Electronic Supplementary Material (ESI) for RSC Advances.

Pharmacokinetic profile and metabolite identification of bornyl caffeate and caffeic acid in rats by high performance liquid chromatography coupled with mass spectrometry

Parent Drug M0: In detail, M0 was believed to be bornyl caffeate by comparing the retention time and accurate mass spectra with those of the authentic reference standard. The drug exhibited a retention time of 78.3 min, a father ion $[M-H]^-$ at m/z 315.1602 ($C_{19}H_{24}O_4$) and daughter ions at m/z 179.0355, m/z 161.0253 and m/z 134.0379.

Metabolites M1a, M1b: Although having different retention times of 73.7 and 74.7 min. M1a and M1b gave rise to identical deprotonated molecular ions at m/z 491.1923 ($C_{25}H_{32}O_{10}$) and m/z 491.1925 ($C_{25}H_{32}O_{10}$). These values were 176 Da higher than the molecular weight of bornyl caffeate. Their MS/MS fragment ions at m/z 113.03 and 175.02 formed from the product of glucuronide, indicated that M1a and M1b were monoglucuronide conjugate of bornyl caffeate. The MS/MS pattern showed an intense daughter ion at m/z 315.15 $[M-H-GlcUA]^-$, which was recognized as quasi-molecular ion of the drug. Therefore, M1a and M1b were tentatively identified as bornyl 3-(3-O-glucuronyl-4-hydroxyphenyl)-2-acrylic acid or bornyl 3-(3-hydroxyphenyl-4-O-glucuronyl)-2-acrylic acid.

Metabolites M2a, M2b:The metabolites M2a and M2b displayed the deprotonated molecule $[M-H]^-$ at m/z 505.2085 ($C_{26}H_{34}O_{10}$) and m/z 505.2086

($C_{26}H_{34}O_{10}$) in their MS spectra. The retention times of M2a and M2b were 73.6 and 74.7 min. The deprotonated molecular ions of the two metabolites were 14 Da higher than 491.19 (M1a and M1b). The MS/MS fragment ions of 113.02 and 175.02, which were product ions of glucuronide. The most abundant daughter ion at m/z 329.17 [M-H-GlcUA]$^-$ was attributed to the neutral loss of glucuronide from the parent ion (m/z 505.2086, $C_{26}H_{34}O_{10}$). The daughter ion at m/z 315.15 [M-H- GlcUA-CH_3] was observed by the loss of 14 Da (CH_2) from the precursor ion at m/z 329.17, suggested that the two ions were methylated conjugate isomers of M2a and M2b. Base on these statements, the two metabolites were identified as bornyl 3-(3-O-glucuronyl-4-O-methyl)-2-acrylic acid or bornyl 3-(3-O- methyl-4-O- glucuronyl)-2-acrylic acid.

Metabolites M3: The metabolite M3, detected at 29.9 min, was observed as an intense quasi-molecular ion at m/z 179.0355 (calculated formula=$C_9H_8O_4$). The major fragment ion was m/z 135.0461, corresponding to loss of carbon dioxide molecule from the precursor ion. It was in accordance with the MS/MS spectra of caffeic acid[24-29]. Accordingly, M3 was assigned as caffeic acid derived from hydrolysis of bornyl caffeate (Fig.6S-1.).

Phase Ⅰ metabolites of caffeic acid

Metabolites M4: M4 was eluted at the retention time of 23.0 min where a molecular ion [M-H]- at m/z 181.0508 was observed. This ion was two mass units higher than M3, confirming the hydrogenation of the double bond. The predominant MS/MS fragment ion at *m/z* 163.0433 represented characteristic ion due to the loss of H_2O from the parent ion[27]. The other fragment ion at *m/z* 136.9874 was generated by

the loss of COOH from the parent ion. The characteristic fragment ions at *m/z* 163.0433 and *m/z* 136.9874 were the typical pattern of dihydrocaffeic acid. With the reference to earlier literature[27,28], this compound was identified as 3-(3,4-dihydroxyphenyl)-propanoic acid (dihydrocaffeic acid) (DHCA). (Fig.6S-1.)

Metabolites M5: M5 presented a retention time of 35.5 min with a molecular ion [M-H]- at m/z 165. 0551. Such m/z value was 16 Da (oxygen atom) less than the ion of M4(m/z 181.0506). The MS/MS ions at *m/z* 121.0647, 106.0408 and 77.0407 represented the characteristic ions originated from the consecutive loss of CO_2, OH, CH_2+OH by sequential. According to previous study[30], M5 was believed to be of 3-(3-hydroxyphenyl) propionic acid(3-HPPA). It was formed by the dehydroxylation of C-4 position on the benzene ring of dihydrocaffeic acid. Booth A N et al and Gonthier et al[31,33] reported that 3-HPPA was the major metabolite of caffeic acid and its ester. (Fig.6S-2.)

Metabolites M6: M6 (39.2 min, m/z 163.0401) generated an ion with m/z value of 16 Da (oxygen atom) lower than M3 (m/z 179.0350). It was an dehydroxylation metabolite from caffeic acid. In addition, M6 was two mass units lower than M5 (m/z 165.0557), confirming the reduction of the double bond. The intense daughter ion of m/z 119.0502 corresponded to a neutral loss of 44 Da (CO_2) from the parent ion. Taking inspiration from previous report[32], we identified M6 as m-Coumaric acid. This metabolite may originate from the hydrogenation of 3-hydroxyphenyl propionic acid. (Fig.6S-2.)

Metabolites M7: M 7 was eluted at the retention time of 20.5 min with a

molecular ion [M-H]- of m/z 194. 0458. It was decarbonization and glycylation of the M6. The major fragment ion at m/z 150.0552 was formed by the loss of one mole of CO_2 from the deprotonated parent ion. The other daughter ion at m/z 93.0348 was generated by the loss of C_2H_4NO from the precursor ion. These characteristic ions were identical to the MS/MS spectra of 3-Hydroxyhippuric acid[32]. Thus, M7 was tentatively characterized as 3-Hydroxyhippuric acid. (Fig.6S-2.)

Metabolites M8: The peak of M 8 (42.9 min, $C_7H_6O_2$) produced a deprotonated ion at m/z 121.0292. The intense daughter ion of m/z 77.0402 was formed due to the neutral loss of 44 Da (CO_2) from the parent ion. It has been reported that hydroxyphenyl propionic acid transforms to benzoic acid when dehydroxylation and beta-oxidation occurred[34]. These results confirmed the identification of M8 as benzoic acid. (Fig.6S-2.)

Metabolites M9a, M9b: M9a and M9b gave different retention times of 33.4 and 37.6 min with identical deprotonated molecular ions at m/z 195.0661($C_{10}H_{12}O_4$) and m/z 195.0663($C_{10}H_{12}O_4$). The deprotonated molecular ions of the two metabolites were 14 Da higher than M4 (DHCA). There was a series of product ions at m/z 151.0751 $[M-H-COO]^-$, 136.0535 $[M-H-COO-CH_3]^-$, and 119.9982 $[M-H-COO-CH_3-OH]^-$. These product ions implied thatsuccessive O-methyl products of M4 (DHCA) and O-methylation conjugation occurred on one of the hydroxyl groups on the phenyl ring of M4. The daughter ion of m/z 136.0535 was the most abundant ion in its MS/MS spectrum. Such characters were in good agreement with the fragmentation pathways of dihydroferulic acid (DHFA) or dihydroisoferulic acid (DHiFA) in

Marmet et al.'s report[28]. M9a and M9b were identified as dihydroferulic acid (DHFA) and dihydroisoferulic acid (DHiFA). (Fig.6S-2.)

Metabolites M10a, M10b and M11a, M11b: M10a and M10b (eluted at 28.2 and 29.9 min, respectively) presented the quasi-molecular ion at m/z 275.0230 [M-H]- and m/z 275.0235 $[M-H]^-$. M11a and M11b, eluted at 24.5 and 27.4 min, exhibited the same deprotonated ion of m/z 371.0986 $[M-H]^-$ and m/z 371.0987 $[M-H]^-$. The characteristic fragment ions at m/z 195.06 and m/z 177.05 were found in the MS/MS spectra of M10 (M10a, M10b) and M11 (M11a, M11b), corresponding to the structural unit information of dihydroferulic acid (DHFA) or dihydroisoferulic acid (DHiFA). According to the composition shift, the elemental formulas of M10 (M10a, M10b) and M11 (M11a, M11b) were deduced as $C_{10}H_{12}O_7S$ and $C_{16}H_{20}O_{10}$. The loss of sulfonic acid and glucuronic acid moiety from deprotonated ion $[M-H]^-$ formed the characteristic ion of m/z 195.06. The fragment ion at m/z 177.05 was formed by the loss of one mole of H_2O from the precursor ion at m/z 195.06. In this case, M10 (M10a, M10b) and M11 (M11a, M11b) were elucidated as the successive sulfated and glucuronidated conjugates isomers of dihydroferulic acid (DHFA) or dihydroisoferulic acid (DHiFA)[28], respectively.

Metabolites M12: The peak of M12 (22.2 min, $C_8H_8O_4$) with a deprotonated ion $[M-H]^-$ at m/z 167.0352 produced a typical fragmentation pattern after collision induced dissociation. This ion was characterized by the loss of methyl radicals (15 Da) from parent ion, leading to a very stable anion radical structure of $[M-H-CH_3]^-$ at m/z 152.0144. In addition, we detected an unusual loss (44 Da) of a carboxylic acid group,

providing an anion of $[M-H-COO]^-$ at m/z 123.0455. Both types of fragment ions may occur simultaneously, producing a distinct anion radical of $[M-H-COO-CH_3]^-$ at m/z 108.0257[35,36]. It has been reported that a small proportion of ingested free ferulic acid may be metabolized through beta-oxidation in liver to form vanillic acid[28]. Taking together, M12 was tentatively characterized as vanillic acid. (Fig.6S-3.)

Metabolites M13: M13 (23.4 min, $C_{10}H_{11}NO_5$) produced a deprotonated ion at m/z 224.0558, which was formedby glycine conjugates with vanillic acid. The daughter ions at m/z 180.0643 and m/z 165.0409 represented the characteristic ions ascribed to the consecutive loss of CO_2 and CH_3. The daughter ion at m/z 123.0433 correspond to the loss of $C_3H_3NO_3$(101 Da). Subsequently, the $C_3H_3NO_3$ group with ion at m/z 100.0040 lose one mole of CO to form a daughter ion at m/z 74.0230. Inspired by the mass data from Pekkinen's research[34,37], we assigned M13 as vanilloylglycine. (Fig.6S-3.)

Metabolites M14a, M14b: M14a and M14b (eluted at 31.7 and 42.5 min, respectively) presented the quasi-molecular ion at m/z 193.0501 $[M-H]^-$ and m/z 193.0505 $[M-H]^-$. The major fragment ions were m/z 178.02 and 149.06, corresponding to loss of methyl or carbon dioxide molecules from the precursor ion. The most abundant fragment ion at m/z 134.03 was generated either by losing a methyl group from the fragment ion m/z 149.05 or by the removal of a carbon dioxide from the fragment ion m/z 178.02. With the reference to the reported data[25,26], M14a and M14b were tentatively assigned as Ferulic acid and Isoferulic acid (Fig. 6S-4.).

Metabolites M15a, M15b: M 15a and M 15b (eluted at 26.8 and 29.9 min)

gengerated quasi-molecular ions at m/z 258.9919 and 258.9914. The two ions were approximate 80 Da heavier than M3 (m/z 179.0350). The daughter ions of m/z 179.03 and m/z 135.04 confirmed the structural unit information of caffeic acid in M13a and M13b. The most abundant fragment ion at m/z 179.03 was formed because the loss of a sulfate group from the quasi-molecular ion. The fragment ion of m/z 135.04 come from the loss of carbon dioxide. Besides these ions, there was a product ion at m/z 96.95 with a formula of O-SO_3H. We tentatively assigned the M 15 a as 3-(3-O-sulphate-4-hydroxy)crylic acid, and identified M 15 b as 3-(3-O-hydroxy-4-sulphate)crylic acid. (Fig.6S-4.)

Metabolites M16a, M16b: M16a, M16b with retention times of 22.8 min and 25.6 min displayed quasi-molecular ion $[M-H]^-$ of m/z 355.0665, 355.0680. Their MS/MS daughter ions included m/z 113.02 and m/z 175.02, indicated that the two metabolites were the isomers of caffeic acid monoglucuronide. Besides these product ions, both M16a and M16b exhibited daughter ions of m/z 135.04[M-H–Glucuronide–CO_2]$^-$, 179.03 [M-H–Glucuronide]$^-$, 311.07 [M-H–CO2]$^-$. This result indicated that glucuronide did not positioned on the carboxyl group. The above-mentioned fragments were similar to those of 3-(3-hydroxy-4-O-glucuronyl) crylic acid or 3-(3-O-glucuronyl-4-hydroxy) crylic acid. The polarity of 3-(3-O-glucuronyl-4-hydroxy) crylic acid was relatively stronger than 3-(3-hydroxy-4-O-glucuronyl) crylic acid. Therefore, M16a with short retention time (22.8 min) was reasonably attributed to 3-(3-O-glucuronyl-4-hydroxy) crylic acid, and M16b (25.6 min) was assigned to 3-(3-hydroxy-4-O-glucuronyl) crylic acid. (Fig.6S-4.)

Metabolites M17a, M17b: M17a and M17b at 33.2 min and 35.2 min showed similar deprotonated molecular ion $[M-H]^-$ of m/z 369.0825 and m/z 369.0826. The fragment ions of m/z 113.02 and m/z 175.02 in their MS/MS spectra indicated that the two metabolites were methylated caffeic acid monoglucuronides. The MS/MS ion at m/z 193.04 was formed by the loss of 176 Da (equivalent to glucuronic acid). The product ions at m/z 178.02$[M-H-CH_3]^-$, m/z 149.06$[M-H-CO_2]^-$, m/z 134.03$[M-H-CO_2-CH_3]^-$ were observed in the MS/MS spectrum of M14a and M14b(m/z 193.05), which confirmed the presence of ferulic acid or isoferulic acid structural unit. The formation of these ions were consistent with the fragmentation behaviors of 3-(3-methoxy-4-O-glucuronyl-phenyl) crylic acid or 3-(3-O-glucuronyl-4-methoxy-phenyl) crylic acid[24,29]. Regarding the polarities of the two metabolites, we tentatively assigned M17a with the short retention time as 3-(3-methoxy-4-O-glucuronyl-phenyl) crylic acid, and M17b as 3-(3-O-glucuronyl-4-methoxy-phenyl) crylic acid. (Fig.6S-5.)

Metabolites M18a,M18b: M18a and M18b (eluted at 31.7 and 34.2 min, respectively) presented the quasi-molecular ion at m/z 273.00 $[M-H]^-$, which were 80 Da heavier than M14a and M14b (m/z 193.05). The characteristic fragment ions at m/z 193.05, m/z 178.02 and m/z 149.06 were found in the MS/MS spectra of M 18a and M18b, which were in good agreement with the fragmentation pathways of M14a and M14b (m/z 193.05). According to the earlier research[28], M18a and M18b were assigned as ferulic-4-O-sulfate or isoferulic-3-O-sulfate. (Fig.6S-5.)

Metabolites M19a, M19b: M19a and M19b with retention times of 20.2 min,

28.2 min) were detected only in urine and presented an identical father ion of m/z 250.07 $[M-H]^-$. The MS/MS profile was characterized by the daughter ions of m/z 206.08 $[M-H-CO_2]^-$, m/z 191.05 $[M-H-CO_2-CH_3]^-$, m/z 149.05 $[M-H-C_3H_3NO_3]^-$, m/z 134.03 $[M-H-C_3H_3NO_3-CH_3]$- and m/z 100.00 $[M-H-C_9H_9O_2]^-$. These ions corresponded to the fragmentation pattern of feruloylglycine and isoferuloylglycine as reported in the literatures[34,38]. Considering such properties, we identified M19a with short retention time as feruloylglycine, and assigned M19b as isoferuloylglycine[34,38]. (Fig.6S-5.)

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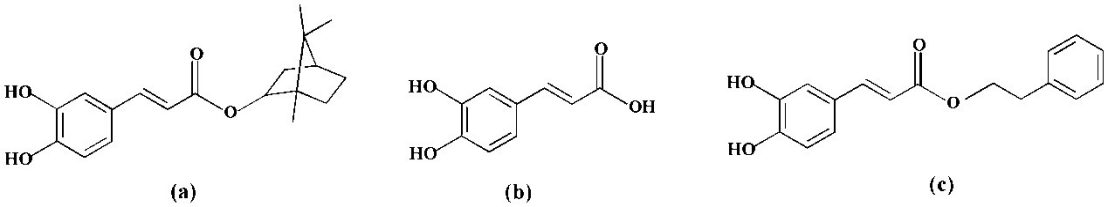

Fig. 1s. Chemical structures of bornyl caffeate (a), caffeic acid (b) and phenethyl caffeate as internal standard (c).

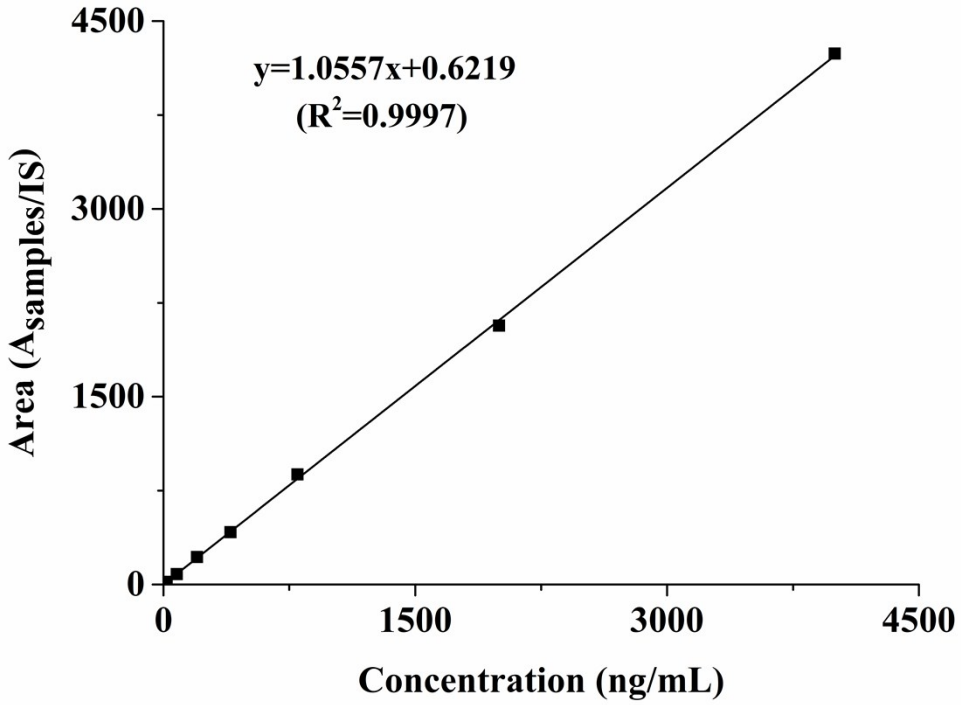

Fig. 2s. Calibration curve of bornyl caffeate

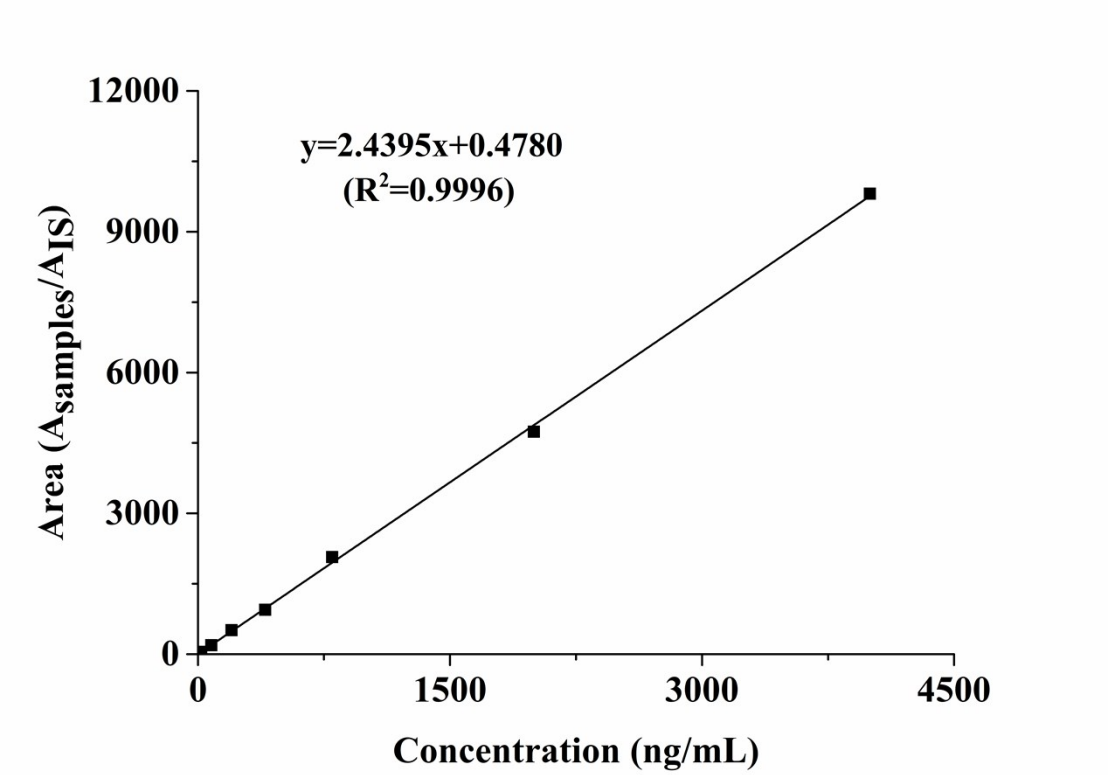

Fig. 3s. Calibration curve of caffeic acid

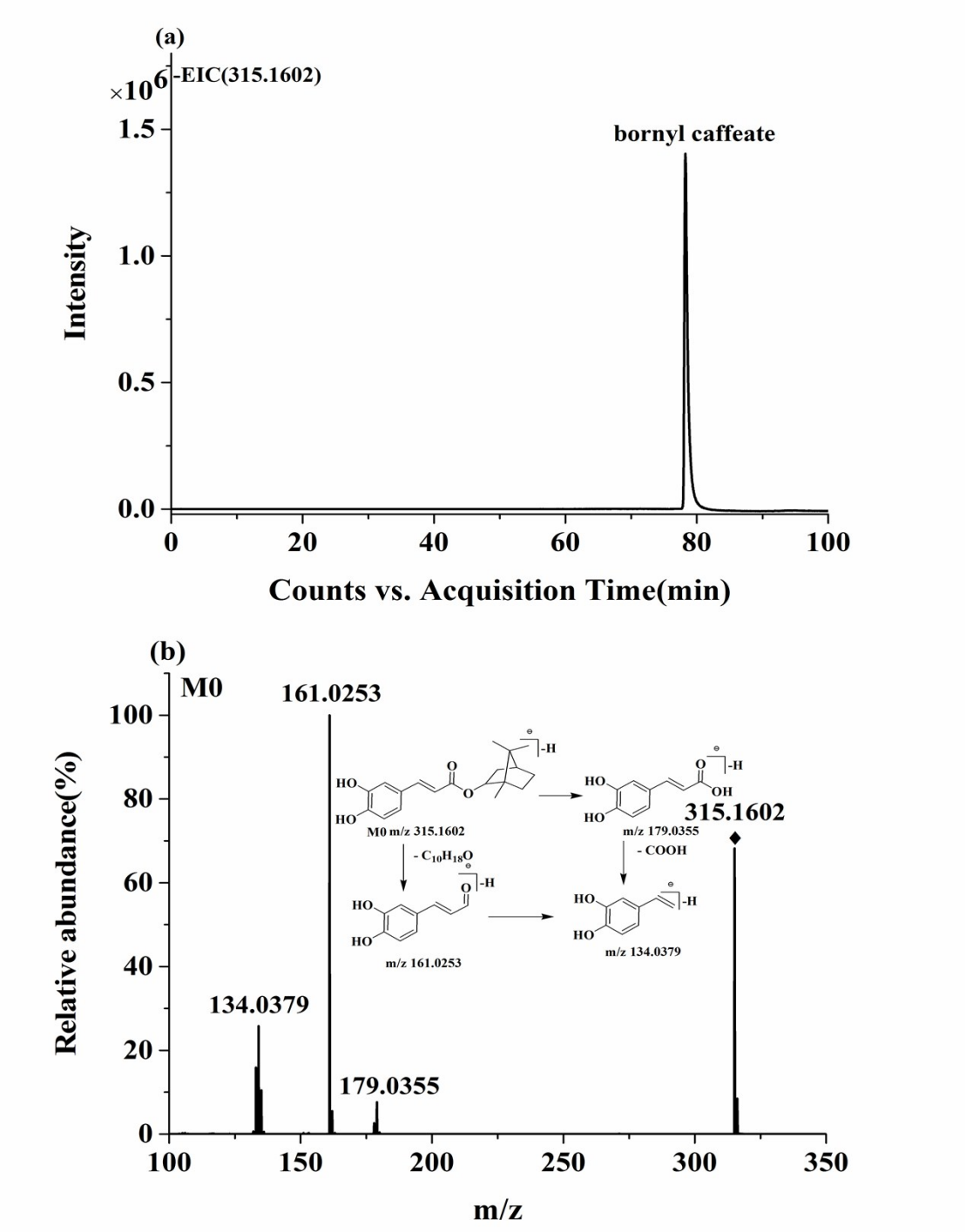

Figure. 4s. Representative profile of extracted ion chromatogram(a) and MS/MS spectrum inserted with the proposed fragmentation pathway of bornylcaffeate (b).

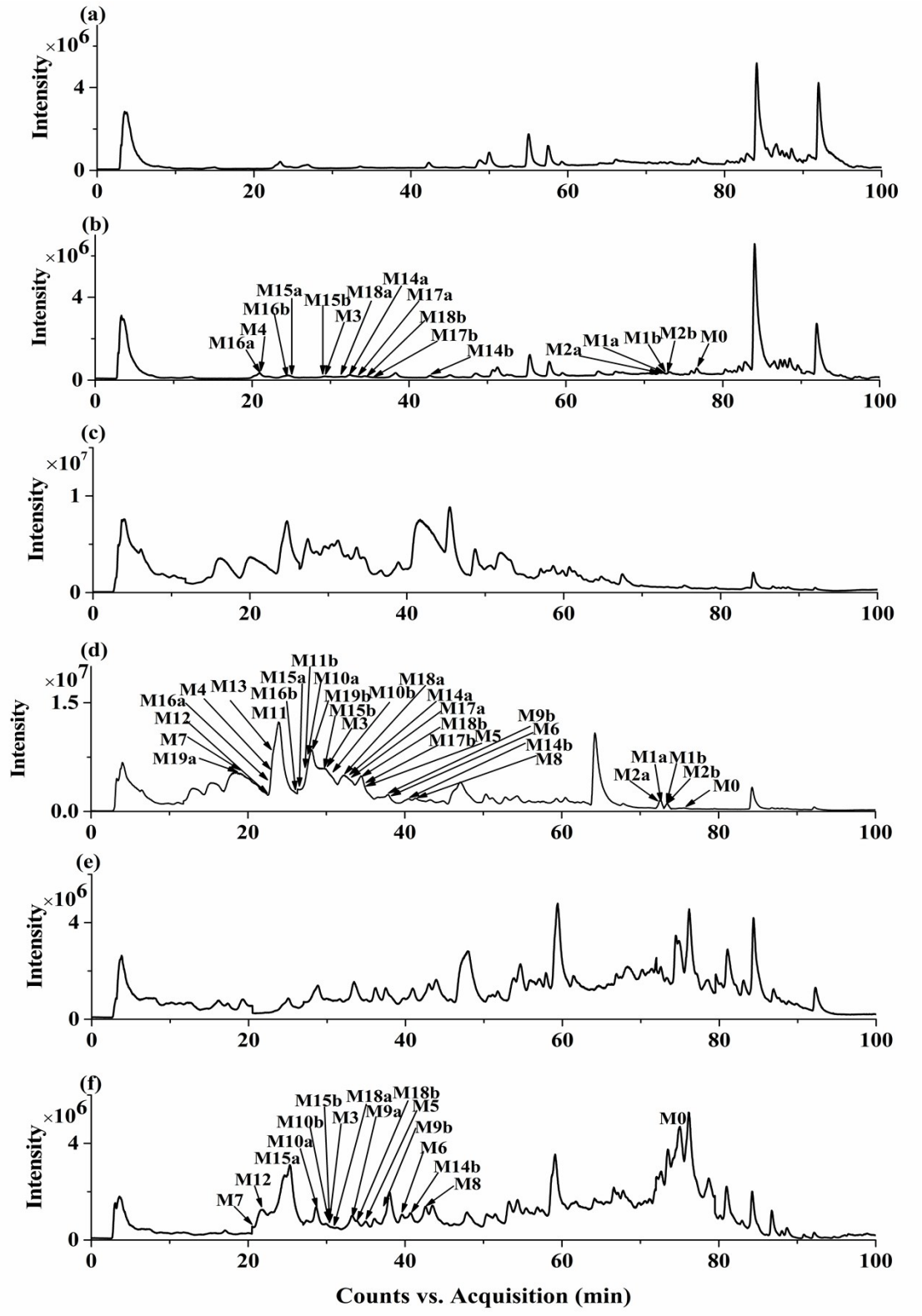

Fig. 5s. The total ion chromatograms(TIC) of (a) blank plasma samples, (b) 1h plasma sample after oral administration at a single dose of 30mg/kg bornyl caffeate, (c) blank urine sample, (d) 0-12h urine sample

after oral administration at a single dose of 30mg/kg bornyl caffeate, (e) blank feces sample, (f) 12-24h feces sample after oral administration at a single dose of 30mg/kg bornyl caffeate.

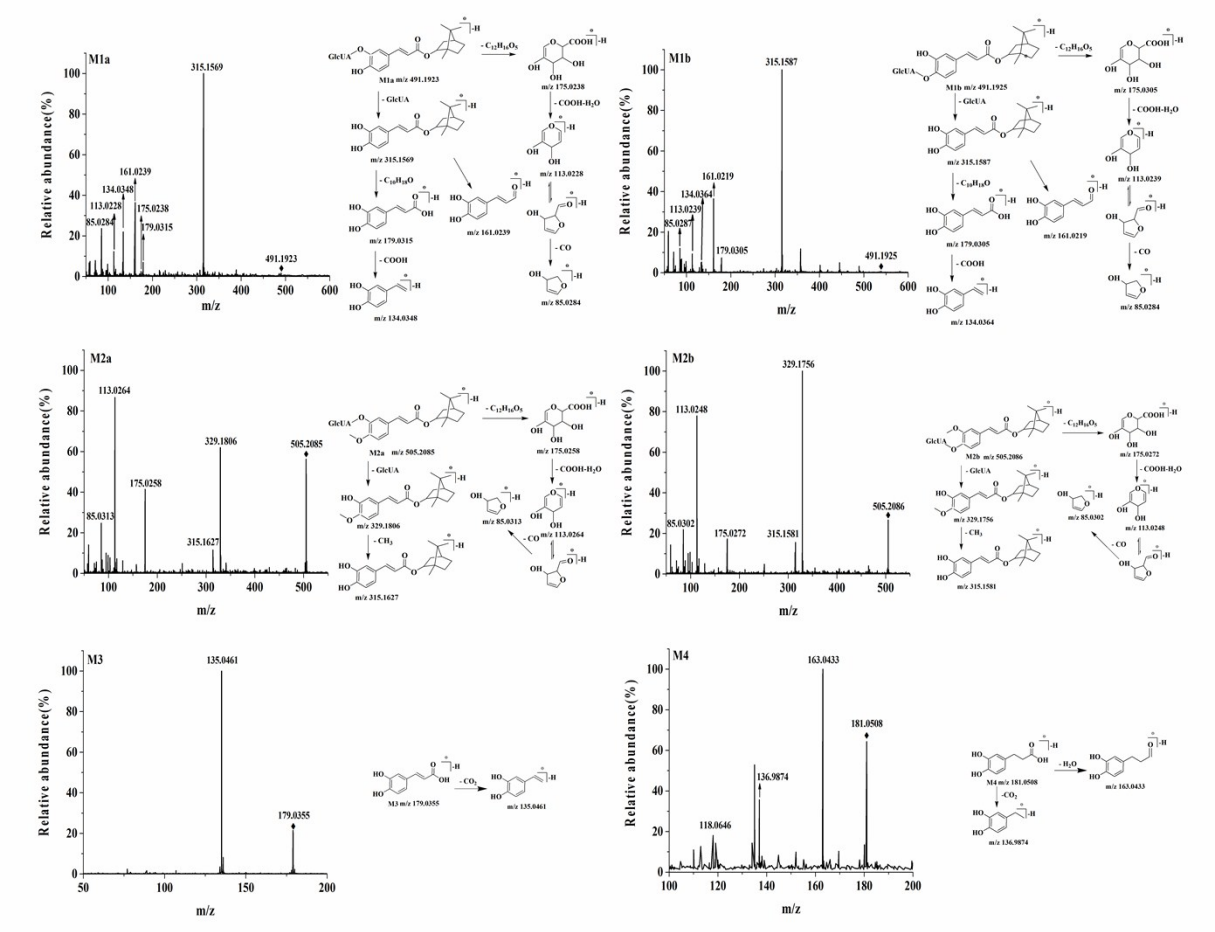

Fig. 6S-1. The representative MS/MS spectra of bornyl caffeate metabolites of M1-M4 in rats. At the right of MS/MS spectra is the respective proposed fragmentation mechanism of M1-M4

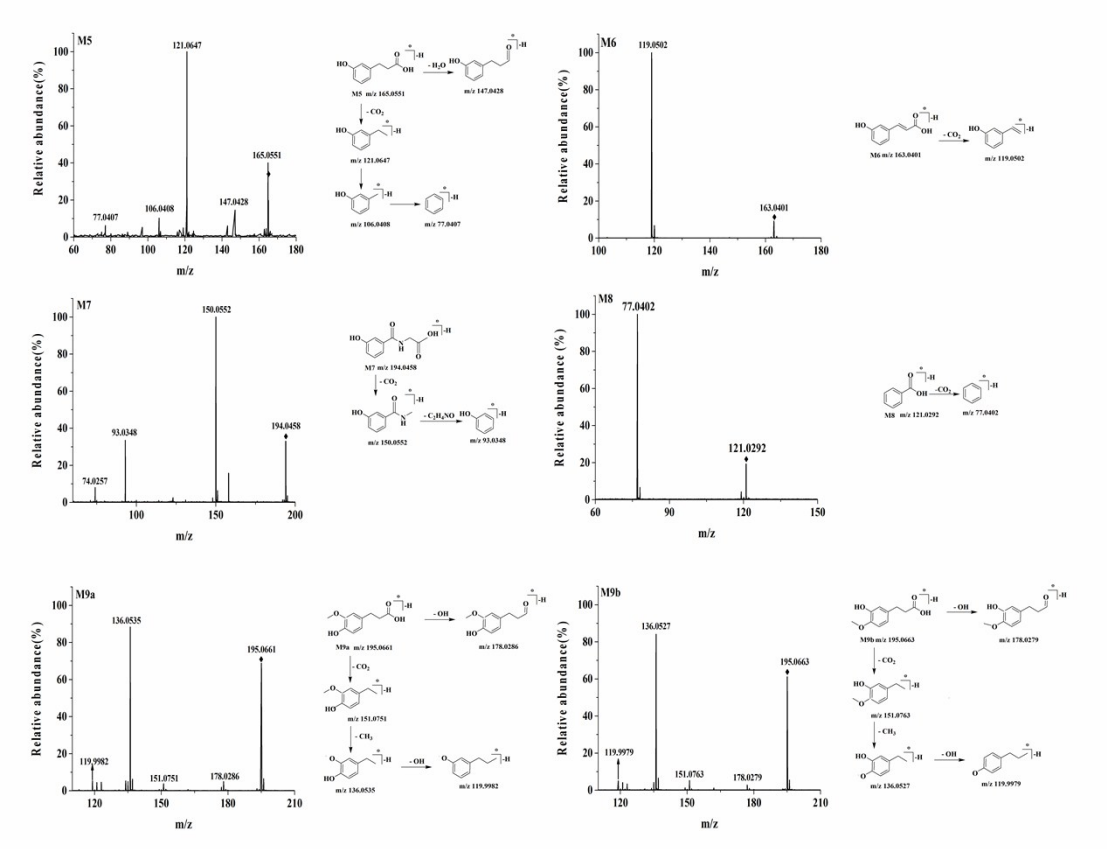

Fig. 6S-2. The representative MS/MS spectra of bornyl caffeate metabolites of M5-M9 in rats. At the right of MS/MS spectra is the respective proposed fragmentation mechanism of M5-M9

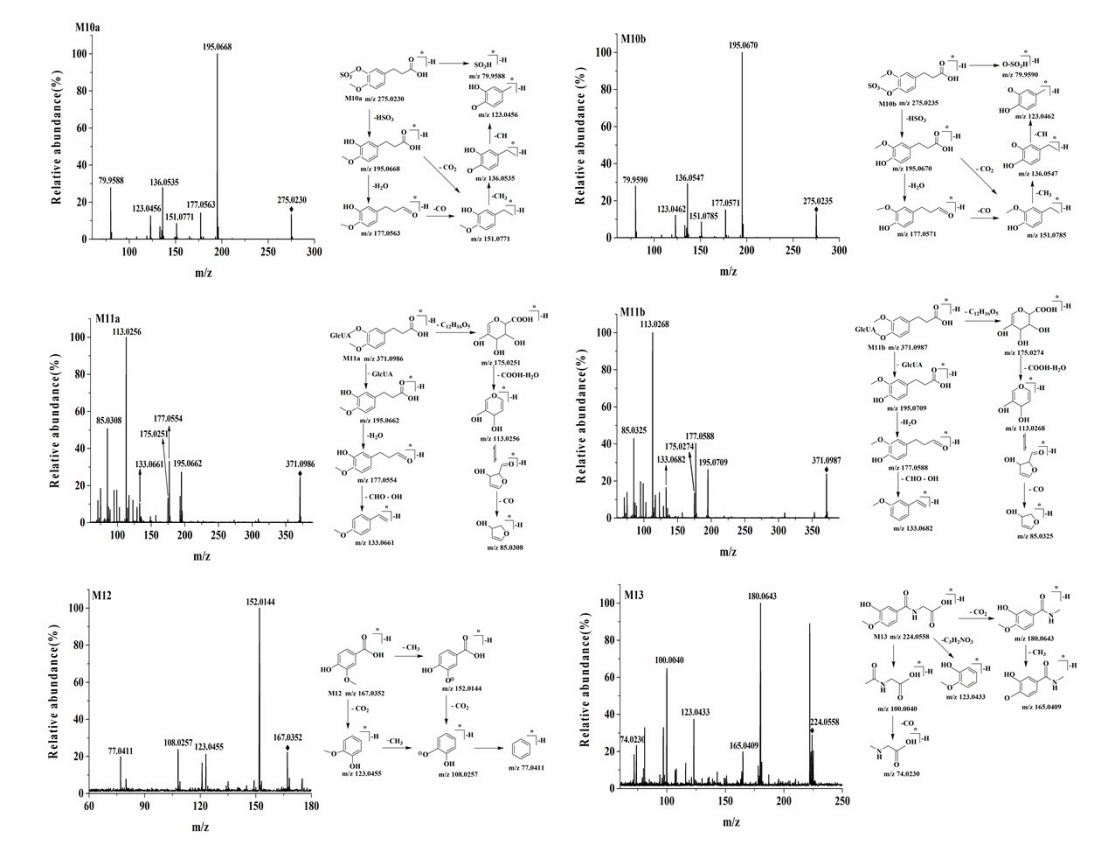

Fig. 6S-3. The representative MS/MS spectra of bornyl caffeate metabolites of M10-M13 in rats. At the right of MS/MS spectra is the respective proposed fragmentation mechanism of M10-M13

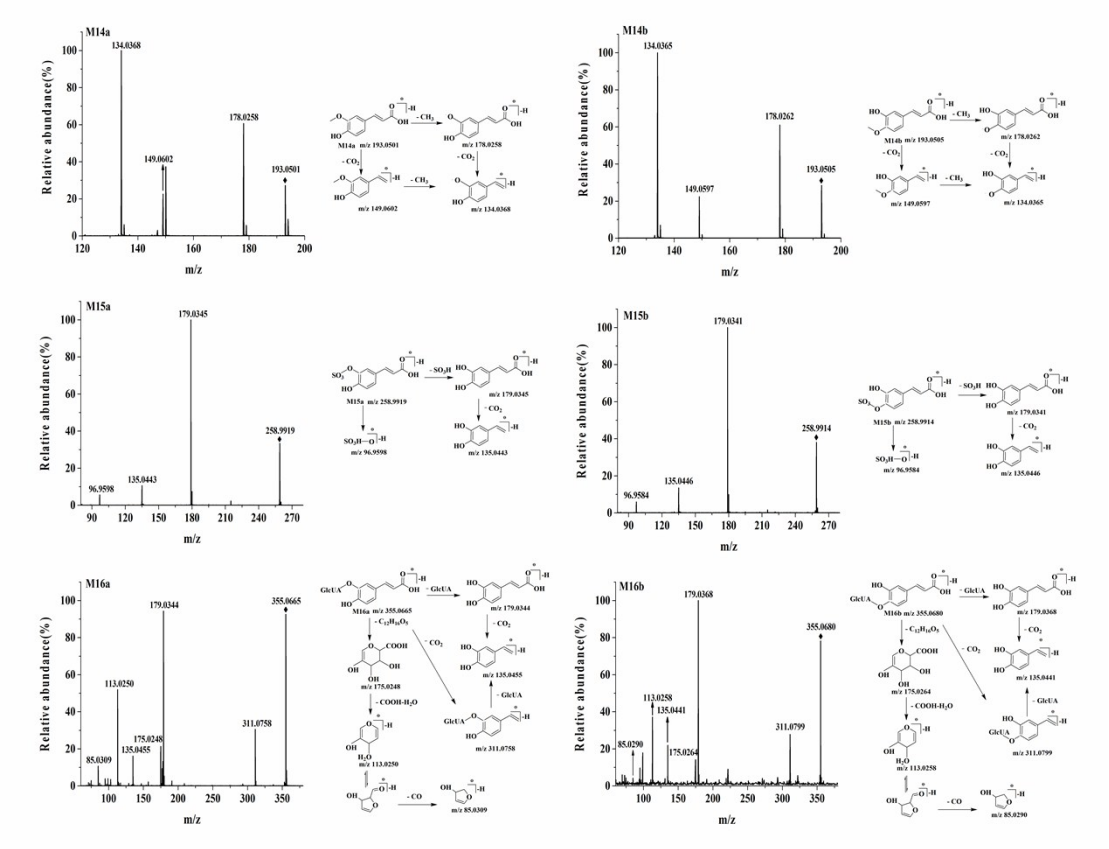

Fig. 6S-4. The representative MS/MS spectra of bornyl caffeate metabolites of M14-M16 in rats. At the right of MS/MS spectra is the respective proposed fragmentation mechanism of M14-M16

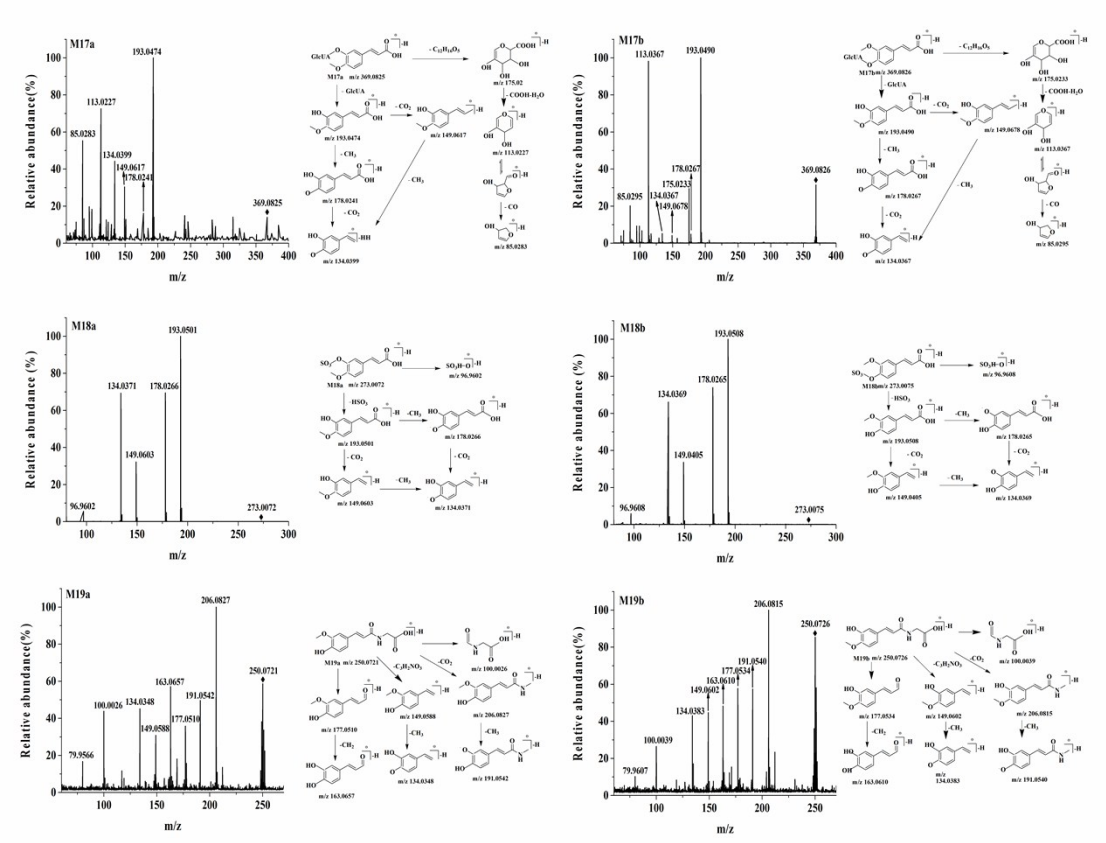

Fig. 6S-5. The representative MS/MS spectra of bornyl caffeate metabolites of M17-M19 in rats. At the right of MS/MS spectra is the respective proposed fragmentation mechanism of M17-M19