_0 type ions. Luteolin-7-O-glucoside (**5**) provides several cross-ring cleavage ions. Many of

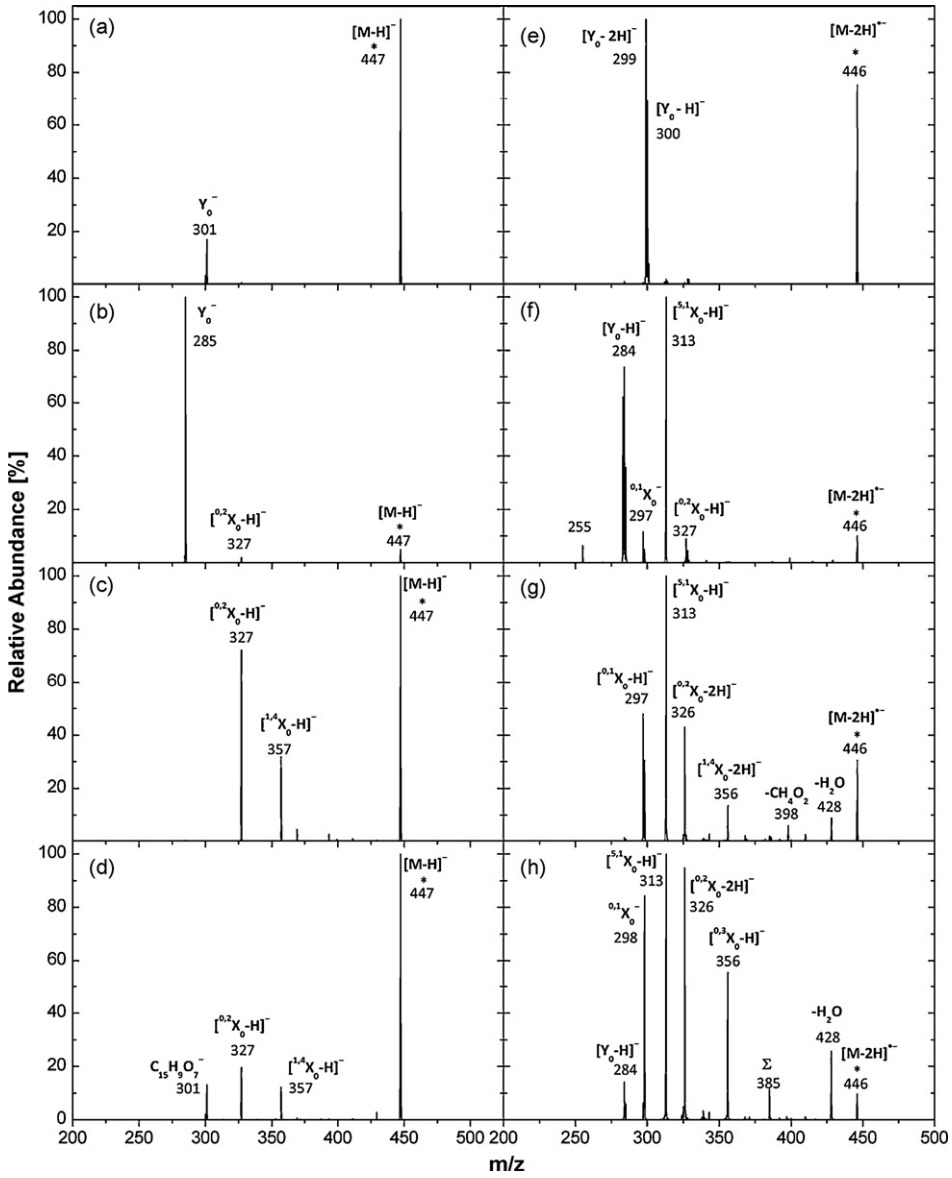


Fig. 2. CID mass spectra of: $[M-H]^-$ of: (a) Quercitrin **4**, (b) Luteolin-7-O-glucoside **5**, (c) Orientin **3**, (d) Homoorientin **2**; $[M-2H]^{\bullet -}$ of: (e) Quercitrin, (f) Luteolin-7-O-glucoside, (g) Orientin, (h) Homoorientin, Σ corresponds to $C_{19}H_{13}O_9^-$, which could be either $[^{0,4}X_0-3H]^-$ or $[^{2,4}X_0-3H]^-$.

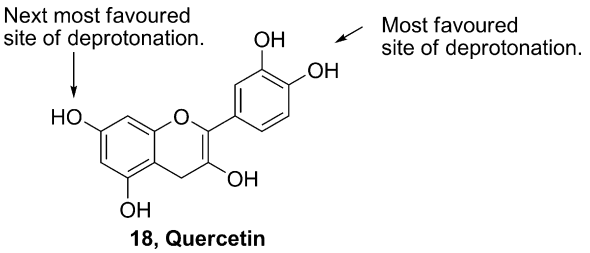
these types of ions are also observed in the Orientin and Homoorientin spectra although differences exist between the three spectra. In summary, all four isomers provide distinct features in the radical anion spectra shown in Fig 2.

Peltatoside (**13**), Hesperidin (**15**) and Rutin (**16**) contain O-linked disaccharides. The CID spectra of their $[M-H]^-$ ions yield only the Y_0 ions. The CID of their corresponding radical anions, $[M-2H]^{\bullet -}$, produces not only a Y_0 type ion corresponding to the loss of the disaccharide but also a Y_1 type ion indicating cleavage between sugars. Additional minor products are also present in all three spectra, which allows the isomeric Rutin and Hesperidin to be distinguished. Naringin (**12**) and Vitexin-2″-O-Rhamnoside (**11**) also produce significantly more complex spectra upon CID of their radical anion than their corresponding even electron counterpart.

3.3. Do flavonoid radical anions undergo radical directed cleavage mechanisms?

In general the CID spectra of the flavonoid $[M-2H]^{\bullet -}$ radical anions are more complex than their corresponding even electron $[M-H]^-$ spectra. Based upon the behaviour of radical sites in other classes of biomolecular radical ions, such as peptides, which have been shown to promote a range of cleavage reactions [12,44,45,63], it is tempting to speculate that the radical sites in the $[M-2H]^{\bullet -}$ radical anions open up new radical driven cleavage reactions. These appear to be important for cross-ring cleavage reactions involving the appended saccharides. Unfortunately we are unable to provide detailed mechanisms for these cross-ring cleavage reactions for several reasons:

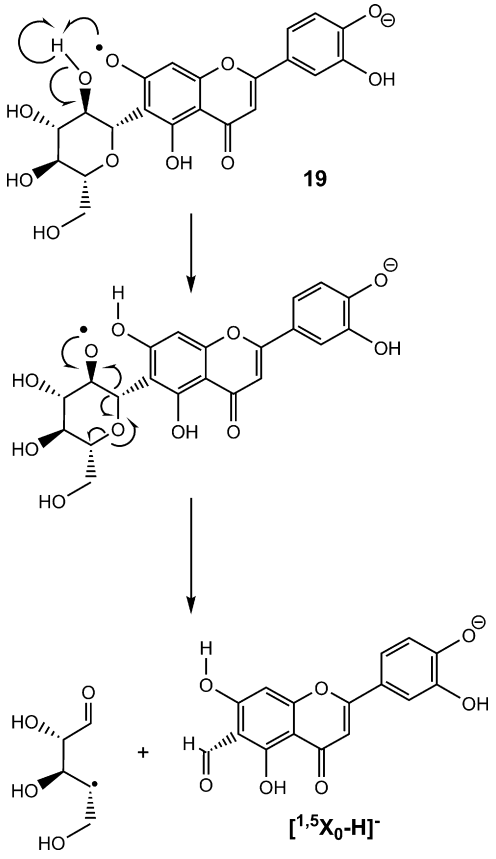
(i) The radical and anion site(s) in these systems are not known/controlled in our experiments. Previous studies on the CID spectra of $[Y_0-]^-$ flavone product ions [56] formed from isomeric flavonoids possessing the same flavone core highlight that anions with different charge sites can fragment differently (i.e., the "mobile proton" [64] does not operate).
(ii) Theoretical calculations to model the fragmentation behaviour of these radical anions are not possible due to the size of the flavonoids and the need to use fairly high levels of theory to accurately predict radical processes.



Scheme 3. Most acidic sites predicted for the model flavonoid quercetin, **18** [65].

(iii) Comparison of the flavonoid structures containing saccharide moieties, **2**–**13**, **15** and **16** and the various cross-ring cleavage product ions does not provide any obvious structure/reactivity correlations.

Despite these challenges, it is worth commenting on possible radical directed cleavage mechanisms for the formation of the unusual $[^{1,5}X_0-H]^-$ cross-ring cleavage product ion. An examination of the CID spectra reveals that this ion is only observed for the $[M-2H]^{\bullet -}$ of the C-glycosides **2** and **3** and the O-glycoside **5**, each of which have the saccharide appended to the A ring of the flavone core. The flavonoids **11**, **12** and **15** possessing disaccharides do not undergo this cross-ring cleavage reaction, suggesting that the presence of the ^{1}OH is important in the fragmentation reaction. Since the formation of the $[M-2H]^{\bullet -}$ require double deprotonation of the flavonoid, it is instructive to consider the most acidic sites in a model flavonoid, quercetin, **18** (Scheme 3). Zhang and Brodbelt have used *ab initio* calculations to show that most acidic site is the 4′ phenol OH followed by the 7 phenol OH [65]. Thus if we assume that both of these sites are deprotonated to bind to the Fe(salen), then a possible resonance structure of the $[M-2H]^{\bullet -}$ of **18** is **19**, which can trigger an intramolecular hydrogen atom transfer reaction to ultimately induce the cross-ring cleavage reaction shown in Scheme 4.



Scheme 4. Possible mechanism for the formation of the $[^{1,5}X_0-H]^-$ cross-ring cleavage product from the $[M-2H]^{\bullet -}$ of Homoorientin **2**.

4. Conclusions

It is clear that CID of appropriately designed ternary metal complexes can be used in the formation of different classes of radical ions of biomolecules. To date, the method has been successfully deployed in the formation of: (i) $[M]^{\bullet +}$ of peptides [40–42,44,45]; (ii) $[M+H]^{\bullet 2+}$ of peptides [66]; (iii) $[M-2H]^{\bullet -}$ of peptides [43]; (iv) $[M]^{\bullet +}$ of nucleobases [19,20]. We have now demonstrated that the method can be extended to the formation of $[M-2H]^{\bullet -}$ of flavonoids. Sixteen different flavonoids from three different groups: flavanone, flavone and flavanol, were investigated. All of them formed desired iron salen complex $[Fe^{III}(salen)(M-2H)]^-$ upon negative mode ESI of methanolic solutions of Fe^{III}(salen)Cl and the flavonoid. CID of the $[Fe^{III}(salen)(M-2H)]^-$ complex produces the flavonoid radical anion, $[M-2H]^{\bullet -}$, as the major product ion in all cases except for Hesperidin, where it is the second most abundant product, and Luteolin-7,3′-di-O-glucoside where the radical was not produced. In general the main competing dissociation pathway to radical formation is proton transfer to form the even electron $[M-H]^-$ ion. For complexes incorporating a flavonoid containing a disaccharide, dissociation via the elimination of salen or fragmentation of the coordinated flavonoid competes with radical formation. Comparison of the CID spectra of the $[M-2H]^{\bullet -}$ radical anions with their corresponding even electron counterparts, $[M-H]^-$, reveals that in general the radical anion provides a richer CID spectrum. For example, the even electron $[M-H]^-$ anions of the O-linked glycosides almost exclusively dissociate via cleavage of the weak glycosidic bond to afford the Y_0 ion. For many of the flavonoids, CID of their radical anion produces not only Y_0 type ions but also extensive cross-ring cleavages of the appended sugars, or where the flavonoid contained a disaccharide, cleavage between sugars to yield Y_1 type ions. Although we have not been able to provide detailed mechanistic insights into the radical anion cleavage reactions, in a number of cases we have demonstrated that CID of the radical anion may provide a means of distinguishing between isomeric flavonoids. Thus further work seems warranted to further delinate how radical anion structure promotes fragmentation in these and other biomolecules.

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Appendix A. Supplementary data

Supplementary materials include Ion Nomenclature used for the fragmentation of flavonoids (S1) and all CID mass spectra: CID of the iron salen complex (Spectra A), CID of radical anion $[M-2H]^{\bullet -}$ (Spectra B), and CID of even electron anion $[M-H]^-$ (Spectra C) for all flavonoids studied (**1**–**16**; S2–S17).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.08.017.

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