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Negative ion ESI–MS analysis of natural yellow dye flavonoids—An isotopic labelling study

Hamish McNab[a,*], Ester S.B. Ferreira[a,b,1], Alison N. Hulme[a,3], Anita Quye[b,2]

[a] School of Chemistry, The University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, UK
[b] National Museums Scotland, Chambers Street, Edinburgh EH1 1JF, UK

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ABSTRACT

Flavonoids are amongst the most commonly used natural yellow colourants in paintings, as lakes, and in historical textiles as mordant dyes. In this paper, evidence from isotopically labelled substrates is used to propose negative ion electrospray collision induced decomposition mechanisms of flavones, flavonols and an isoflavone. These mechanisms include a retro-Diels-Alder fragmentation (observed for flavones and flavonols) and an M-122 fragmentation (characteristic of 3′,4′-dihydroxyflavonols). In addition, the presence of a *m*/*z* 125 fragment ion is shown to be characteristic of 2′-hydroxyflavonols and an ion at *m*/*z* 149 is shown to be characteristic of 4′-hydroxyflavones. Applications of these methods are exemplified by the identification of a minor component of Dyer's camomile (*Anthemis tinctoria* L.) and the identification of the dye source in green threads sampled from an 18th Century Scottish tartan fragment.

1. Introduction

Flavonoid derivatives are the most common yellow natural colorants and were used until the late nineteenth century [1] in paintings, as lakes, and in textiles, as mordant dyes. Mixed with blue (*e.g.*, indigo), they were also used to produce green colours. Although flavonoids suffer more from degradation (*e.g.*, by photo-oxidation) than most other natural dyes, their presence can still be detected in well-preserved historical textiles such as tapestries. Several historical examples are quoted in ref. [1].

Many historical sources mention the preparation of yellow lakes from flavonoid rich plants. For example, the *jaune d'avignon* (Yellow of Avignon) was mentioned by Turquet De Mayerne in his manuscript of 1620 [2], as follows: "*Un beau jaune constant et qui ne meurt point* (. . .). *Je crois que avec le bleu il peut faire un vert très excellent.* (. . .) *Le jaune d'Avignon se fait avec le suc des baies vertes de Nepruns, en y meslant un peu d'Alum*" [A beautiful permanent yellow that does not fade. (. . .) I believe that with blue it can make an excellent green. (. . .) The yellow of Avignon is made with the juice of the berries mixed with a little alum.] The *baies vertes d'Avigon* or *de Nepruns* are the unripe berries of *Rhamnus* sp. The combined use of these berries with weld (*Reseda luteola* L.) and old fustic (*Chlorophora tinctoria* L.) for the preparation of "schiet-yellow" or Dutch pink lake is also mentioned in the "Pekstok" papers, a late seventeenth century treatise on the preparation of pigments and other artists' materials, possibly compiled and written by Willem Pekstok [3]. Other dye sources and recipes for lakes are also described in ref. [3] and the authors conclude that yellow lake pigments in the 17th Century "seem to have been in great demand", suggesting production on an industrial scale. Despite this wealth of evidence for their use, surprisingly few flavonoid-containing lakes have been unequivocally identified by the scientific analysis of paintings, due to the relative instability of lakes compared with other pigments and because of the low organic colourant quantity relative to total weight. However, flavonoids have been positively identified in a dark green lake derived from dyer's broom (*Genista tinctoria* L.) in Sir Nathaniel Bacon's (1858–1927) *Cookmaid with Still Life of Vegetables and Fruit* [4] and in a lake containing the flavone luteolin in Vermeer's *Girl with a Pearl Earring* [5]. An additional problem in identifying flavonoids is that comparatively large sample sizes are required for the methods usually employed in lake analysis. The direct injection MS method described here, combined with tandem MS, would allow lake colourant identification using much smaller samples.

The mass spectral characteristics of flavonoids – in particular negative ion electrospray ionisation (ESI–MS), coupled with liquid chromatography (LC) – have been extensively studied [6], and preliminary reports of our own work in this field have appeared

* Corresponding author. Tel.: +44 131 650 4718; fax: +44 131 650 4743.
E-mail addresses: H.McNab@ed.ac.uk (H. McNab), ester.ferreira@sikart.ch (E.S.B. Ferreira), Alison.Hulme@ed.ac.uk (A.N. Hulme), A.Quye@nms.ac.uk (A. Quye).
[1] Present address: Leiterin Labor und Technologieforschung, Zollikerstrasse 32, CH - 8032 Zürich, Switzerland. Tel.: +41 44 388 51 51; fax: +41 44 381 50 52.
[2] Tel.: +44 131 247 4376; fax: +44 131 247 4306.
[3] Tel.: +44 131 650 4711; fax: +44 131 650 4743.

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in specialist journals devoted to conservation science [7–12]. In this paper, we present full details of our use of isotopic labelling, supported by synthetic studies, to track flavonoid breakdown mechanisms in negative ion LC–MS under collision induced decomposition (CID) conditions. These results provide additional evidence for the mechanisms proposed by ourselves [7–13] and others [6,14] and have assisted in the structural assignment of isomeric flavonoids which may act as marker compounds in the identification of dye plants [11,13].

2. Experimental—materials and methods

2.1. Chemicals

[2-^{13}C]-labelled quercetin-*O*-glycoside **12** was kindly supplied by Dr. R. C. Hartley [15]. Flavonoids and other chemicals were purchased from chemical suppliers and were used as received.

2.2. Apparatus

Mass spectrometry and tandem mass spectrometry (MS–MS) analyses of the flavonoid solutions and dye extracts were performed on a Thermo-Finnigan LCQ^{TM} quadrupole ion trap mass spectrometer in negative electrospray ionisation mode. The parameters were optimised by tuning the detector to a reference flavonoid solution.

Dye extraction and PDA-HPLC analyses were performed as described previously [9].

2.3. Mass spectrometric conditions

Parameters for electrospray ionisation in negative mode were: spray voltage 4.50 kV; capillary temperature 175 °C; capillary voltage −17 V; sheath gas (N_2) flow rate 70–80 arb units; auxiliary gas (N_2) flow rate 5–10 arb units; tube lens offset −10 V; first octapole offset 2.5 V; second octapole offset 5.5 V; lens voltage 28 V; relative collision energy 20.1%; helium collision gas; sample injection flow rate 8 μL min^{-1}.

Data were collected and processed by LCQ^{TM} Navigator software.

2.4. Synthesis of 3,5,7,3′-tetrahydroxyflavone *23*

2.4.1. 2′,4′,6′-Trihydroxy-1-methoxyacetophenone *21* [16]

In a 250 mL 3-neck flask, zinc chloride (6.5 g) was dried by reflux in thionyl chloride for 6 h. The thionyl chloride was removed under vacuum. A solution of phloroglucinol (18.9 g) in dry ether (30 mL) was added followed by a solution of methoxyacetonitrile (10.7 g) in dry ether (30 mL). The solution was continuously stirred by an overhead stirrer. The solution was cooled in an ice-bath and dry HCl gas was passed through for approximately 5 h. A white precipitate was formed. The ether was removed under vacuum and the remaining solid was dissolved in warm deionised water (400 mL). The solution was heated under reflux for 90 min and the title compound **21** (24.0 g, 81%) crystallised on cooling; mp 197–198 °C; *m*/*z* 198 Da ($C_9H_{10}O_5$); ^{1}H NMR ([D_6]-acetone, 250 MHz): δ_H = 11.75 (2H, br), 9.51 (1H, br), 5.94 (2H, s), 4.66 (2H, s) and 3.60 (3H, s); ^{13}C NMR ([D_6]-acetone, 63 MHz); δ_C 200.8 (quat), 164.0 (quat), 163.5 (quat), 102.1 (quat), 94.0, 76.6 and 57.5. An X-ray crystal structure of this compound has been published [17].

2.4.2. 5,7-Dihydroxy-3,3′-dimethoxyflavone *22* [16]

m-Anisoyl chloride was prepared by heating *m*-anisic acid (3.2 g, 20 mmol) in an excess of thionyl chloride (4.5 mL, 58 mmol) at 60 °C for 40 min. The remaining thionyl chloride was removed at reduced pressure and the residual brown oil was dissolved in toluene (100 mL), along with tetrabutylammonium bromide (4.8 g, 15 mmol), 2′,4′,6′-trihydroxy-1-methoxyacetophenone (2.0 g, 10 mmol) and anhydrous potassium carbonate (8.3 g, 60 mmol). The solution was heated under reflux for 5.5 h. After overnight cooling the impure residue was filtered, the toluene filtrate was hydrolysed in K_2CO_3 (5% aqueous solution) for 4 h. CO_2 (gas) was passed through the solution until the pH dropped from 12 to 7, and a yellow precipitate was formed (0.70 g) which was the relatively pure flavone **22** used directly for the next stage.

2.4.3. 3,5,7,3′-Tetrahydroxyflavone *23* (c.f. ref. [18])

5,7-Dihydroxy-3,3′-dimethoxyflavone (0.28 g, 0.89 mmol) was dissolved in dry dichloromethane (CH_2Cl_2, 45 mL). The solution was cooled to −78 °C and boron tribromide (0.400 mL) was added slowly under nitrogen. The reaction was kept at −78 °C for 15 min and allowed to warm to room temperature overnight. Water (30 mL) was added and the products were extracted with ether and ethyl acetate. The combined organic layers were washed, dried ($MgSO_4$) and the solvent removed by rotary evaporation. Dry flash chromatography gave 3,5,7,3′-tetrahydroxyflavone **23** (44%); mp 257–259 °C; *m*/*z* 286 Da ($C_{15}H_{10}O_6$); ^{1}H NMR ([D_6]-acetone, 250 MHz): δ_H = 7.75 (2H, m), 7.39 (1H, dd, *J* 7.9 and *J* 8.0 Hz), 7.01 (1H, dd, *J* 1.0 and *J* 2.5 Hz), 6.55 (1H, d, *J* 2.0 Hz) and 6.29 (1H, d, *J* 2.0 Hz); ^{13}C NMR ([D_6]-acetone, 63 MHz): δ_C = 175.2 (quat), 163.5 (quat), 160.6 (quat), 156.6 (quat), 156.2 (quat), 144.4 (quat), 136.2 (quat), 131.5 (quat), 128.7, 118.1, 116.3, 113.6, 102.6 (quat), 97.5 and 92.8.

3. Results

3.1. Scope and methodology

Compounds **1–11** (Fig. 1) used in this study were chosen to reflect the major structural types of flavonoids encountered in historical colorants; they include the flavones chrysin **1**, apigenin **2** (C.I. 75580; the major colorant of a species of wild camomile, *Anthemis chia* [1]) and luteolin **3** (C.I. 75580; the major constituent of many dye plants [1] including weld, *R. luteola* L. [9] and sawwort, *Serratula tinctoria* L. [13]). The following flavonols were studied; galangin **4** (C.I. 75630; present in the leaves and stems of bastard hemp, *Datisca cannabina* L. [1]), kaempferol **5** (C.I. 75640; present in many dye plants including Persian berries, *Rhamnus* spp. [1,10]), datiscetin **6** (C.I. 75630; present along with **4** in the leaves and stems of bastard hemp, *D. cannabina* L. [1]), morin **7** (C.I. 75660; present in the wood of old fustic, *C. tinctoria* (L.) Gaud [1]), quercetin **8** (C.I. 75670; present in many dye plants including Persian berries, *Rhamnus* spp. and onion skins, *Allium cepa* L. [1,10]), fisetin **9** (C.I. 75620; present in the wood of young fustic, *Cotinus coggygria* Scop. [1]), myricetin **10** (C.I. 75620; present in the branches and leaves of young fustic *C. coggygria* Scop. [1]), isorhamnetin **11** (an *O*-methylflavonol, C.I. 75680; present in Persian berries, *Rhamnus* spp. [1,10]) and the ^{13}C-labelled quercetin glucoside **12** [15]. The isoflavone genistein **13** (C.I. 75610; present, for example, in dyer's greenweed, *G. tinctoria* L. [1]) has also been studied.

All measurements were made using a quadrupole ion trap in negative ion electrospray mode with ion breakdown under CID conditions as we have previously described [7–13]. As an important means of establishing the structures of the fragment ions, spectra were recorded for samples dissolved in MeOD solution, as well as in MeOH. Under the former conditions, all the hydrogen atoms in the phenolic groups (except the one which had ionised under negative ion conditions) exchanged with deuterium. Comparison of the MS^n spectra in the two isotopomeric solvents

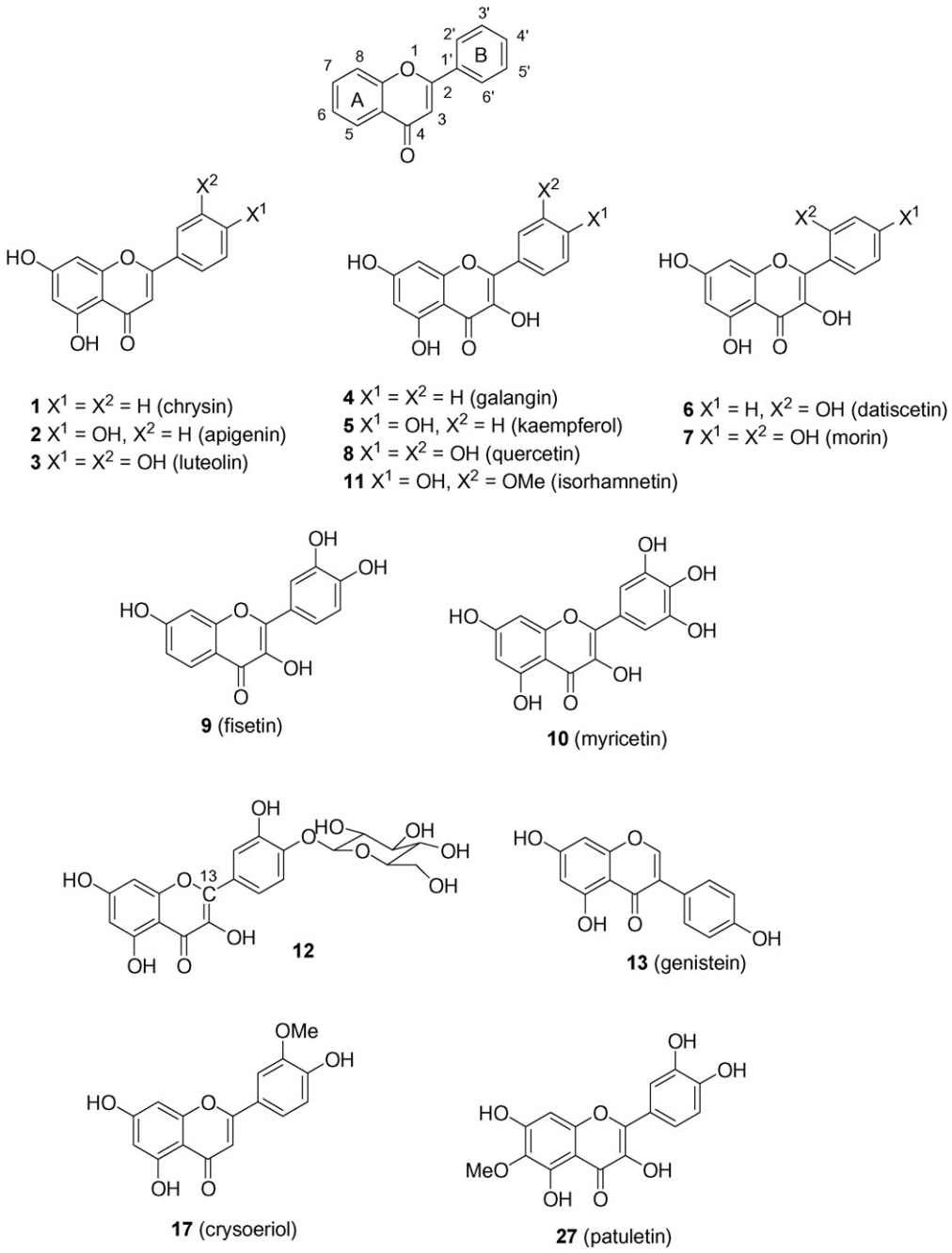

Fig. 1. The flavonoids studied.

therefore provides evidence of the number of hydrogen atoms derived from phenolic groups in any fragment and this information assists with the assignment of fragment structures and breakdown mechanisms.

The results are summarised in Table 1 and many of the MeOH results broadly mirror those obtained by other authors under similar conditions (*e.g.*, [14]). However, the ESI–MS behaviour of datiscetin **6**, morin **7** and other 2′-hydroxyflavonols is less well documented and so these results will be particularly emphasised in this paper.

In all our spectra, most of the negatively charged fragment ions originate from the A ring. This suggests that the 7-OH is the most acidic site, in agreement with the work of Georgievskii [19] and with more recent studies by Zhang and Brodbelt [20].

3.2. Flavonoid breakdown by loss of small molecules

As found by Fabre et al. [14] MS^2 spectra of flavonoids often show loss of small neutral fragments [*e.g.*, 28 Da (CO), 18 Da (H_2O), 44 Da (CO_2) and/or combinations of these] and this behaviour proved to be common to all the flavonoids analysed (Table 2). These breakdown peaks contribute to the mass spectral fingerprint (Table 1), but generally provide little structural information. Partial assignments have nevertheless been provided by Fabre et al. [14] and the loss of 2CO is particularly important in the positive ion ESI spectra of flavonoids [21]. Flavonoids which contain methylated phenol groups often show loss of a methyl group $(M-H-CH_3)^{\bullet-}$ as the initial breakdown (Table 1); for example the base peak in the MS^2 spectrum of the $(M-H)^-$ ion of isorhamnetin **11** resulted from

Table 1
MS^2 results for selected flavones and flavonols in MeOH and MeOD, shown in order of fragment ion abundance

Compound	$(M-H)^-/(M-D)^-$	MS^2
Chrysin **1**	253	209 (−44, 100%), 185 (−68, 10%), 181 (−72, 9%), 211 (−42, 8%), 210 (−43, 5%), 225 (−28, 5%), 165 (−88, 5%), 151 (−102, 4%)
	254	210 (−44, 100%), 186 (−68, 12%), 182 (−72, 10%), 211 (−43, 3%), 212 (−42, 2%), 226 (−28, 7%), 166 (−88, 8%), 152 (−102, 3%)
Apigenin **2**	269	225 (−44, 100%), 149 (−120, 27%), 201 (−68, 25%), 227 (−42, 17%), 151 (−118, 13%), 183 (−76, 9%), 241 (−28, 5%), 181 (−78, 5%), 197 (−72, 5%), 223 (−46, 4%), 117 (−152, 2%), 107 (−162, 1%)
	271	227 (−44, 100%), 151 (−120, 8%), 203 (−68, 18%), 228 (−43, 4%), 229 (−42, 2%), 152 (−119, 8%), 150 (−121, 5%), 185 (−76, 3%), 183 (−78, 4%), 199 (−72, 2%), 108 (−163, 2%), 252 (−19, 2%)
Luteolin **3**	285	241 (−44, 100%), 243 (−42, 60%), 217 (−68, 60%), 199 (−86, 52%), 175 (−110, 47%), 151 (−134, 15%), 257 (−28, 22%), 267 (−18, 11%), 213 (−72, 17%), 223 (−62, 8%), 201 (−84, 13%), 197 (−88, 9%)
	288	244 (−44, 100%), 245 (−43, 27%), 246 (−42, 18%), 220 (−68, 55%), 201 (−87, 25%), 202 (−86, 20%), 200 (−88, 10%), 203 (−85, 4%), 177 (−111, 21%), 178 (−110, 10%), 176 (−112, 5%), 152 (−136, 12%), 260 (−28, 22%), 269 (−19, 7%), 270 (−18, 3%), 225 (−63, 4%)
Galangin **4**	269	197 (−72, 100%), 213 (−56, 86%), 227 (−42, 81%), 241 (−28, 53%), 223 (−46, 29%), 199 (−70, 18%), 225 (−44, 30%), 169 (−100, 14%), 211 (−58, 11%), 198 (−71, 10%), 171 (−98, 9%), 240 (−29, 7%), 195 (−74, 7%), 173 (−96, 3%)
	271	199 (−72, 100%), 215 (−56, 97%), 198 (−73, 12%), 200 (−71, 10%), 228 (−43, 45%), 227 (−44, 40%), 243 (−28, 53%), 242 (−29, 10%) 224 (−47, 39%), 223 (−48, 15%), 171 (−100, 21%), 172 (−99, 6%), 240 (−31, 6%), 175 (−96, 3%), 174 (−97, 2%), 173 (−98, 4%)
Kaempferol **5**	285	257 (−28, 100%), 213 (−72, 63%), 151 (−134, 63%), 241 (−44, 49%), 239 (−46, 60%), 169 (−116, 48%), 229 (−56, 60%), 243 (−42, 45%), 185 (−100, 39%), 267 (−18, 30%), 173 (−112, 27%), 187 (−98, 20%), 256 (−45, 30%), 199 (−86, 16%), 211 (−74, 20%), 215 (−70, 26%), 255 (−30, 15%), 163 (−122, 15%)
	288	260 (−28, 100%), 216 (−72, 59%), 214 (−74, 12%), 152 (−136, 50%), 241 (−47, 47%), 244 (−44, 32%), 240 (−48, 20%), 245 (−43, 13%), 232 (−56, 65%), 172 (−116, 25%), 188 (−100, 35%), 269 (−19, 12%), 170 (−118, 8%), 173 (−115, 6%), 189 (−99, 9%), 200 (−88, 6%), 164 (−124, 6%), 258 (−30, 4%)
Datiscetin **6**	285	213 (−72, 100%), 257 (−28, 32%), 267 (−18, 30%), 151 (−134, 27%), 241 (−44, 23%), 171 (−114, 19%), 125 (−160, 18%), 143 (−142, 13%), 199 (−86, 9%), 163 (−122, 9%), 229 (−56, 5%), 169 (−116, 5%), 197 (−88, 2%), 185 (−100, 3%), 133 (−152, 3%), 107 (−178, 2%)
	288	216 (−72, 100%), 260 (−28, 42%), 244 (−44, 30%), 268 (−20, 25%), 269 (−19, 3%), 152 (−136, 23%), 153 (−135, 3%), 128 (−160, 16%), 173 (−115, 10%), 172 (−116, 6%), 174 (−114, 1%), 145 (−143, 5%), 201 (−87, 5%), 200 (−88, 4%), 202 (−86, 1%), 232 (−56, 6%), 164 (−124, 2%), 165 (−123, 2%)
Morin **7**	301	257 (−44, 100%), 229 (−72, 98%), 151 (−150, 71%), 273 (−28, 50%), 283 (−18, 45%), 125 (−176, 33%), 239 (−62, 18%), 163 (−138, 18%), 175 (−126, 16%), 187 (−114, 11%), 215 (−86, 10%), 169 (−132, 9%), 213 (−88, 9%), 147 (−154, 6%), 159 (−142, 5%), 179 (−122, 4%)
	305	261 (−44, 92%), 233 (−72, 100%), 152 (−153, 48%), 277(−28, 62%), 285 (−20, 29%), 286 (−19, 4%), 128 (−176, 20%), 242 (−63, 8%), 241 (−64, 12%), 164 (−141, 10%), 175 (−130, 10%), 217 (−88, 9%), 170 (−135, 4%), 147 (−154, 6%), 162 (−143, 1%), 180 (−125, 1%)
Quercetin **8**	301	179 (−122, 100%), 151 (−150, 41%), 273 (−28, 14%), 257 (−44, 13%), 229 (−72, 6%), 239 (−62, 4%), 193 (−108, 4%), 169 (−132, 1%), 211 (−90, 2%), 283 (−18, 1%), 213 (−88, 1%)
	305	180 (−125, 100%), 152 (−153, 47%), 277 (−28, 24%), 261 (−44, 17%), 233 (−72, 6%), 241 (−64, 3%), 242 (−63, 3%), 195 (−110, 4%), 170 (−135, 3%)
Fisetin **9**	285	163 (−122, 100%), 135 (−150, 41%), 257 (−28, 22%), 153 (−132, 17%), 241 (−44, 19%), 229 (−56, 9%), 213 (−72, 4%), 223 (−62, 3%), 149 (−136, 2%), 267 (−18, 1%)
	288	163 (−125, 100%), 135 (−153, 18%), 260 (−28, 20%), 270 (−18, 16%), 269 (−19, 6%), 244 (−44, 2%), 232 (−56, 6%), 216 (−72, 1%), 153 (−135, 8%)
Myricetin **10**	317	179 (−138, 100%), 151 (−166, 21%), 192 (−125, 11%), 193 (−126, 6%), 191 (−126, 3%), 137 (−180, 4%), 299 (−18, 4%), 289 (−28, 3%), 255 (−62, 4%), 245 (−72, 2%), 227 (−90, 3%), 169 (−148, 2%)
	322	180 (−142, 100%), 179 (143, 30%), 152 (−170, 21%), 151 (−171, 4%), 193 (−124, 9%), 194 (−123, 6%), 195 (−122, 3%), 140 (−182, 3%), 302 (−20, 2%), 303 (−19, 1%), 294 (−28, 3%), 258 (−64, 4%), 259 (−63, 2%), 250 (−72, 1%), 231 (−91, 1%), 230 (−92, 1%), 170 (−152, 1%)
Isorhamnetin **11**	315	300 (−15, 100%), 287 (−28, 0.1%), 271 (−44, 0.1%), 151 (−164, 0.1%) [MS^3 (300): 271 (−29, 100%), 272 (−28, 51%), 151 (−149, 33%), 255 (−45, 34%), 256 (−44, 8%), 254 (−46, 6%), 227 (−73, 29%), 228 (−72, 5%), 283 (−17, 25%), 188 (−112, 11%), 243 (−57, 8%), 244 (−56, 5%), 215 (−85, 7%), 216 (−84, 7%), 164 (−136, 3%)]
23	285	239 (−46, 100%), 257 (−28, 90%), 229 (−56, 66%), 213 (−72, 50%), 211 (−74, 11%), 241 (−44, 10%), 185 (−100, 10%), 243 (−42, 8%), 199 (−86, 9%), 256 (−29, 6%), 195 (−90, 4%), 169 (−116, 2%), 267 (−18, 2%), 151 (−134, 1%)
Genistein **13**	269	225 (−44, 100%), 224 (−45, 35%), 201 (−68, 75%), 241 (−28, 42%), 240 (−29, 15%), 197 (−72, 32%), 196 (−73, 8%), 181 (−88, 22%), 199 (−60, 18%), 213 (−56, 13%), 212 (−57, 3%), 133 (−166, 11%), 135 (−164, 2%), 159 (−110, 8%), 169 (−100, 8%), 157 (−112, 7%), 155 (−114, 2%), 180 (−89, 6%), 167 (−102, 2%), 173 (−96, 3%), 251 (−18, 5%), 176 (−83, 2%), 208 (−61, 3%)
12	464	302 (−162, 100%) [MS^3 (302): 179 (−123, 100%), 151 (−151, 37%), 274 (−28, 13%), 258 (−44, 8%), 230 (−72, 2%), 240 (−62, 3%), 194 (−108, 2%), 169 (−133, 1%)]

Table 2
Commonly observed loss of small fragments in negative ion ESI spectra of flavonoids

m/z	Fragment	Structure
18	H_2O	2, 3, 5, 6, 7, 8, 9, 10, 11
28	CO	All structures analysed
42	CH_2CO	1, 2, 3, 4, 5
44	CO_2	1, 2, 3, 4, 5, 6, 7, 8, 9, 11
46 (18 + 28)	$CO + H_2O$	2, 4, 5
56 (28 + 28)	CO + CO	4, 5, 6, 11
62 (44 + 18)	$CO_2 + H_2O$	3, 7, 8, 10
68	C_3O_2 (or C_4H_4O)	1, 2, 3, 4
72 (44 + 28)	CO_2 + CO	1, 2, 4, 5, 6, 7, 8, 10, 11
86	$C_4H_6O_2$ (or $C_3H_2O_3$)	3, 5, 6, 7
88	$CO_2 + CO_2$ (or $C_3H_4O_3$)	1, 3, 6, 7

this mechanism [*m/z* 300 (100%)]. Other, more structurally characteristic breakdown mechanisms of the resulting anion radical $(M-H-CH_3)^{\bullet -}$ can take place in MS^3 spectra [9,22] (see below).

Our deuterium exchange experiments have revealed a highly specific water loss mechanism which takes place only in 2′-hydroxyflavonols (datiscetin **6** and morin **7**) which has not previously been investigated. These data show that water loss in most flavonoids takes place as HOD [*e.g.*, kaempferol **5** (MeOH), $(M-H)^-$ *m/z* 285; $(M-H-H_2O)^-$ *m/z* 267: kaempferol **5** (MeOD), $(M-D)^-$ *m/z* 288; $(M-D-HOD)^-$ *m/z* 269] corresponding to loss of OD and one H-atom, the latter deriving from a CH. However, in the 2′-hydroxyflavonols datiscetin **6** and morin **7**, specific loss of D_2O takes place—*i.e.*, both hydrogen atoms lost are originally bound to O [*e.g.*, datiscetin **6** (MeOH), $(M-H)^-$ *m/z* 285; $(M-H-H_2O)^-$ *m/z* 267: datiscetin **6** (MeOD), $(M-D)^-$ *m/z* 288; $(M-D-D_2O)^-$ *m/z* 268, with 269 *ca.* 10% of the intensity of 268. Morin **7** (MeOH), $(M-H)^-$ *m/z* 301; $(M-H-H_2O)^-$ *m/z* 283: morin **7** (MeOD), $(M-D)^-$ *m/z* 305; $(M-D-D_2O)^-$ *m/z* 285, with 286 <20% of the intensity of 285]. The proposed cyclisation mechanism shown in Fig. 2, only possible for 2′-hydroxyflavonols, accounts for these observations.

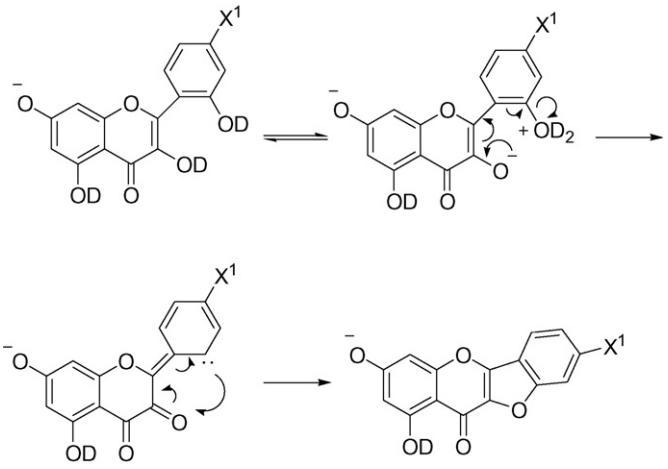

Fig. 2. Specific $(M-H_2O)^-$ breakdown mechanism for 2′-hydroxyflavonols.

3.3. Flavonoid breakdown by the retro-Diels-Alder (RDA) mechanism

The well-known retro-Diels-Alder (RDA) fragmentation mechanism (exemplified by the scheme shown in Fig. 3) (*e.g.*, refs. [6,8,14,22], *etc.*) was found to be common to almost all the flavones and flavonols analysed (including datiscetin **6** and morin **7**), but with variable intensity. This pathway was not observed for the isoflavone genistein **13**. The RDA pathway can give information relevant to the number of hydroxy groups present in the A ring of flavones and flavonols. For example the major fragment ions produced by the RDA mechanism for kaempferol **5** (two OH groups in the A ring) and its isomer fisetin **9** (one OH group in the A ring) are *m/z* = 151 (65%) and *m/z* = 135 (41%), respectively.

Importantly, the RDA mechanism is supported by the MS^n results of the MeOD experiments (Table 3). These show that only the ionisation site and OD units of the A ring remain after the fragmentation.

MS^3 of the fragment ion **14** (*m/z* = 151) (Fig. 3) of quercetin **8** shows a further loss of CO_2 (−44, *m/z* = 107, 100%) suggesting an electrocyclisation to the lactone **16** prior to fragmentation as proposed by Fabre et al. [14]. In addition, small peaks at *m/z* 107 are observed in the MS^2 spectra of most of the flavonoids which show the RDA mechanism.

The RDA fragmentation path can provide information concerning the nature of the functional groups present in the A ring (*e.g.*, if any of the phenolic groups have been methylated (*c.f.* refs. [9,12]). In the case of isorhamnetin **11** the fragment resulting from RDA fragmentation mechanism (*m/z* = 151) was detected but at very low relative abundance (0.1%) in the MS^2 spectrum. However, the MS^3 spectrum of the ion *m/z* = 300 showed a significant peak at *m/z* 151 (31%) from the RDA breakdown pathway with the incorporation of the radical centre in the neutral fragment. Similarly, we have used MS^3 analysis of a minor, luteolin-like constituent of weld (*R. luteola* L.), to show that its methoxy-substituent is located in the B-ring [9]; this compound was subsequently identified as crysoeriol **17** [12] (Fig. 1).

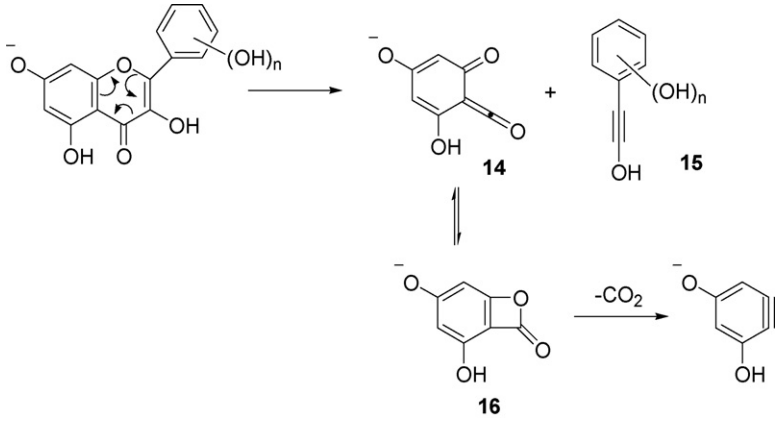

Fig. 3. The RDA fragmentation mechanism.

Table 3
MS^2 results for RDA mechanism for selected flavones and flavonols in MeOH and MeOD

Flavonoid	Parent ion (*m*/*z*)		Daughter ion 14 *m*/*z* (relative abundance)		Neutral fragment lost 15 (*m*)	
	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]
Chrysin **1**	253 $[M-H]^-$	254 $[M-D]^-$	151 (4%)	152 (3%)	102	102
Apigenin **2**	269 $[M-H]^-$	271 $[M-D]^-$	151 (13%)	152 (8%)	118	119
Luteolin **3**	285 $[M-H]^-$	288 $[M-D]^-$	151 (15%)	152 (12%)	134	136
Kaempferol **5**	285 $[M-H]^-$	288 $[M-D]^-$	151 (63%)	152 (50%)	134	136
Datiscetin **6**	285 $[M-H]^-$	288 $[M-D]^-$	151 (27%)	152 (23%)	134	136
Morin **7**	301 $[M-H]^-$	305 $[M-D]^-$	151 (71%)	152 (48%)	150	153
Quercetin **8**	301 $[M-H]^-$	305 $[M-D]^-$	151 (41%)	152 (47%)	150	153
Fisetin **9**	285 $[M-H]^-$	288 $[M-D]^-$	135 (41%)	135 (18%)	150	153
Myricetin **10**	317 $[M-H]^-$	322 $[M-D]^-$	151 (21%)	152 (21%)	166	170
Isorhamnetin **11**	315 $[M-H]^-$		151 (0.1%)		164	

[a] Solvent.

Table 4
MS^2 results for 3′,4′-dihydroxyflavonols in MeOH and MeOD

Flavonoid	Parent ion (*m*/*z*)		Daughter ion 18 *m*/*z* (relative abundance)		Neutral fragment 19/20 (*m*)	
	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]
Quercetin **8**	301 $[M-H]^-$	305 $[M-D]^-$	179 (100%)	180 (100%)	122	125
Fisetin **9**	285 $[M-H]^-$	288 $[M-D]^-$	163 (100%)	163 (100%)	122	125
Myricetin **10**	317 $[M-H]^-$	322 $[M-D]^-$	179 (100%)	180 (100%)	138	142

[a] Solvent.

In the case of the [2-^{13}C]-labelled quercetin-*O*-glucoside **12**, similar behaviour was observed with the base peak in the MS^2 study resulting from the cleavage of the *O*-glucoside bond [23]. In the MS^3 spectrum a peak at *m*/*z* = 151 (37%) was detected, showing, as predicted for the RDA mechanism, that an isotopic label at C(2) of the ring system (Fig. 1) is incorporated in the neutral fragment.

3.4. The M-122 fragmentation of 3′,4′-dihydroxyflavonols

The unusual loss of a fragment of 122 Da in the negative ion ESI spectra of 3′,4′-dihydroxyflavonols provides the base peak of the MS^2 spectra of quercetin **8** (*m*/*z* 179), fisetin **9** (*m*/*z* 163) and (the 3′,4′,5′-trihydroxyflavonol) myricetin **10** (M-138; *m*/*z* 179). This behaviour has been highlighted by us [7] and subsequently noted by Fabre et al. [14]. The source of this fragment [being essentially the B-ring + the carbon atom C(2) + H, corresponding to $C_7H_6O_2$] is supported by our labelling experiments (Table 4) [7]. In particular, C(2) was proved to be in the neutral fragment by the MS^3 spectrum of the ^{13}C-labelled compound **12** which shows a charged fragment of the same mass as the unlabelled species (Table 1). In addition, the results of the MeOD experiments on compounds **8–10** show (Table 4) that 3 deuterium atoms have been incorporated into the neutral fragment (4 in the case of myricetin **10**, which has three hydroxy-groups in the B-ring), *i.e.*, there must be a transfer of one D-atom from ring A or from the heterocyclic ring, into the neutral fragment. The structure of the charged fragment is best shown as a benzofuran-2,3-dione **18** (Fig. 4). This structure is supported by further breakdown of **18**, which shows sequential loss of CO and CO_2 as described by Fabre et al. [14] [from **8**, MS^3 (*m*/*z* 179) 151 (100%); MS^3 (*m*/*z* 151) 107 (100%)].

It is clear from the spectrum of kaempferol **5** that this breakdown route does not take place with 4′-monohydroxyflavonols; in our original mechanism [7] the cleavage of the $C_7H_6O_2$ fragment was postulated to take place by a carbene mechanism which requires only the 3′-hydroxy group to activate hydrogen transfer from the 3-OH position. It was therefore important to establish if the presence of a 3′-hydroxy group was sufficient for this mechanism to take place. Therefore, 3,5,7,3′-tetrahydroxyflavone **23** was synthesised by the method shown in Fig. 5. First, 5,7-dihydroxy-3,3′-dimethoxyflavone **22** was made in two steps as described by Deng et al. [16]. The known acetophenone **21** [16] was prepared from phloroglucinol and methoxyacetonitrile by a Houben–Hoesch reaction. In the second step several reactions occur: perbenzoylation of the acetophenone **21** is followed by cyclisation and

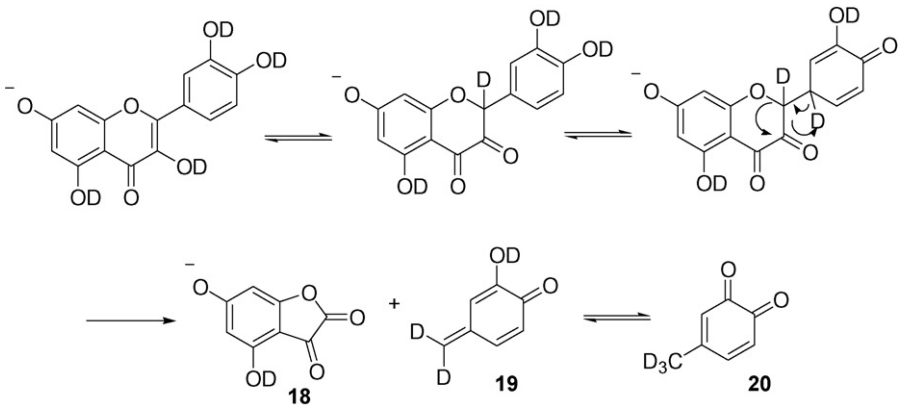

Fig. 4. The $(M-122)^-$ mechanism for 3′,4′-dihydroxyflavonols.

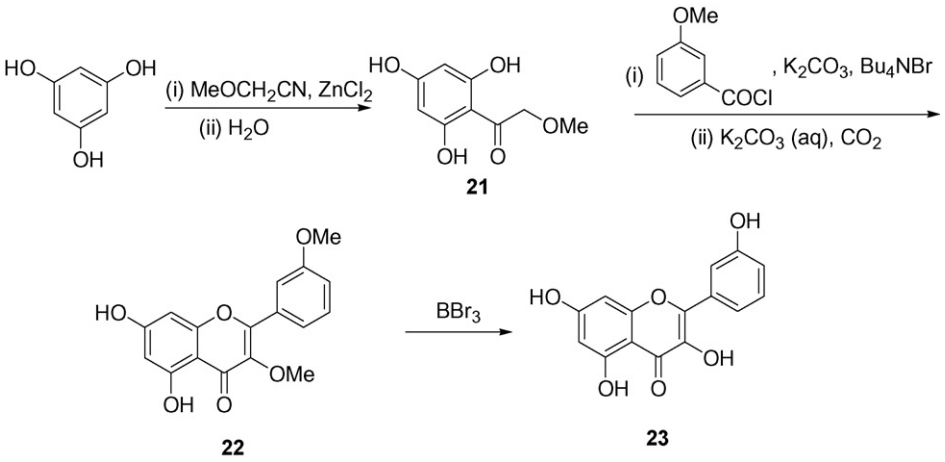

Fig. 5. Synthesis of **23**.

Table 5
MS^2 results for morin **7** and datiscetin **6** in MeOH and MeOD

Flavonoid	Parent ion (*m/z*)		Daughter ion 24 *m/z* (relative abundance)		Combined neutral fragment (*m*)	
	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]
Morin **7**	301 $[M-H]^-$	305 $[M-D]^-$	125 (33%)	128 (20%)	176	176
Datiscetin **6**	285 $[M-H]^-$	288 $[M-D]^-$	125 (18%)	128 (16%)	160	160

[a] Solvent.

hydrolysis to the flavone **22**. Deprotection of the 3- and 3′-hydroxy groups to provide **23** was performed by the method of Grieco et al. [18] (see Section 2).

The MS^2 analysis of the $(M-H)^-$ peak (*m/z* = 285) of 3,5,7,3′-tetrahydroxyflavone **23** showed no evidence of a peak at *m/z* 179 resulting from the loss of 106 Da due to the [B-ring + the carbon atom C(2) + H] fragment. The main breakdown peaks are *m/z* = 239 (−46, 100%), *m/z* = 257 (−28, 92%), *m/z* = 229 (−56, 69%) and *m/z* = 213 (−72, 50%). It can therefore be concluded that both the 4′-OH and the 3′-OH groups are important in the M-122 breakdown mechanism. This may be because the $C_7H_6O_2$ fragment is relatively stabilised, by being able to adopt a quinonoid [14] or quinone structure **19** or **20**, respectively (Fig. 4). In either case a sequence of hydrogen shifts to the original C(2) atom is required to generate the neutral fragment. One possible mechanism is shown in Fig. 4. We believe that the intense MS^2 peak at M-122 is therefore uniquely diagnostic for 3′,4′-dihydroxyflavonols.

Unlike the RDA mechanism, this pathway was not observed in the MS^3 spectrum of the 3′-methoxy-4′-hydroxyflavonol isorhamnetin **11** after loss of Me•.

3.5. The m/z 125 fragment ion of 2′-hydroxyflavonols

A fragment ion at *m/z* = 125, corresponding to the anion of phloroglucinol **24**, was detected exclusively in the MS^2 spectra of the 2′-hydroxyflavonols [datiscetin **6** (18%) and morin **7** (33%)]. The MeOD spectra (Table 5) show clearly that three deuterium atoms are incorporated into the product ion **24**, which suggests that one deuterium must be bonded to C rather than O. The proposed mechanism (Fig. 6) accounts for this observation.

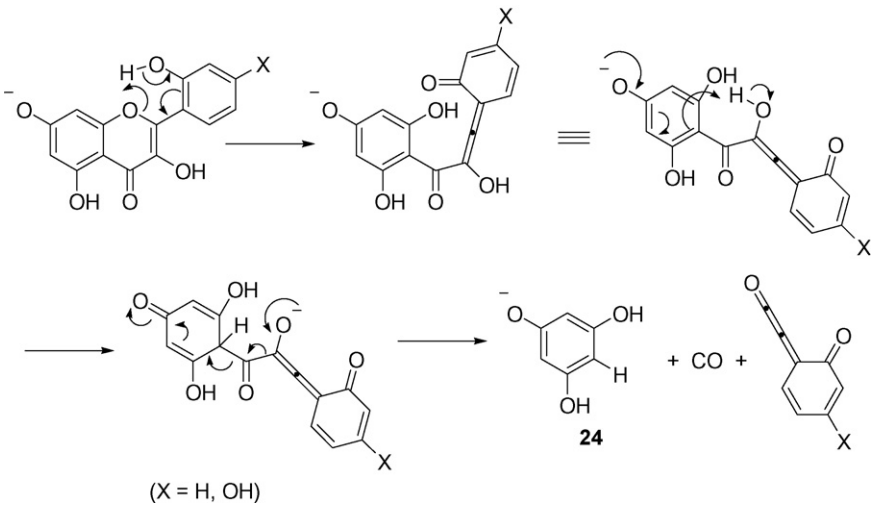

Fig. 6. Formation of *m/z* 125 fragment ion in the negative ion ESI mass spectra of 2′-hydroxyflavonols.

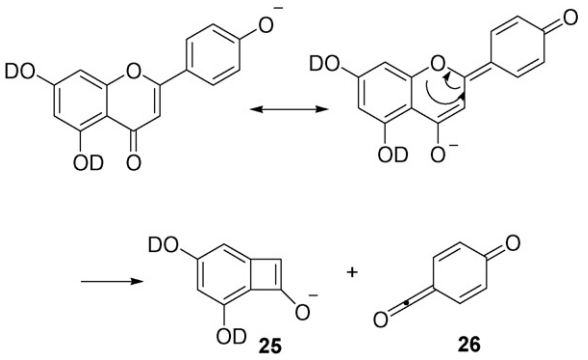

Fig. 7. Formation of the *m*/*z* 149 fragment ion of 4′-hydroxyflavones.

3.6. The m/z 149 fragment ion of 4′-hydroxyflavones

The fragment ion *m*/*z* = 149 (27%), corresponding to loss of a neutral fragment of 120 Da, ($C_7H_4O_2$) was detected in the MS^2 spectrum of apigenin **2**. The following proposed mechanism (Fig. 7) is supported by the MS^2 analysis of apigenin **2** in MeOD, which shows that 2 deuterium atoms remain in the anionic fragment (Table 6). MS^3 studies of this fragment **25** showed that a further loss of CO (−28, *m*/*z* = 121, 100%); CO_2 (−44, *m*/*z* = 105, 84%) and C_2H_2O (−42, *m*/*z* = 105, 72%) were the main pathways observed. This is the only mechanism in which ionisation of a hydroxy group in the B-ring is required.

4. Discussion

The mechanisms presented above, supported by isotopic labelling experiments, provide a basis for the structural assignments of known and unknown flavonoids. Thus, 2′-hydroxyflavonols can be recognised by a specific $(M\text{-}H_2O)^-$ mechanism which is revealed by deuterium exchange; such compounds also show a characteristic peak at *m*/*z* 125. The RDA mechanism provides an assay of the number and types of substituents in the A- and B-rings; importantly, this mechanism is also shown in MS^2 and, particularly, MS^3 spectra of *O*-methyl flavonoids after loss of the Me group. 3′,4′-Dihydroxyflavonols show a consistently intense $(M\text{-}122)^-$ peak, which appears to be characteristic of this class of flavonoids; other characteristic fragment ions are shown by 4′-hydroxyflavones.

As an example of the application of this methodology to the identification of a minor dye component, PDA-HPLC analysis of an extract from a sample of alum mordanted wool, dyed with Dyer's chamomile (*Anthemis tinctoria* L.), shows the presence of about 10 significant components, with quercetin **8** (30%) the dominant chromophore. One minor component, which elutes just before quercetin, shows *m*/*z* = 331 $[(M-1)^-]$ The MS^2 spectrum shows a base peak (*m*/*z* = 316) resulting from the loss of CH_3 consistent with the presence of a methoxy substitutent. The $(M\text{-}122)^-$ peak characteristic of 3′,4′-dihydroxyflavones is present [*m*/*z* = 209 (7%)] and the peak resulting from an RDA pathway [*m*/*z* = 181 (4%)] indicates that the methoxy substitutent is situated in the A ring in addition to two hydroxy substituents. A peak at *m*/*z* = 166 (1%) results from the consecutive loss of CH_3 and RDA breakdown. As shown in Fig. 8, these results suggest that this component is patuletin **27**, the only isomer of an A-ring methylated flavonol which fits these data and is known to be present in *A. tinctoria* L. [24]. Other examples of our applications of this methodology have been published [9,10,12].

MS^n analysis of flavonoid samples from historical artefacts can also be used to substantiate preliminary results from PDA-HPLC. As one example, green wool fibres from a mid 18th Century tartan fabric [which now forms part of the collection of the West Highland Museum, Scotland (WHM 156)] were said to originate from local sheep and to be dyed with plants from Glencoe [25]. Quercetin (warp: 94%; weft 94%), apigenin (warp: 3%; weft 4%) and kaempferol (warp: 3%; weft 2%) were identified by PDA-HPLC as the main flavonoid components of dark green threads indicating

Table 6
MS^2 results for apigenin **2** in MeOH and MeOD

Flavonoid	Parent ion (*m*/*z*)		Daughter ion 25 *m*/*z* (relative abundance)		Neutral fragment 26 (*m*)	
	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]	CH_3OH[a]	CH_3OD[a]
Apigenin **2**	269 $[M-H]^-$	271 $[M-D]^-$	149 (27%)	151 (8%)	120	120

[a] Solvent.

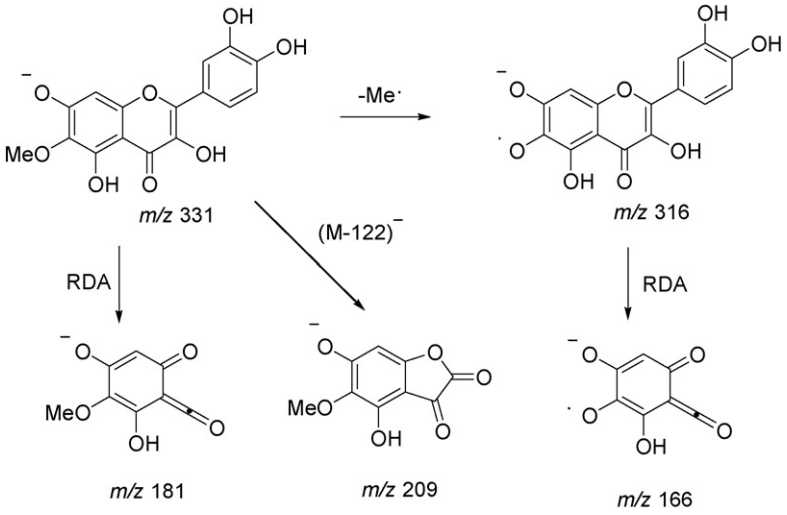

Fig. 8. MS^2 fragmentation of patuletin **27**.

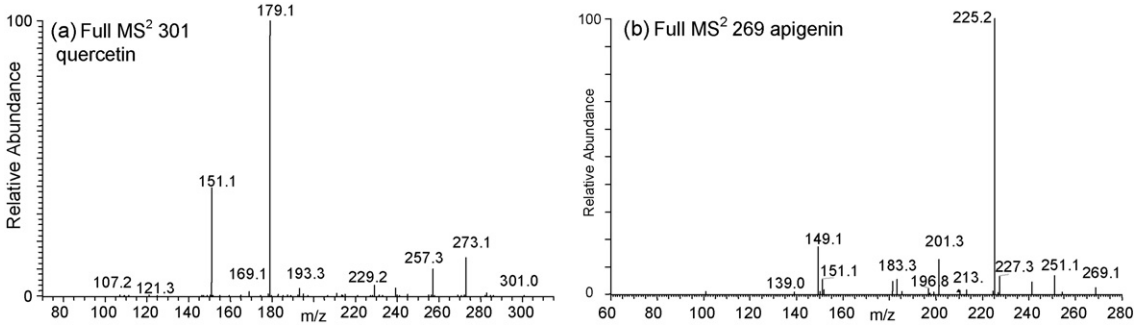

Fig. 9. LC–ESIMS analysis of green wool warp thread from the Glencoe tartan fabric. (a) MS^2 $m/z = 301$ (b) MS^2 $m/z = 269$.

that the same dyestuff was used for both warp and weft. The presence of quercetin and apigenin was confirmed by LC–ESI–MS analysis (Fig. 9 – *c.f.* Table 1). These results suggest that heather (*Calluna vulgaris* L.) (typically containing quercetin 80%, apigenin 14%, kaempferol 6%) was used to dye this sample, thereby providing scientific confirmation of the historical record. This result proved to be of particular importance, because most "quality" tartans dating from this period were dyed with imported colourants [25].

5. Conclusions

Electrospray ionisation mass spectrometry in negative ion mode is a highly sensitive technique for the analysis of flavonoids in solution and is therefore suitable for the study of natural yellow dye extracts from textiles, and, potentially, from lakes. The MS^2 and MS^3 spectra can provide important information in flavonoid identification both by comparison and matching to those of known standards and by detection of fragment ions characteristic of certain structural features.

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