**Identification of formononetin metabolites**

**1 Primary branching metabolites of formononetin(A1-A20)**

With the retention time of 6.92 min, **A1** was 162 Da more massive than formononetin, which possessed the theoretical [M+H]+ ion at 431.13365 (C22H23O9, mass error within ±5 ppm) in positive ion mode. In ESI-MS2 spectrum, the fragment ions at *m/z* 269 [M+H-Glc]+, *m/z* 253 [M+H-Glc-CH2]+, and *m/z* 237 [M+H-Glc-OCH2]+ were observed. Additionally, the DPIs of formononetin at *m/z* 107, *m/z*136, and *m/z* 161 were also detected. According to the literature [1], **A1** was identified as ononin.

**A2** yielded significant [M-H]-/ [M+H]+ ions at 253.05043 (C15H9O4, mass error of -0.799 ppm) and *m/z* 255.06427 (C15H11O4, mass error of -0.915 ppm) in negative and positive ion modes, respectively. It was 14 Da less massive than formononetin. And then, the fragment ions at *m/z* 237 [M-H-O]-, *m/z* 225 [M-H-CO]-, *m/z* 221 [M-H-2O]-, and *m/z* 209 [M-H-CO2]- were generated by a series of neutral losses of O, CO, and CO2. All the above fragment ions suggested that **A2** was a demethylated metabolite of formononetin, daidzein.

**A3**, **A4,** and **A15** showed [M-H]- ions at *m/z* 347.02319 (C16H11O7S, mass error of 0.269 ppm), *m/z* 443.09888 (C22H19O10, mass error of 0.045 ppm), and *m/z* 423.16559 (C22H23N4O5, mass error of -1.261 ppm), which were 80 Da, 176 Da, and 156 Da more massive than formononetin, indicating that **A3**, **A4**, and **A15** could be deduced as sulfation metabolite, glucuronidation metabolite, and arginine conjugation metabolites of formononetin, respectively. In their ESI-MS2 spectra, the significant fragment ion at *m/z* 267 was formed by the neutral loss of SO3, GluA, and Arg, respectively. Other DPIs of formononetin at *m/z* 91, *m/z* 107, *m/z* 135, *m/z* 147, *m/z* 209, *m/z* 223, and *m/z* 252 were observed. Therefore, **A3**, **A4**, and **A15** were deduced as formononetin-7-O-sulfate, formononetin-7-O-GluA, and formononetin-7-O-Arg, respectively.

Eight isomeric metabolites, **A5-A12** were 16 Da more massive than formononetin, which had the same theoretical [M-H]-/[M+H]+ ions at *m/z* 283.06009 (C16H11O5, mass errors within ±5 ppm) and *m/z* 285.07575 (C16H13O5, mass errors within ±5 ppm). Therefore, **A5-A12** were tentatively identified to be the mono-hydroxylated metabolites of formononetin. Among them, the MS2 spectra of **A5-A9** all showed complementary fragment ions at *m/z* 135 (C7H3O3) and *m/z* 147 (C9H7O2), which were generated by cleavage pathway (c), indicating hydroxylation in the B-ring or C-ring. Notably, the pathway cleavage (b) of **A7** produced fragment ions at *m/z* 163 (C8H3O4) and *m/z* 120 (C8H8O), indicating hydroxylation in the C-2 position. In general, the larger the Clog*P* value, the larger the retention time. The Clog*P* values for the substituted hydroxyl groups of C-2', C-6', C-3', and C-5' were 1.11784, 1.11784, 1.48784, and 1.48784, respectively. Therefore, the hydroxyl substitution of C-2' and C-6' were **A5** and **A6** (or **A6** and **A5**), and the hydroxyl substitution of C-3' and C-5' were **A8** and **A9** (or **A9** and **A8**). In addition, the MS2 spectra of **A10**, **A11**, and **A12** all showed fragment ions at *m/z* 152 (C7H4O4) and *m/z* 132 (C9H8O), which were typical DPIs produced by RDA cleavage. The *m/z* 152 indicated the presence of two hydroxyl groups in the A-ring. This was also confirmed by the fragment ions at *m/z* 136, *m/z* 114, *m/z* 91, and *m/z* 71 produced by the cleavage pathway (c)/(e). In other words, the hydroxyl substitution in the A-ring has three positions: C-5, C-6, and C-8. The substituted hydroxyl group in the C-5 position readily formed an intramolecular hydrogen bond with the carbonyl group in the C-4 position, which significantly reduced the polarity of the metabolite, and its retention time was larger. Thus, **A12** was tentatively identified as 5-hydroxyl-formononetin (Biochanin A). The Clog*P* values of **A10** and **A11** were both 1.57529, so it was difficult to distinguish between C-6 and C-8 substituted hydroxyl groups.

**A13** showed [M-H]-/ [M+H]+ ions at *m/z* 281.08194 (C17H13O4, mass error of 0.028 ppm) and *m/z* 283.09564 (C17H15O4, mass error of -2.986 ppm), and it was 14 Da more massive than formononetin. In negative ion mode, the product ions at *m/z* 253 [M-H-CO]-, *m/z* 223 [M-H-CO-OCH2]-, and *m/z* 221 [M-H-2OCH2]- were observed. Additionally, the characteristic ions at *m/z* 136 and *m/z* 132 were produced by RDA cleavage, indicating that the A-ring hydroxyl group underwent methyl substitution. Therefore, **A13** was inferred to be 7-methoxy-formononetin.

**A14** showed the chromatographic peak at 7.21 min and possessed [M-H]- ion at 269.08200 (C16H13O4, mass error of 0.252 ppm), which was 2 Da more massive than formononetin. In the ESI-MS2 spectrum, several dominant fragment ions at *m/z* 253 [M-H-O]-, *m/z* 225 [M-H-CO2]-, and *m/z* 209 [M-H-O-CO2]- were observed. The significant fragment ions at *m/z* 135 (C7H3O3) and *m/z* 133 (*m/z* 103+OCH3) confirmed the occurrence of hydrogenation in the C-2 and C-3 positions. Thus, **A14** was 2,3-dihydro-formononetin.

**A16** and **A17** had the same theoretical [M+H]+ ions at *m/z* 241.08516 and *m/z* 241.08516, which were 28 Da less massive than formononetin. It was speculated that **A16** and **A17** were the decarbonylated metabolites of formononetin. Through cleavage pathway (a), **A16** and **A17** yielded fragment ions at *m/z* 93 and *m/z*109, indicating that the decarbonylation of **A16** and **A17** were in the C-2 and C-3 positions, respectively.

**A18** showed [M+H]+ ion at *m/z* 257.08005 (C15H13O4, mass error of -0.785 ppm), which was 12 Da less massive than formononetin. The fragment ions at *m/z* 209 [M+H-CO]+, *m/z* 137 [M+H-C8H9O]+, *m/z* 121 [M+H-C7H5O3]+, and *m/z* 109 [M+H-C8H9O-CO]+ were all observed in the ESI-MS2 spectrum. It was indicated that RDA cleavage and loss of C-2 had occurred. **A18** could be deduced as decarbonized metabolite of formononetin.

The theoretical [M-H]- ions of **A19** and **A20** were at *m/z* 267.06519, the same as formononetin. It was presumed that both metabolites were isomers of formononetin. In negative ion mode, **A19** yielded characteristic ions at *m/z* 72 (C4H8O) and *m/z* 121 (C7H5O2), suggesting that the carbonyl group at the C-4 position might be condensed with C-6' in the C-ring. **A20** showed fragment ions at *m/z* 253.05 [M-H-CH2]-, *m/z* 225 [M-H-CH2-CO]-, and *m/z* 193 [M-H-OCH2-CO2]- were observed. Therefore, it was speculated that **A20** was an isomerized aurone metabolite.

**2 Secondary branching metabolites of formononetin(B1-B42)**

**B1** yield [M-H]- ion at *m/z* 447.13028 (C22H23O10, mass error of 1.364 ppm), which was 18 Da more massive than **A1**. In the ESI-MS2 spectrum, the fragment ions at *m/z* 429 [M-H-H2O]- and *m/z* 202 [M-H-H2O-Glc-CO2-OCH3]- were generated. Other DPIs at *m/z* 175, *m/z* 135, and *m/z* 121 were observed. Thus, **B1** was a secondary branching metabolite resulting from the hydrolysis of **A1**. Similarly, **B2**, **B3**, and **B4** had the same DPIs as **A1**. But, **B3** was 2 Da more massive than **A1**. **B2** and **B4** were 18 Da and 2 Da less massive than **A1**, respectively. Therefore, **B2**, **B3**, and **B4** were dehydrated, hydrogenated, and dehydrogenated metabolites of **A1**, respectively.

Five isomeric metabolites, **B5-B9** were 14 Da less massive than **A5-A12**. And thus,

they were tentatively inferred to be demethylated products of **A5-A12**. In their ESI-MS2 spectra, the DPIs at *m/z* 251 [M-H-O]-, *m/z* 241 [M-H-CO]-, and *m/z* 225 [M-H-CO2]- were obtained after the sequential loss of O, CO, and CO2, respectively. Based on the structural characteristics of **A5-A12** and the Clog*P* values, the structures of **B5-B9** were inferred (**Fig.4**).

**B10** and **B11** afforded [M-H]- ion at *m/z* 333.00634 (C15H9O7S, mass errors within ±5 ppm), which were 80 Da more massive than **A2**. The product ions of **B10** and **B11** at *m/z* 305 [M-H-CO]- and *m/z* 253 [M-H-SO3]- were observed in negative ion mode. In addition, the ions of *m/z* 135 and *m/z* 117 (*m/z* 198-SO3) were generated in **B10**; *m/z* 214 and *m/z* 217 were generated in **B11**. The Clog*P* values for **B10** and **B11** were 0.4985 and 0.879, respectively. Therefore, **B10** was daidzin-4’-O-sulfate, **B11** was daidzin-7-O-sulfate.

With the retention time of 4.66 and 5.31 min, **B12** and **B13** were 14 Da less massive than **A4**, afforded the [M-H]- ions at *m/z* 429.08377 and *m/z* 429.08231 (C21H17O10, mass errors within ±5 ppm) in negative ion mode. Therefore, **B12** and **B13** were deduced as glucuronidation and demethylation metabolites of formononetin. Based on the RDA cleavage, the B-ring fragment ions of **B12** and **B13** were *m/z* 117 and *m/z* 293 (117+GluA), respectively. It was determined that **B12** was daidzin-7-O-GluA and **B13** was daidzin-4’-O-GluA.

In negative ion mode, **B14** and **B15** showed [M-H]- ions at *m/z* 445.11426 and *m/z* 445.11523 (C22H21O10, mass errors within ±5 ppm), which were 2 Da more massive than **A4**. The DPIs at *m/z* 91, *m/z* 107, *m/z* 135, *m/z* 147, *m/z* 209, *m/z* 223, and *m/z* 252 all were observed. They were speculated to be the hydrogenated metabolites of **A4**. Meanwhile, **B16-B21** showed the same DPIs, but 16 more massive than **A4**, indicating that **B16-B21** were the hydroxylated metabolites of **A4** and the secondary branching metabolites of formononetin.

**B22-B25** were 32 Da more massive than formononetin and 16 Da more massive than **A5-A12**, which displayed [M-H]- ion at *m/z* 299.05501 (C16H11O6, mass errors within ±5 ppm) and [M+H]+ ion at *m/z* 301.07066 (C16H13O6, mass errors within ±5 ppm). In their ESI-MS2 spectra, the fragment ions at *m/z* 284 [M-H-CH3]-, *m/z* 268 [M-H-OCH3]-, *m/z* 256 [M-H-CH3-CO]-, and *m/z* 240 [M-H-CH3-CO2]- were generated in negative ion mode. Notably, the DPIs of the parent nucleus of formononetin were observed at *m/z* 107, *m/z* 132, *m/z* 135, and *m/z* 161. Therefore, **B22-B25** were the dihydroxylated metabolites of formononetin. Similarly, **B26-B28** were 30 Da more massive than **A5-A12**, **B29** was 42 Da more massive than **A5-A12**, and **B30** was 14 Da more massive than **A13**. They all showed DPIs of the parent nucleus of formononetin. Thus, **B26-B28**, **B29**, and **B30** were speculated to be hydroxylated and methoxylated, hydroxylated and acetylated, and dimethylated metabolites of formononetin, respectively.

**B31** eluted at 7.49 min and possessed [M+H]+ ion at *m/z* 255.10184, which was 14 Da more massive than formononetin in positive ion mode, with a mass error of 1.055 ppm. The fragment ions at *m/z* 237 [M+H-O]+, *m/z* 222 [M+H-OCH3]+, *m/z* 194 [M+H-OCH3-CO]+, *m/z* 121 [M+H-C10H8O3]+, and *m/z* 107 [M+H-C11H10O3]+ were observed. Two key ions at *m/z* 119 (C7H3O2) and *m/z* 133 (C9H9O) were generated by RDA cleavage, indicating dehydroxylation of the A-ring and hydrogenation of the C-ring. Based on this, **B31** was deduced to be the dehydroxylated and hydrogenated secondary branching metabolite of formononetin.

**B32** (*m/z* 243.10086, C15H15O3, mass error of -0.711 ppm) was 28 Da less massive than **A14** with a retention time of 6.18 min in positive ion mode, suggesting it could be decarbonylated metabolites of **A14**. The significant fragment ions at *m/z* 227 [M+H-O]+, *m/z* 197 [M+H-O-OCH3]+, *m/z* 109 (C6H5O2), and *m/z* 135 (C9H11O) were observed. They confirmed the loss of the carbonyl group at the C-4 position. Thus, **B32** was a decarbonylated metabolite of dihydro-formononetin. Furthermore, **B33** possessed the theoretical [M+H]+ ion at *m/z* 328.11768 (C18H18NO5, mass error of -0.820 ppm), which was 57 Da more massive than **A14**. The fragment ions at *m/z* 270 [M+H-Gly]+, *m/z* 254 [M+H-Gly-O]+, *m/z* 242 [M+H-Gly-CO]+, and *m/z* 226 [M+H-Gly-CO2]+ were produced, indicating that **B33** could be tentatively concluded as glycine conjugation metabolite of dihydro-formononetin.

**B34** eluted at 12.67 min and afforded the deprotonated molecular ion at *m/z* 421.15543 (C22H21N4O5, mass error of 0.687 ppm). It was 2 Da less massive than **A15**. It was generated fragment ions at *m/z* 265 [M-H-Arg]-, *m/z* 249 [M-H-Arg-O]-, *m/z* 221 [M-H-Arg-CO]-, *m/z* 205 [M-H-Arg-CO2]-, and *m/z* 235 [M-H-Arg-OCH2]-. Hence, **B34** could be temporarily speculated as a dehydrogenated metabolite of **A15**. **B35-B39** showed their theoretical deprotonated molecular ions at *m/z* 407.17138 (C22H23N4O4, mass errors within ±5 ppm), which were 16 Da less massive than **A15**. According to the DPIs of **A15**, they might be deduced as deoxygenated metabolites of **A15**. Moreover, **B40** and **B41** yielded significant [M+H]+ ion at *m/z* 407.17023 (C22H23N4O4, mass errors within ±5 ppm), which were 18 Da less massive than **A15**. Therefore, **B40** and **B41** were extrapolated to dehydrated metabolites of **A15**. All the metabolites mentioned above were also secondary branching metabolites of formononetin.

**B42** was 271 Da (GSH-H2O) more massive than formononetin, which showed [M-H]- ion at *m/z* 538.13043 (C26H24N3O8S, mass error of 2.734 ppm). In the ESI-MS2 spectrum, four notable ions at *m/z* 249 [M-H-GSH]-, *m/z* 233 [M-H-GSH-O]-, *m/z* 221 [M-H-GSH-CO]-, and *m/z* 217 [M-H-GSH-CO2]- were formed. Therefore, **B42** might be inferred as a glutathione conjudation and dehydration metabolite of formononetin.

**3 Tertiary branching metabolites of formononetin (C1-C31)**

**C1** showed theoretical deprotonated molecular ion at *m/z* 449.14703 (C22H25O10, mass error of 1.807 ppm), which was 2 Da more massive than **B1**. Two predominant fragment ions at *m/z* 405 [M-H-CO-H2O]- and *m/z* 374 [M-H-CO2-H2O-CH3]- were detected. Additionally, high abundance ions at *m/z* 447 (**B1**) and *m/z* 431 (**B1**-O) were generated. Based on this, **C1** was identified as the hydrogenated metabolite of **B1**, a tertiary branching metabolite of formononetin.

**C2** generated its [M-H]- ion at *m/z* 271.06100 (C15H11O5, mass error of 0.132 ppm) with the retention time of 7.20 min. It was 2 Da more massive than **B5-B9**, extrapolating that **C2** was a hydrogenated metabolite of **B1**. The DPIs at *m/z* 251, *m/z* 241, and *m/z* 225 were observed. **C3** afforded the deprotonated molecular ion at *m/z* 431.09869 (C21H19O10, mass error of 0.320 ppm), which was 162 Da more massive than **B5-B9**, and generated fragment ions at *m/z* 269 [M-H-Glc]-, *m/z* 255 [M-H-Glc-CH2]-, *m/z* 241 [M-H-Glc-CO]-, and *m/z* 225 [M-H-Glc-CO2]-. Therefore, **C3** was characterized as a glucosylation product of **B5-B9**. Moreover, **C4-C6** possessed the same theoretical [M-H]- ion at *m/z* 349.00238 (C15H9O8S, mass errors within ±5 ppm), which were 80 Da more massive than **B5-B9**. The fragment ions at *m/z* 269 [M-H-SO3]-, *m/z* 241 [M-H-SO3-CO]-, and *m/z* 225 [M-H-CO2]- were exhibited in their ESI-MS2 spectra. So, **C4-C6** were speculated to be sulfated metabolites of **B5-B9**. Similarly, the dominant ion at *m/z* 269 [M-H-GluA]- was detected in the ESI-MS2 spectra of **C7-C9**. They were 176 Da more massive than **B5-B9**, inferring that they were glucuronided metabolites of **B5-B9**.

**C10** gave rise to [M+H]+ ion at *m/z* 317.06604 (C16H13O7, mass error of 1.453 ppm), which was 16 Da more massive than **B22-B25**. In the ESI-MS2 spectrum, the product ions at *m/z* 299 [M+H-H2O]+, *m/z* 271 [M+H-H2O-CO]+, and *m/z* 225 [M+H-H2O-CO2-OCH2]+ were generated in positive ion mode. The DPIs of formononetin appeared at *m/z* 93, *m/z* 109, *m/z* 121, *m/z* 134, *m/z* 137, and *m/z* 161. These results suggested that **C10** was a tri-hydroxylated metabolite of formononetin.

The retention time of **C11** was 6.96 min, with a mass 2 Da less than that of **B34**, giving [M-H]- ion at *m/z* 419.13513 (C22H19O5N4, mass error of -0.963 ppm). The fragment ions at *m/z* 373 [M-H-CO-H2O]-, *m/z* 263 [M-H-Arg]-, and *m/z* 221 [M-H-Arg-CO-CH2]- were detected, which indicated that **C11** might be dehydrogenated metabolite of **C11**. In addition, **C12-C17** showed their deprotonated molecular ions at *m/z* 409.18703 (C22H25N4O4, mass errors within ±5 ppm), which was 2 Da more massive than **B35-B39**. Through the continuous loss of Arg, CO, and CH2, the characteristic product ions at *m/z* 253 [M-H-Arg]-, *m/z* 225 [M-H-Arg-CO]-, and *m/z* 211 [M-H-Arg-CO-CH2]- were generated. Therefore, they might be deduced as hydrogenated metabolites of **B35-B39**.

**C18** showed its [M-H]- ion at *m/z* 417.11948 and eluted at 6.86 min. Its molecular formula was speculated as C21H21O9 with a mass error of 0.898 ppm, which was 164 (162+2) Da more massive than **A2**. In the ESI-MS2 spectrum, high abundance fragment ions at *m/z* 255 (C12H15O6) and *m/z* 163 (C9H7O3) were generated through cleavage pathway (f), suggesting that glycosylation reaction has occurred in the B-ring. Other ions at *m/z* 241 (255-CH2) and *m/z* 147 (163-O) have also been observed. Hence, **C18** was a demethylated, hydrogenated, and glycosylated metabolite of formononetin.

Six isomeric, tertiary branching metabolites, **C19-C24** displayed their [M-H]- ion at *m/z* 321.04273 (C15H13O6S, mass errors within ±5 ppm) in negative ion mode, which were 26 (28-2) Da less massive than **A3**. They showed meaningful fragment ions at *m/z* 241 [M-H-SO3]- and *m/z* 80 (SO3). Other fragment ions at *m/z* 188 (C6H5O5S) and *m/z* 133 (C9H9O) produced by RDA cleavage demonstrated the loss of double bonds at C-2 and C-3 positions, as well as the loss of CO at C-4 position. Therefore, **C19-C24** were all presumed to be decarbonylated and hydrogenated products of **A3**.

In negative ion mode, **C25** possessed the deprotonated molecular ion at *m/z* 287.09274 (C16H15O5, mass error of 0.847 ppm) with the retention time of 5.71 min. It was 4 Da more massive than **A5-A12**, indicating that a dihydrogenation reaction might have occurred. The fragment ions at *m/z* 257 [M-H-OCH2]-, *m/z* 241 [M-H-OCH2]-, *m/z* 229 [M-H-OCH2]-, and *m/z* 213 [M-H-OCH2]- were detected. Thus, **C25** could be attributed to be a hydroxylated and dihydrogenated metabolite of formononetin.

**C26-C28** were 60 (30+30) Da more massive than **A14**, which showed the theoretical [M+H]+/[M-H]- ions at *m/z* 331.11761 (C18H19O6, mass errors within ±5 ppm) and *m/z* 329.10196 (C18H17O6, mass errors within ±5 ppm), respectively. In negative ion mode, the fragment ions at *m/z* 311 [M-H-H2O]-, *m/z* 284 [M-H-CO2]-, *m/z* 269 [M-H-2OCH2]-, and *m/z* 239 [M-H-3OCH2]- were observed. Given the above results, **C26-C28** were dimethoxylated metabolites of **A14**. Similarly, **C29** was 87 (103-16) Da more massive than **A14**. It possessed the same DPIs as **A14** and appeared as a fragment ion at *m/z* 103 (Cys). Therefore, **C29** was presumed to be a deoxygenation and cysteine combination metabolite of **A14**.

**C30** and **C31** eluted at 9.98 min and 11.01 min, afforded the deprotonated molecular ions at *m/z* 570.11871 and *m/z* 570.11920 (C26H24O10N3S, mass errors within ±5 ppm), respectively. In their ESI-MS2 spectra, the emphatic fragment ion at *m/z* 307 suggested that GSH has been lost. However, **C30** and **C31** were 303 (307-4H) Da more massive than formononetin. Based on this, we hypothesized that **C30** was the glutathioned and didehydrogenated metabolite of formononetin.

**4 Mmulti-level branching metabolites of formononetin (D1-D37)**

**D1** had [M-H]- ion at *m/z* 433.11414 (C21H21O10, mass error of 0.120 ppm) in the ESI-MS2 spectrum. It was 2 Da less massive than **C3**. Besides, glucosylation reaction characterization was presented. The fragment ions at *m/z* 415 [M-H-H2O]-, *m/z* 271 [M-H-Glc]-, *m/z* 257 [M-H-Glc-CH2]-, and *m/z* 239 [M-H-Glc-CH2-H2O]- were presented. Based on the analysis above, **D1** was a dehydrogenated metabolite of **C3**. However, it could be clearly stated that demethylation occurred at the C-4' position.

**D2-D7** with experimental [M-H]- ion at *m/z* 393.19211 (C22H25N4O3, mass errors within ±5 ppm) were observed. They were 16 Da less massive than **C12-C17**, suggesting that **D2-D7** were the deoxygenated products of **C12-C17**. Noteworthy ions at *m/z* 133 and *m/z* 104 (260-Arg) from RDA cleavage, and fragment ions at *m/z* 147 and *m/z* 246 from cleavage pathway (e), suggesting that arginine conjugation occurred in the C-7 position and two oxygen deletions in the C-ring, with hydrogenation of C-2 and C-3. Moreover, **D22**, **D24-D28**, and **D32-D34** were also arginine conjugation metabolites with only a few structural changes. Their structures were predicted based on the results of fragment ions analysis and the DPIs of the primary and secondary branching arginine conjugation metabolites, as shown in **Fig.4**.

**D8-D10** were 44 Da more massive than **C18**, indicating a neutral loss of CO2. They generated the [M-H]- ion at *m/z* 373.12817 (C20H21O7, mass errors within ±5 ppm) and [M+H]+ ion at *m/z* 375.14382 (C20H23O7, mass errors within ±5 ppm). For instance, a series of diagnostic fragment ions at *m/z* 357 [M+H-H2O]+, *m/z* 213 [M+H-Glc]+, and *m/z* 199 [M+H-Glc-CH2]+ were produced in positive ion mode. Similarly, based on the parent nucleus structure of **C18**, **D11-D15,** and **D17** were 30 (OCH2) Da and 18 (H2O) Da less massive than **C18**, their molecular formula were predicted to be C20H19O8 and C21H21O8 in negative ion mode, respectively. Moreover, **D16**, **D18-D21**, and **D23** were 2 (2H) Da, 80 (SO3) Da, and 96 (SO4) Da more massive than **C18**, their molecular formulas were predicted to be C21H23O9, C21H21O12S, and C21H21O13S in negative ion mode, respectively. And thus, **D11-D15**, **D16**, **D17**, and **D18-D21** were temporarily assigned as demethoxylated, hydrogenated, dehydrated, and sulfated metabolites, respectively. **D23** was provisionally designated as a hydroxylated and sulfated metabolite of **C18**. They were all defined as multi-level branching metabolites of formononetin.

By extrapolation of the recursive tree analysis strategy, **B31** was produced by deoxygenation and hydrogenation metabolism. Continuing the recursion, if **B31** underwent two deoxygenations and hydrogenations, **D29-D31** with the molecular formula C16H19O were produced. **D29**, **D30**, and **D31** were eluted at 5.79 min, 7.65 min, and 11.16 min, and possessed the protonated molecular ions at *m/z* 227.14249, *m/z* 227.14310, and *m/z* 227.14328 (C16H19O, mass errors within ±5 ppm), respectively. They were 28 Da less massive than **B31**, but this was not due to the loss of CO, but rather to deoxygenation and hydrogenation twice. In their ESI-MS2 spectra, a pair of DPIs at *m/z* 95 (C7H11) and *m/z* 135 (C9H11O) were produced by the cleavage pathway (c). Other remarkable fragment ions at *m/z* 121 (135-CH2) and *m/z* 105 (135-OCH2) were detected, indicating the integrity of the B-ring. Therefore, **D29-D31** were speculated to be multi-level branching metabolites of formononetin.

**D35** gave rise to the [M+H]+ ion at *m/z* 219.04480 (C15H7O2, mass error of 3.397 ppm) with a retention time of 7.29 min in positive ion mode, which was 50 (CO2+3H2) Da less massive than formononetin. In the ESI-MS2 spectrum, it produced the base peak ion at *m/z* 219. And then, the product ions at *m/z* 203 [M+H-O]+ and *m/z* 201 [M+H-H2O]+ also further confirmed that dehydrogenation in the A-ring occurred leading to dehydration. In the end, **D35** was identified as a CO2-loss and tri-dehydrogenated metabolite of formononetin.

The taurine conjugation reaction often occurs during metabolic processes. By recursive tree analysis strategy, we did not find direct formononetin metabolites bound to taurine but found the multi-level metabolites **D36** and **D37**. Firstly, **D36** lost three oxygen based on taurine conjugation. It showed theoretical deprotonated ion at *m/z* 326.08807 (C18H16NO3S, mass error of 2.433 ppm) and protonated ion at *m/z* 328.10138 (C18H18NO3S, mass error of 3.625 ppm). The characteristic product ion at *m/z* 125 was generated by the loss of Tau. Other DPIs of formononetin such as *m/z* 93 (91+2H), *m/z* 109 (107+2H), *m/z* 137 (135+2H), *m/z* 146, and *m/z* 175 were also observed in negative ion mode. Furthermore, **D37** underwent double hydrogenation and double deoxygenation after taurine conjugation, which was displayed [M-H]- ion at *m/z* 346.10828 (C18H20NO4S, mass error of -3.572 ppm). A battery of fragment ions at *m/z* 316 [M-H-OCH2]-, *m/z* 284 [M-H-OCH2-2O]-, and *m/z* 125 (C2H7NO3S) were showed. Finally, **D36** and **D37** were concluded to be multi-level branching metabolites of formononetin with taurine conjugation.

**Reference**

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